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PLUSH TACSy: Homonuclear planar TACSy with two-band selective shaped pulses applied to C^α, C' transfer and $C^\beta, C^{\text{aromatic}}$ correlations

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Summary

A new homonuclear Hartmann–Hahn-type mixing scheme is introduced that effects coherence transfer between resonances in two separated frequency bands. The mixing scheme relies on the irradiation of two-band selective shaped pulses that are expanded in an MLEV-16 supercycle. Similar to heteronuclear Hartmann–Hahn experiments, a planar effective coupling tensor is created. This novel mixing scheme is applied to C^α, C' transfer and to the transfer between C^β and aromatic carbon spins.

Introduction

Macromolecular NMR spectroscopy in solution relies on fast correlations between spins. Large interactions like the one-bond $^1\text{H}, ^{13}\text{C}$ or $^{13}\text{C}, ^{13}\text{C}$ coupling constants have proven to be valuable even for large biomacromolecules. Heteronuclear Hartmann–Hahn experiments based on continuous wave (CW) irradiation at the resonance frequencies of two heteronuclear spins (Hartmann and Hahn, 1962; Müller and Ernst, 1979; Chingas et al., 1981) form an interesting alternative to INEPT-type polarization-transfer experiments (Morris and Freeman, 1979). However, heteronuclear Hartmann–Hahn transfer became only important in practice when the limitations imposed by the use of CW irradiation were lifted by the application of multiple-pulse sequences (Bearden and Brown, 1989; Canet et al., 1990; Zuiderweg, 1990; Artemov, 1991; Brown and Sanctuary, 1991; Ernst et al., 1991; Schwendinger et al., 1994; Sunitha Bai et al., 1994). In heteronuclear Hartmann–Hahn experiments the effective coupling constant $J_{ij}^{\text{eff}} = S_{ij} J_{ij}$ is invariably scaled down and the largest possible effective coupling constant $J_{ij}^{\text{eff}} = J_{ij}/2$ (i.e. $S_{ij} = 1/2$) is found if an effective planar coupling tensor is created (Ernst et al., 1991).

Homonuclear Hartmann–Hahn-type experiments (Braunschweiler and Ernst, 1983; Bax and Davis, 1985) also offer a number of advantages compared to COSY-type coherence-transfer experiments (Aue et al., 1976) and have found numerous applications in high-resolution NMR. In broadband homonuclear Hartmann–Hahn sequences, such as DIPSI (Shaka et al., 1988) or FLOPSY (Kadkhodaei et al., 1991), Hartmann–Hahn matching conditions are created within a contiguous spectral range $\Delta\nu$ by irradiating the multiple-pulse sequence with a radio-frequency (rf) amplitude $\nu_1 = \gamma B_1/2\pi$ in the center of the spectral range $\Delta\nu$. Ideally, isotropic effective coupling tensors are created by these sequences, but the scaling factor S_{ij} of homonuclear coupling constants is in general smaller than $\cos \theta_{ij}$ (Shaka et al., 1988; Bax et al., 1990) where θ_{ij} is the angle between the effective fields created by CW irradiation with amplitude ν_1 at the offsets of the spins i and j . Hence, $S_{ij} < 1/2$ if $\Delta\nu/2$ is larger than the rf amplitude ν_1 . If the separation $\Delta\nu$ between two directly coupled homonuclear spins of interest is larger than reasonable rf amplitudes, it can therefore be of advantage to use pseudo-heteronuclear Hartmann–Hahn experiments in which the two homonuclear spins of interest are simultaneously irradiated. For example, Konrat et al. (1991) used

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doubly selective CW irradiation with $\nu_1 \ll \Delta\nu$ to create effective planar mixing conditions with $J_{ij}^{\text{eff}} = J_{ij}/2$ for homonuclear spins. Shirakawa et al. (1995) used doubly selective WALTZ-16 sequences for homonuclear C^α, C' transfer.

In addition to the bandwidth to be covered by a Hartmann–Hahn sequence, it is important to consider the effective size of the spin system during the mixing process. While in *total* correlation spectroscopy (TOCSY) (Braunschweiler and Ernst, 1983; Bax and Davis, 1985) coherence is transferred between *all* spins that are part of a common J-coupling network, Hartmann–Hahn transfer is deliberately restricted within a well-defined subset of a spin system in *tailored* correlation spectroscopy (TACSy) (Glaser and Drobny, 1989, 1991; Eaton et al., 1990; Konrat et al., 1991; Mohebbi and Shaka, 1991; Kupče and Freeman, 1992; Glaser, 1993; Richardson et al., 1993; Grzesiek and Bax, 1995; Quant et al., 1995). In these experiments, the transfer efficiency is in general enhanced if the effective size of the spin system is restricted to a minimum during the mixing process (Glaser, 1993). This is often possible if the nuclei to be connected by coherence transfer lie in well-separated regions of the spectrum. For example, in ^{13}C -labelled proteins, the C' and C^α regions are well separated from the rest of the resonances. Hence, it is possible to switch off the $^1J(C^\alpha, C^\beta)$ coupling and to achieve a reduction to an effective two-spin system consisting only of C^α and C' . If this reduction can be realized, optimal transfer between C^α and C' resonances is expected.

The C^β resonances of aromatic amino acids are also well separated from the other resonances of these amino acids. The assignment of the aromatic ring resonances, which is of vital importance for the correct evaluations of the NOE contacts of the hydrophobic core of the protein, can be easily achieved if these resonances are correlated to the C^β . For this purpose, INEPT-type experiments have been previously proposed (Yamazaki et al., 1993). However, the $C^\beta, C^{\text{aromatic}}$ transfer can also be obtained via Hartmann–Hahn-type sequences (Grzesiek and Bax, 1995). In this case, optimal transfer between C^β and C^{aromatic} is expected if the $^1J(C^\alpha, C^\beta)$ coupling can be effectively eliminated. If this is possible, C^β coherence flows into the aromatic ring without any loss to C^α or C' .

We and the authors of the accompanying paper (Zuiderweg et al., 1996) introduce novel double-band selective TACSy sequences. These sequences are based on shaped pulse trains and implement a planar average coupling Hamiltonian within two bands of the same width $\Delta\nu_b$ separated by an adjustable frequency $\Delta\nu$ (e.g. 120 ppm for the C^α/C' and 100 ppm for the $C^\beta/C^{\text{aromatic}}$). In addition, the Hartmann–Hahn condition is matched for spins in both rotating frames, simultaneously. We will refer to this class of planar double-band selective homonuclear TACSy experiments as PLUSH TACSy.

Design of PLUSH TACSy sequences

Two-band selective sequences can be conveniently created by a $\cos(\pi\Delta\nu t)$ amplitude modulation of a multiple-pulse train. The effect of an amplitude-modulated sequence is identical to the application of the multiple-pulse sequence at $\pm\Delta\nu/2$ with an amplitude that is reduced for each band by a factor 2 compared to the maximum amplitude of the modulated sequence. Since the two corresponding rotating frames coincide only at integer multiples of $\Delta\nu^{-1}$, an average Hamiltonian defined in the doubly rotating frame is only equivalent to the effective Hamiltonian in a single rotating frame if the cycle time τ_c is a multiple of $\Delta\nu^{-1}$. If this condition is not taken into account in practice, coherences acquire additional phase terms that must be considered if the coherences are to be further manipulated by hard or selective pulses. If the amplitude-modulated multiple-pulse sequence is implemented on the spectrometer by repetition of amplitude and possibly phase-modulated constituent pulses R , it is convenient to adjust also the duration of these constituents to an integer multiple of $\Delta\nu^{-1}$. This ensures that the x-axes of the two rotating frames are aligned whenever a new pulse constituent R of the multiple pulse sequence starts.

Under the assumption that the multiple pulse sequence irradiated at either band does not affect resonances that are off-resonance by approximately $\Delta\nu$, the corresponding amplitude-modulated sequence will behave as two multiple-pulse sequences irradiated independently at $\pm\Delta\nu/2$. Thus, it is expected that amplitude modulation of a band-selective multiple-pulse sequence, which is designed to remove heteronuclear couplings and therefore also the chemical shift (Waugh, 1982), will be a good implementation for a double-band selective sequence that zeroes the chemical shift within the two bands and therefore fulfills the Hartmann–Hahn condition. For example, all shaped inversion pulses R with a sharp inversion profile subjected to amplitude modulation and expanded into an MLEV-4 (RRRR) or MLEV-16 supercycle (Levitt et al. 1983) are expected to fulfill the Hartmann–Hahn condition within the two bands. The second requirement is to achieve the desired planar coupling Hamiltonian of the form:

$$H_{J,\text{eff}} = \pi J(I_{1y}I_{2y} + I_{1z}I_{2z}) \quad (1)$$

for two spins I_1 and I_2 that are located in two different frequency bands. This imposes two additional constraints on the shaped inversion pulse R . These constraints can be derived in a straightforward way from the form of the coupling Hamiltonian in the toggling frame. In the limit $\Delta\nu \gg \nu_1^{\text{max}}$ which is perfectly realized for two heteronuclear spins, the coupling term H_J^d in the doubly rotating frame is given by:

$$H_J^d = 2\pi J I_{1z} I_{2z} \quad (2)$$

In this frame the rf Hamiltonian has the form:

$$H_{rf}^d = 2\pi v_1(t) (F_x \cos \varphi(t) + F_y \sin \varphi(t)) \quad (3)$$

where $v_1(t)$ is the rf amplitude in each band and $\varphi(t)$ is a possible phase modulation. In the toggling frame which is defined by the rf propagator:

$$U_{rf}^d(t) = \exp\{-iH_{rf}^d t\} \quad (4)$$

the coupling Hamiltonian:

$$\tilde{H}_J(t) = (U_{rf}^d(t))^{-1} H_J^d U_{rf}^d(t) \quad (5)$$

is time-dependent. The zero-order average Hamiltonian \bar{H}_0 in the toggling frame is given by:

$$\bar{H}_0 = \frac{1}{\tau} \int_0^\tau \tilde{H}_J(t) dt \quad (6)$$

In order to create the desired effective planar coupling tensor with a minimum scaling of the J coupling, the rf field must be applied only along the x -axis of the doubly rotating frame, i.e. the shaped pulse R must be purely amplitude-modulated with $\varphi(t)=0$ or π (Ernst et al. 1991). In addition, the trajectories of the operators $\tilde{I}_{1z}(t)$ and $\tilde{I}_{2z}(t)$ must spend equal amounts of time along the z - and y -axis of the toggling frame. This condition is not fulfilled for most band-selective inversion pulses such as Gaussian pulses, G3 (Emsley and Bodenhausen, 1990) or I-BURP-2 (Geen and Freeman, 1991). If the shaped inversion pulse is composed of any two identical selective 90° pulses, the

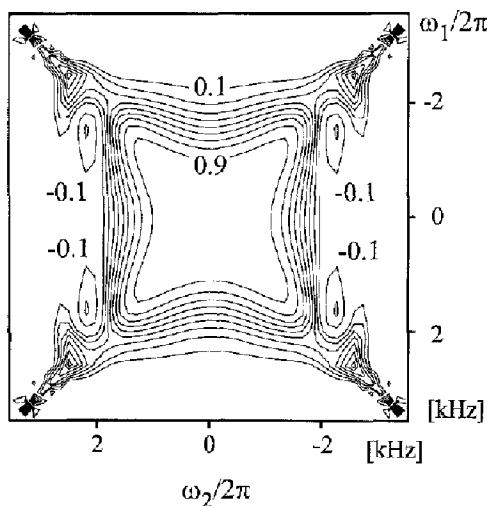


Fig. 1. Simulated offset profile for the heteronuclear transfer ($I_x \rightarrow S_x$) using the G2 sequence, which is an MLEV-16 expansion of $R = G^{270^\circ} \cdot G^{270^\circ}$ pulses truncated at 15% of their maximum with $\tau_r = 533.33 \mu\text{s}$ on each channel with a $v_1^{\text{max}} = \gamma B_1^{\text{max}}/2\pi$ of 4.608 kHz. The coupling constant is $J_{IS} \approx 120$ Hz and the mixing time used equals $1/J_{IS}$. The theoretical maximum is 1. The contour levels are indicated.

desired planar mixing Hamiltonian is always achieved on resonance by the following symmetry argument.

The average Hamiltonian during the first shaped 90° pulse of duration τ is calculated from:

$$\bar{H}_{0,1} = \frac{1}{\tau} \int_0^\tau 2\pi J_{12} \{ \tilde{I}_{1z}(t) \tilde{I}_{2z}(t) \} dt \quad (7)$$

Since $\tilde{I}_x(\tau) = I_y$ and $\tilde{S}_x(\tau) = S_y$, the contribution of the second 90° pulse to the average Hamiltonian is:

$$\bar{H}_{0,2} = \frac{1}{\tau} \int_0^\tau 2\pi J_{12} \{ \tilde{I}_{1y}(t) \tilde{I}_{2y}(t) \} dt \quad (8)$$

The average Hamiltonian $\bar{H}_0 = (\bar{H}_{0,1} + \bar{H}_{0,2})/2$ is:

$$\begin{aligned} \bar{H}_0 &= \frac{1}{2\tau} \int_0^\tau 2\pi J_{12} \{ \tilde{I}_{1z}(t) \tilde{I}_{2z}(t) + \tilde{I}_{1y}(t) \tilde{I}_{2y}(t) \} dt \\ &= \pi J_{12} (I_{1z} I_{2z} + I_{1y} I_{2y}) \end{aligned} \quad (9)$$

due to the invariance of zero-quantum operators about the x -axis ($I_{1z} I_{2z} + I_{1y} I_{2y}$) with respect to x rotations. Then, \bar{H}_0 is the desired planar coupling Hamiltonian in the toggling frame. The same argument also holds for the combination of two 270° pulses which, due to the 'self-refocussing', offers better phase characteristics within the band. Since the MLEV-16 expansion of any inversion pulse is cyclic, the toggling-frame average Hamiltonian created by the expanded sequence can be used to describe the effective evolution of the spin system in the doubly rotating frame. As a simple implementation of this recipe, we propose the use of an MLEV-16 expansion of amplitude-modulated shaped inversion pulses R , consisting of two Gaussian 270° pulses, i.e. $R = G^{270^\circ} \cdot G^{270^\circ}$, which henceforth we will refer to as the G2 sequence. The characteristic properties of the G2 sequence for heteronuclear and homonuclear Hartmann-Hahn transfer will be analysed below. In contrast to the qualitative zero-order average Hamiltonian arguments used above, the following simulations were performed with an extended version of the simulation program SIMONE (Glaser and Drobny, 1990) in which the exact evolution of the density operator is calculated based on the Liouville-Von Neuman equation, neglecting relaxation.

If the G2 sequence is applied to a heteronuclear spin system, the irradiation at the two bands does not interfere and the ideal planar Hamiltonian (Eq. 1) is achieved on resonance. The offset profile for the sequence applied to a heteronuclear two-spin system with a coupling of $J_{IS} \approx 120$ Hz, a mixing time of $8.33 \text{ ms} \approx 1/J_{IS}$, an $R = G^{270^\circ} \cdot G^{270^\circ}$ pulse module of $533.33 \mu\text{s}$ duration and a 15% truncation level is shown in Fig. 1. The maximum transfer of 100%

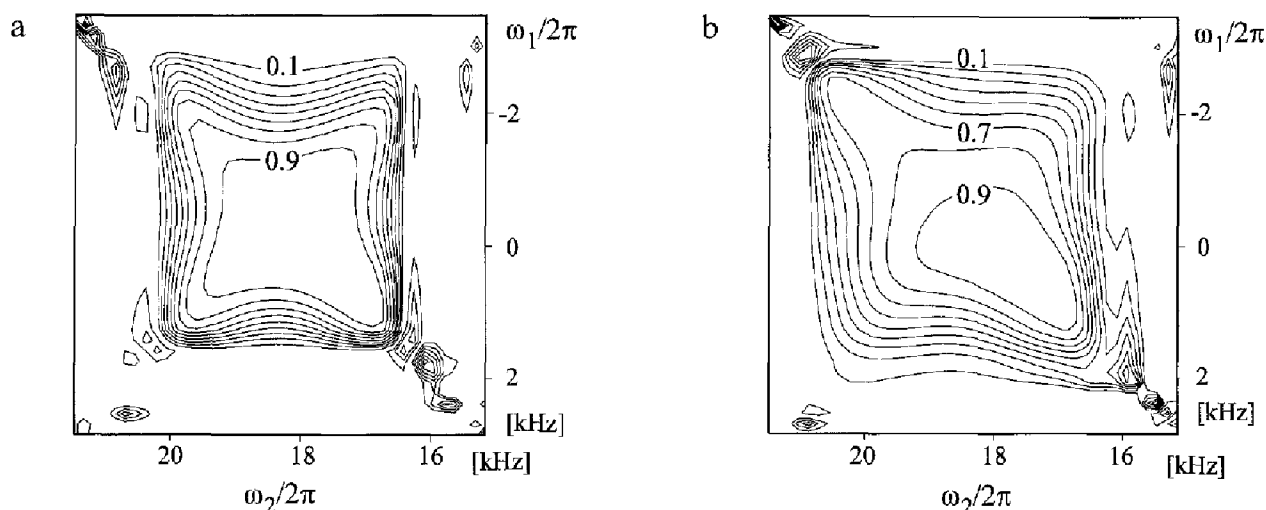


Fig. 2. Simulated offset profiles for the C^α, C' transfer ($I_{1x} \rightarrow I_{2x}$) ($J = 56$ Hz) using the G2 sequence based on amplitude- and phase-modulated $R = G^{270} \cdot G^{270}$ pulses with $\tau_R = 559.91 \mu\text{s}$; $\Delta\nu/2 = 8.93$ kHz for both amplitude and phase modulation; mixing time = 17.9 ms (two cycles of MLEV-16). The pulse was truncated at 15% (a) and 5% (b) of its maximum, resulting in the following values: (a) 15% truncation, $2\nu_1^{\text{max}} = 8.204$ kHz, $\langle 2(\nu_1)^2 \rangle^{1/2} = 4.147$ kHz; (b) 5% truncation, $2\nu_1^{\text{max}} = 9.516$ kHz, $\langle 2(\nu_1)^2 \rangle^{1/2} = 4.472$ kHz.

is achieved on resonance, as expected. The offset profiles for an rf amplitude scaled by -5% and $+5\%$ were calculated as well and they demonstrate the robustness of the sequence with respect to rf inhomogeneity (Supplementary Material).

When this sequence is applied to a homonuclear system, mutual interference of the two-band selective irradiations is expected since the condition $\Delta\nu \gg \nu_1^{\text{max}}$ is not strictly fulfilled. Therefore it was necessary to optimize the length and the truncation level of the composite-shaped pulse R in order to obtain minimum interference between the two bands.

Here we should note some properties of two-band selective sequences: the maximum field of a two-band selective sequence $\nu_{\text{rf}}^{\text{max}}$ is given by $2\nu_1^{\text{max}}$. Furthermore, the rf power $P_{\text{rf}} = \langle (\nu_{\text{rf}})^2 \rangle = \langle 2(\nu_1)^2 \rangle$ of a two-band selective sequence is twice that of the individual bands. It should be mentioned also that, for convenience, the transmitter does not need to be in the middle of the two bands, but can be shifted by phase modulation of the whole pulse with a constant time-proportional phase increment of $\Delta\phi/\Delta t = 2\pi \Delta\nu'$ by exactly this frequency $\Delta\nu'$. If the transmitter is put to the center of either band $\Delta\nu' = \pm \Delta\nu/2$.

For the C^α, C' transfer, the separation of the two bands $\Delta\nu$ at 600 MHz is 18 kHz and we find optimal transfer for the G2 sequence based on Gaussian G^{270} pulses with $2\nu_1^{\text{max}} = 2\gamma B_1^{\text{max}}/(2\pi) = 8.204$ kHz (4.1 kHz per band) truncated at 15% of the maximum amplitude, with $\langle 2(\nu_1)^2 \rangle^{1/2}$ of 4.147 kHz, a duration τ_R of 559.91 μs (Fig. 2a) and a width $\Delta\nu_b$ of each band of ≈ 2.5 kHz. The parameter $\Delta\nu/(\nu_1^{\text{max}})$ (Zuiderweg et al., 1996) equals 3.8. In Fig. 2b the offset profile is shown for Gaussian pulses of the same length, but with 5% truncation level, $2\nu_1^{\text{max}} = 9.516$ kHz and $\langle 2(\nu_1)^2 \rangle^{1/2}$ of 4.472 kHz. With both parameter sets the C^α and C' regions are covered while the C^β reson-

ances are effectively decoupled, with the exception of serine C^β atoms.

For the coherence transfer between C^β and $C^{\gamma, \text{aromatic}}$ atoms, the separation of the two bands at 600 MHz is 15 kHz. This reduced offset with respect to the C^α, C' case imposes stronger restrictions on some of the pulse parameters. Only with a truncation level of 15% and a pulse length $\tau_R = 533.33 \mu\text{s}$ we have obtained good transfer within the whole band of interest ($\Delta\nu_b \approx 2.5$ kHz) with minimal interference between the two bands (Fig. 3). Note that due to this interference the maximum transfer is not exactly on resonance. The maximum rf amplitude is $2\nu_1^{\text{max}} = 9.216$ kHz (4.61 kHz per band) resulting in

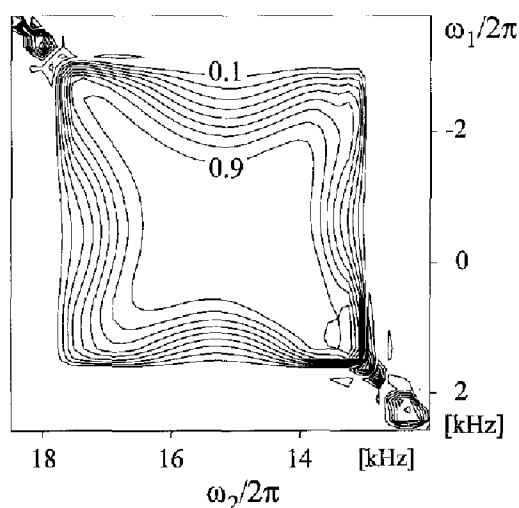


Fig. 3. Theoretical offset profiles for $C^\beta, C^{\gamma, \text{aromatic}}$ transfer ($I_{1x} \rightarrow I_{2x}$) ($J = 44$ Hz) using the G2 sequence based on amplitude- and phase-modulated $G^{270} \cdot G^{270}$ pulses with $\tau_R = 533.33 \mu\text{s}$; a truncation level of 15%; $\Delta\nu/2 = 7.5$ kHz for both amplitude and phase modulation; mixing time = 25.6 ms (three cycles of MLEV-16); $2\nu_1^{\text{max}} = 9.216$ kHz; $\langle 2(\nu_1)^2 \rangle^{1/2} = 4.336$ kHz.

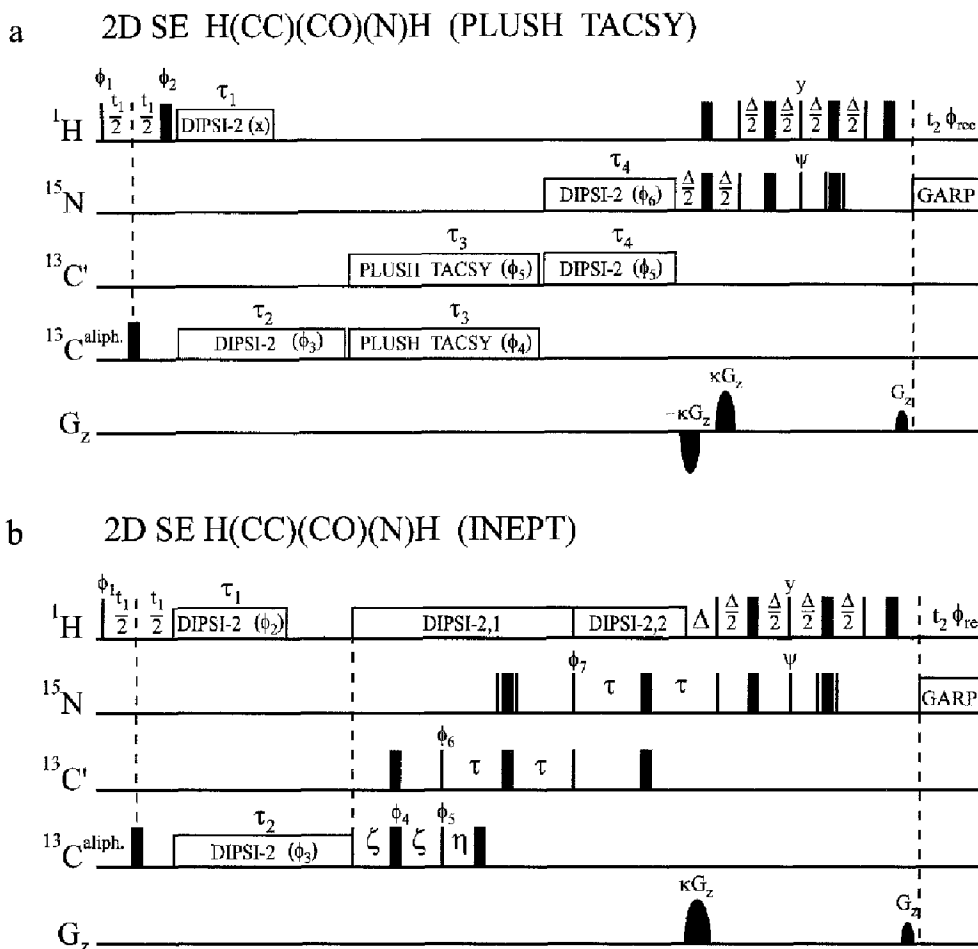


Fig. 4. (a) Pulse sequence for H(CC)(CO)(N)H correlation using PLUSH TACS Y for the C^α, C' transfer. Three heteronuclear Hartmann–Hahn transfers are used in addition to homonuclear C,C TOCSY. The DIPSI-2 sequence (Shaka et al., 1988) for the C,H transfer had a duration of 6 ms and used an average $\gamma B_1/2\pi$ of 7.2 kHz. The homonuclear C,C TOCSY was achieved by the same DIPSI-2 sequence on the carbon channel, applied -20 kHz off-resonance with the carrier placed in the middle of the carbonyl region. The G2 PLUSH TACS Y of 18 ms duration for C^α, C' coherence transfer was implemented with an $R = G^{270} \cdot G^{270}$, $\tau_R = 559.91 \mu\text{s}$ and 5% truncation level expanded in an MLEV-16 supercycle. The C', N transfer was accomplished by heteronuclear Hartmann Hahn mixing using DIPSI-2. Either G3 (180° , 250 μs) or G4 (90° , 400 μs)-type pulses were used for selective C-pulses; the proton frequency was switched after C', N transfer from H^{aliph} to H^N . $\kappa = 5, -5$, $\psi = y, -y$; $\phi_1 = 8(x), 8(-x)$; $\phi_2 = x$; $\phi_3 = 2(x), 2(-x)$, $\phi_4 = x$; $\phi_5 = x$; $\phi_6 = 4(x)$; $\phi_{\text{rec}} = 2(x), 4(-x), (2x), 2(-x), 4(x), 2(-x)$; $\Delta = 5.4$ ms, $\tau_1 = 6$ ms; $\tau_2 = 12$ ms, $\tau_3 = 18$ ms, $\tau_4 = 48$ ms. (b) INEPT version of the sequence in Fig. 4a. The C^α, C' and C', N transfers have been replaced by selective coherence transfer. The C^{aliph} pulses were either G3 (180°) or G4 (90°) pulses of duration 250 μs and 400 μs , respectively. The carbon carrier was in the middle of the aliphatic region. $\kappa = 10, -10$, $\psi = y, -y$; $\phi_1 = 2(y), 2(-y)$ and TPPI(t_1), $\phi_2 = x$; $\phi_3 = x$, $\phi_4 = x$ (plus 25° to correct for zero-order and a delay of 15 μs to correct for a first-order Bloch–Siegert phase shift); $\phi_5 = 8(x), 8(-x)$; $\phi_6 = x$ (-75° to correct for zero-order and a delay of 25 μs to correct for a first-order Bloch–Siegert phase shift); $\phi_7 = 4(x), 4(-x)$; $\phi_{\text{rec}} = 2(x), 4(-x), (2x), 2(-x), 4(x), 2(-x)$; $\Delta = 5.4$ ms, $\tau = 13.5$ ms; $\tau_1 = 6$ ms, $\tau_2 = 12$ ms, $\zeta = 3.4$ ms, $\eta = 4.5$ ms.

$\langle 2(v_1)^2 \rangle^{1/2}$ of 4.336 kHz. The parameter $\Delta v/(v_1^{\text{max}})$ equals 3.2. The sequence is robust with respect to B_1 inhomogeneities, as demonstrated by the offset plot calculations for a 5% enhanced and reduced B_1 field (Supplementary Material).

The mutual interference of the two bands of MLEV-16 expanded $G^{270} \cdot G^{270}$ pulses in the G2 sequence is also reflected in the on-resonance effective coupling Hamiltonian which slightly deviates from the ideal planar coupling term of Eq. 1 that is found for heteronuclear applications. For the C^α, C' G2 sequence with a 15% truncation level the on-resonance effective coupling Hamiltonian is:

$$H_{J,\text{eff}} = \pi J(0.985 I_{1y} I_{2y} + 0.944 I_{1x} I_{2z}) \quad (10)$$

while for the $C^\beta, C^{\gamma, \text{aromatic}}$ transfer it amounts to:

$$H_{J,\text{eff}} = \pi J(-0.141 I_{1x} I_{2x} + 0.986 I_{1y} I_{2y} + 0.831 I_{1z} I_{2z}) \quad (11)$$

For this latter case, the offset transfer profiles from I_{1x} to the undesired coherences (I_{2y} , I_{2z} and antiphase coherences) have been calculated, as well as transfers from I_{1y} to I_{2y} and from I_{1z} to I_{2z} . (Supplementary Material). They show that there is very little transfer from I_{1x} to the undesired coherences. While the Hamiltonian yields very fast $I_{1x} \rightarrow I_{2x}$ transfer (Fig. 3), the transfer is much slower for $I_{1z} \rightarrow I_{2z}$ and for $I_{1y} \rightarrow I_{2y}$, as expected from the form of the effective coupling terms (Eq. 10) on resonance.

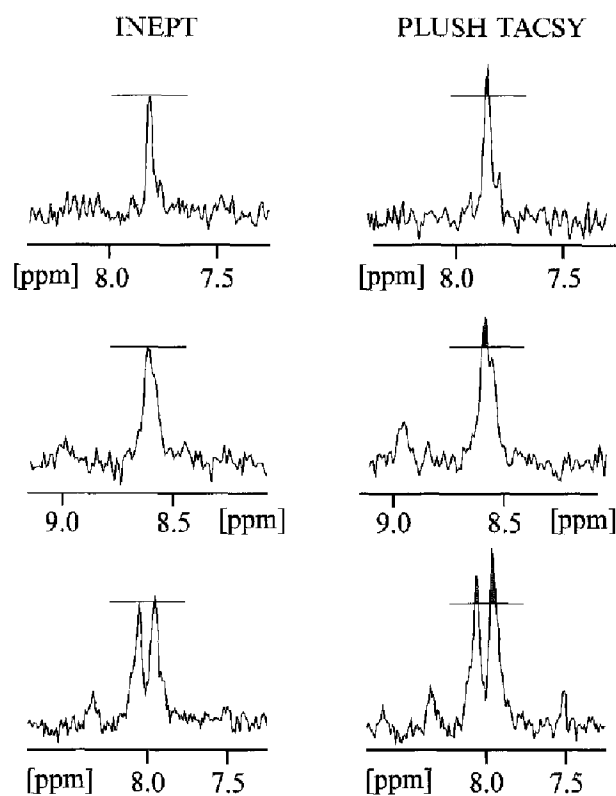


Fig. 5. Representative traces from 2D H(CC)(CO)(N)H experiments acquired with the pulse sequences of Fig. 4 on a 1.5-mM ^{13}C , ^{15}N -labelled calmodulin–C20W complex sample. The INEPT-type experiment and the PLUSH TACS experiment are shown on the left and on the right side, respectively. The observed sensitivity-enhancement factor is ≈ 1.2 .

C^{α} , C^{\prime} PLUSH TACS

The G2 PLUSH TACS sequence for the C^{α} , C^{\prime} coherence transfer was incorporated into a H(CC)(CO)(N)H experiment (Fig. 4a) and compared with the conventional sequence based on INEPT-type polarization transfer (Fig. 4b) (Montelione et al., 1992; Grzesiek et al., 1993; Logan et al., 1993). In order to compare the transfer efficiency, traces are taken through three representative cross-peaks originating from the calmodulin–C20W complex (Fig. 5). The G2 sequence was used with a truncation level of 5%, a maximum $2\gamma\text{B}_1^{\text{max}}/2\pi$ of 9.516 kHz and a τ_{r} of 559.91 μs . It can be seen from Fig. 5 that the sensitivity of this PLUSH TACS experiment is approximately 20% better than the sensitivity of the conventional experiment. The main reason for the sensitivity enhancement is the fact that most of the C^{β} resonances are not affected by the G2 sequence. This results effectively in a two-spin system. In the pulsed version, $\text{C}^{\text{aliphatic}}$ carbon pulses were used that do not confine the coherences to the $\text{C}^{\alpha}/\text{C}^{\prime}$ spin system, thus yielding a lower signal-to-noise ratio. If in the pulsed version the C^{α} are decoupled from the C^{β} during the $\text{C}^{\alpha}/\text{C}^{\prime}$ transfer, using for example a selective C^{α} inversion pulse, the difference in sensitivity between the PLUSH TACS experiment and the conventional experiment

decreases. Nevertheless, an improvement in signal to noise can still be observed, which can be attributed to the better performance of the Hartmann Hahn sequence in the presence of B_1 field inhomogeneities.

Concatenation of the two consecutive Hartmann–Hahn mixing processes between C^{α} , C^{\prime} and C^{β} , N is able to further reduce the duration of the pulse sequence by approximately 10 ms by application of the PLUSH TACS sequence also for the latter transfer (Majumdar and Zwieterweg, 1995), although it has to be considered that not all the probes can operate in optimal conditions during the application of a 50-ms TOCSY sequence with a peak rf field of 4.4 kHz on the nitrogen channel. If the concatenation of the two consecutive TOCSY sequences cannot be realized due to hardware reasons, the G2 PLUSH TACS sequence turns out to be longer than the INEPT-type polarization-transfer sequence and therefore may not be the best choice for very large proteins. Nevertheless, with respect to B_1 field inhomogeneities and to offset behaviour the performance of the G2 PLUSH TACS sequence is still expected to be better than that of the pulsed analog.

A modified 3D sequence has been applied for the side-chain assignment of the 103 amino acid protein rhodniin, which is a thrombin inhibitor. Figure 6 shows representative strips belonging to residues 79–89 of this protein by which the carbon side-chain resonances of the protein can be assigned.

C^{β} , $\text{C}^{\text{aromatic}}$ PLUSH TACS

Several experiments have been proposed to achieve transfer between C^{β} and $\text{C}^{\text{aromatic}}$ atoms, among which are the polarization-transfer-based experiments proposed by Yamazaki et al. (1993) and the AMNESIA sequence recently proposed by Grzesiek and Bax (1995). While the former experiments transfer coherence from the C^{β} atom via polarization transfer to the C^{γ} atom and then further via polarization transfer to C^{δ} and C^{ϵ} atoms, the AMNESIA sequence implements a homonuclear TACS with an effective coupling scaled by almost a factor 2 (Grzesiek and Bax, 1995) between the C^{β} and $\text{C}^{\text{aromatic}}$ protons, even if relatively high levels of rf power are used.

We present here an application of the G2 sequence to correlate C^{β} and $\text{C}^{\text{aromatic}}$ carbons. The C^{β} and $\text{C}^{\text{aromatic}}$ carbons form two narrow (≈ 2.5 kHz) bands of resonances (except for the C^{ϵ} and the C^{δ} resonances of the tyrosine) that are approximately 15 kHz apart on a 600 MHz spectrometer. For these residues it is desirable to transfer coherence from the C^{β} to the aromatic carbons and detect the aromatic protons. The transfer requires multiple steps: first a PLUSH TACS transfer between C^{β} and C^{γ} and then transfer within the aromatic ring systems (Fig. 7). The latter carbon resonances are within a relatively small chemical shift region and isotropic mixing can therefore

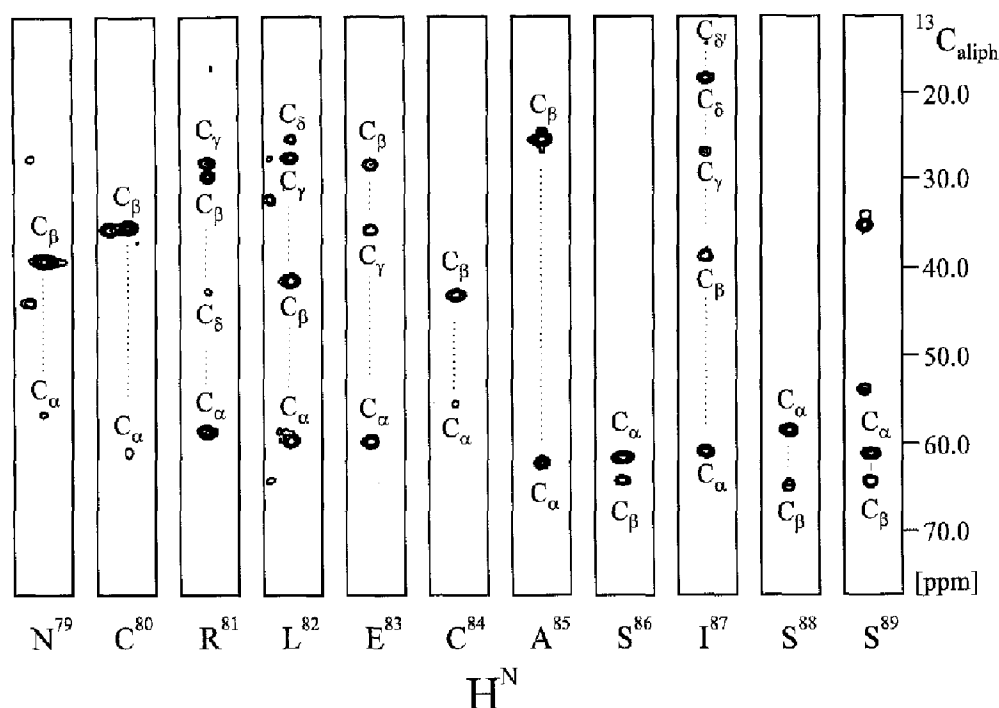


Fig. 6. Side-chain carbon resonances of amino acids Asn⁷⁹ to Ser⁸⁶ from a 3D (H)CCCO(N)H experiment on a 2.5-mM completely ¹³C,¹⁵N-labelled rhodniin sample. Complete transfer to the whole carbon spin system can be observed for all the residues. The data size was 1024(*t*₂) × 64(*t*₂) × 160(*t*₁) (real points), resulting in an acquisition time of 2.5 days; after Fourier transformation, linear prediction and zero-filling the resulting matrix size was 1024(*t*₂) × 128(*t*₂) × 256(*t*₁) (real points).

be applied to transfer coherence along the aromatic spin system. During the G2 sequence between C^β and C^{aromatic} isotropic mixing TOCSY transfer within the aromatic carbon spin system starts immediately when coherence reaches the C^γ carbon. For extended transfer within the tyrosine ring system a DIPSI-2 (Shaka et al., 1988) mixing period was added. The pulse sequence used is derived from the aromatic side chain experiments originally introduced by Yamazaki et al. (1993) which after a C^β evolution with refocussing of the heteronuclear ¹J(C^β,H^β) coupling and refocussing of the ¹J(C^α,C^β) coupling constant by the 1-1 pulse, transfer coherence to the C^γ carbons via an INEPT step, via a RELAY transfer to the C^δ

and even further in a second RELAY step to C^ε. In the proposed sequence (Fig. 8), the INEPT step from C^β to C^{aromatic} is substituted by a PLUSH TACS Y which also effects coherence transfer within the aromatic carbon spin system. The successive DIPSI-2 sequence enhances the latter transfer. Here the DIPSI-2 sequence of ν₁^{max} = 7.2 kHz acts as an isotropic TACS Y sequence since it only transfers coherence within the aromatic carbon spin system. Hence, the experiment is an example of a two-step homonuclear TACS Y experiment (Glaser and Drobny, 1991; Konrat et al., 1991; Kupče and Freeman, 1992, 1993; Majumdar and Zuiderweg, 1995).

The simulated coherence-transfer curves for the differ-

2D (HB)CB(CCaro)H

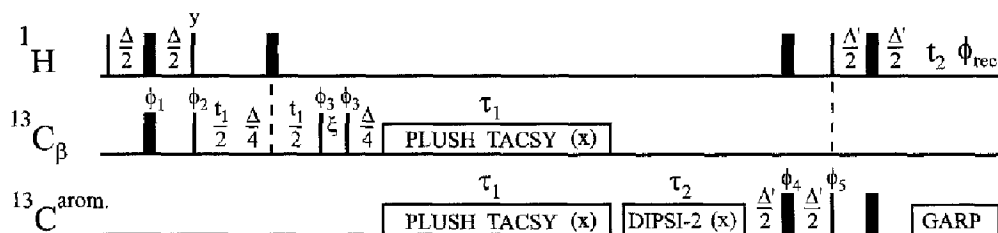


Fig. 7. Pulse sequence for (HB)CB(CCaro)H correlation using PLUSH TACS Y for C^β,C^{aromatic} transfer. The G2 sequence used for PLUSH TACS Y transfer was the same as described in Fig. 3 with a mixing time of 34 ms; further transfer within the aromatic ring was achieved via 11 ms of the DIPSI-2 mixing scheme with a γB/2π ratio of 7.2 kHz. Selective G3 (180°) and G4 (90°) carbon pulses were used with lengths of 333 μs and 400 μs, respectively. The carbon carrier was positioned at 36 ppm in the beginning of the sequence and switched to 136 ppm for the C^{aromatic},H INEPT transfer. φ₁ = 32(x),32(-x), φ₂ = 8(x),8(-x) and States-TPPI; φ₃ = (x,-x,y,-y); φ₄ = 16(x),16(-x); φ₅ = 4(x),4(-x); φ_{rec} = 2(x,-x),4(-x,x),2(x,-x); Δ = 3.8 ms; Δ' = 2.5 ms; τ₁ = 25.6 ms, τ₂ = 11.2 ms, ξ = 1/(2Δ_{opt}) - (4/π) τ₉₀ = 61.43 ms.

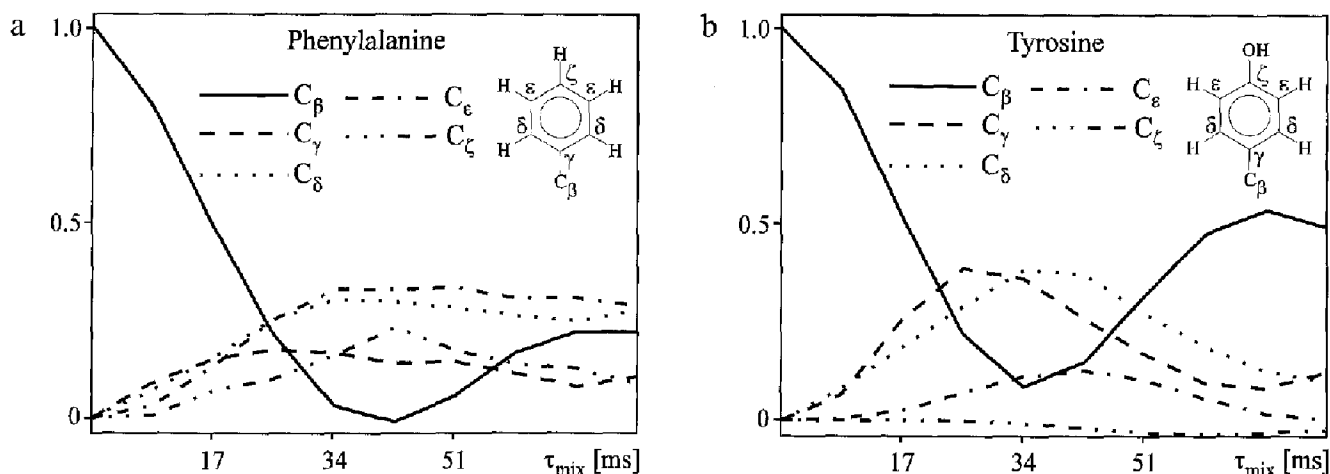


Fig. 8. Theoretical build-up curves for the reduced spin systems of (a) phenylalanine and (b) tyrosine consisting of the C^β and C^{aromatic} spins. The same pulse sequence was used as in Fig. 3. An optimal mixing time of four MLEV-16 cycles was found to give maximum transfer of C^β magnetization into the aromatic ring.

ent carbons in phenylalanine and tyrosine are depicted in Fig. 8. It can be appreciated that after 34 ms almost all the magnetization originating from the C^β atoms has been transferred to the aromatic carbons. While for the phenylalanine spin system all the aromatic carbons lie in a range of less than 1 kHz, the tyrosine aromatic carbons are spread over a range of 6 kHz. Therefore, transfer along the tyrosine ring is not complete. From simulations (not shown) we infer that after an additional isotropic mixing sequence for the aromatic carbons of 11 ms duration, the transfer within the aromatic carbon spin system is optimized. Simulations show that for histidine residues even the ideal PLUSH TACSU sequence does not show en-

hancement of sensitivity compared to the pulsed versions by Yamazaki et al. (1993). Therefore, histidine residues have been disregarded during the remainder of the paper.

In order to compare the results with the previously published sequences of Yamazaki et al. (1993) we recorded the INEPT-type correlation experiments in the version that reaches the C^δ carbon and in the version that transfers up to the C^ϵ carbons. The 2D $C^\beta, C^{\text{aromatic}}$ PLUSH TACSU spectrum of rhodniin is shown in Fig. 9. The cross-peaks are assigned and represent all the aromatic residues present in the protein. Traces are taken through the respective spectra (Fig. 10). The traces are scaled as if the two INEPT-type experiments had run as long as the

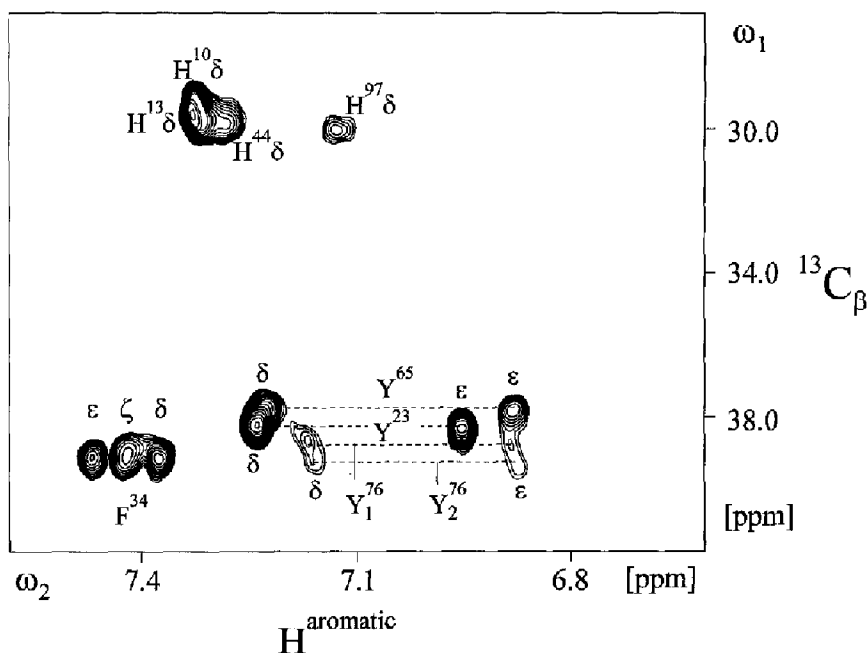


Fig. 9. 2D spectrum showing the $C^\beta, H^{\text{aromatic}}$ correlations of rhodniin. The spectrum was acquired using the same pulse sequence as shown in Fig. 8. $2048(1_t) \times 88(1_t)$ real points were recorded, resulting in a total measuring time of 12 h. After linear prediction, zero-filling and Fourier transformation, a matrix of 2048×256 points was obtained.

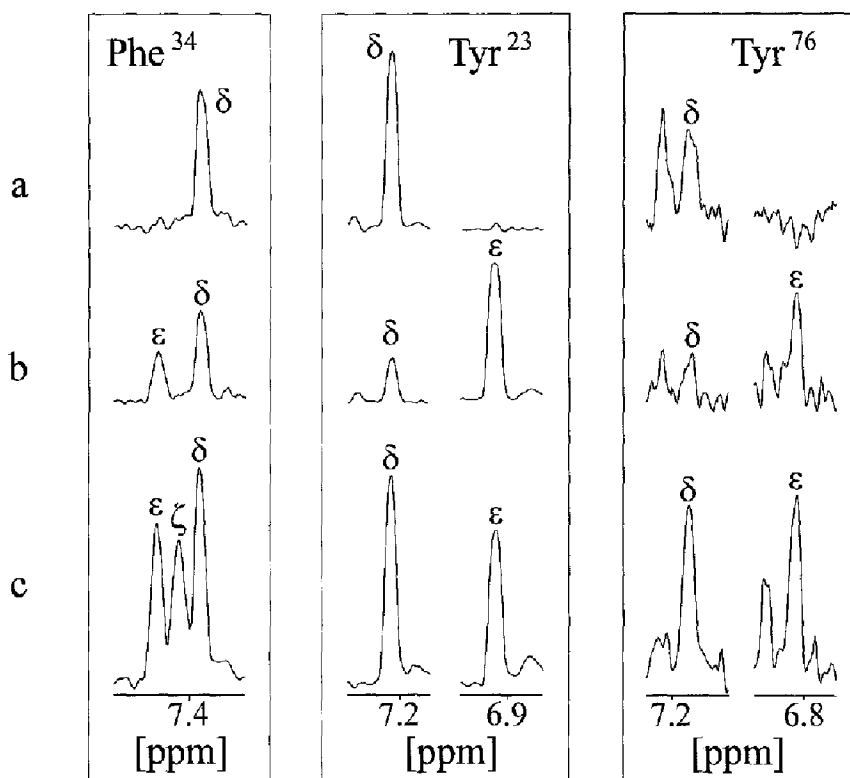


Fig. 10. In (a) and (b), the C^β, H^β and the C^β, H^ϵ correlations, obtained from the pulse sequences proposed by Yamazaki et al. (1993) using INEPT-type transfer steps from the C^β to the C^α and then further, in multiple steps, into the aromatic ring, are shown, respectively. The $C^\beta, H^{\text{aromatic}}$ correlations recorded using the pulse sequence of Fig. 6 are shown in (c). The data size of each experiment was $2048(t_2) \times 88(t_1)$ real points with 128 scans per increment, resulting in a measuring time of ≈ 12 h. Since each of the INEPT-type experiments gives ideally only one type of correlation (either C^β, C^δ or C^β, C^ϵ), the measuring time necessary to obtain the C^β, H^β and C^β, H^ϵ correlations via INEPT transfers is twice as long as for the experiment in Fig. 7, in which all $C^\beta, C^{\text{aromatic}}$ correlation are seen. For this reason, in order to have a fair comparison, traces in (c) were multiplied by a factor $2^{1/2}$. Enhancement in sensitivity is obtained for both phenylalanine and tyrosine. In addition, the H^ζ residue of phenylalanine can be observed.

TABLE 1
COMPARISON OF SENSITIVITIES OF PULSED $C^\beta, C^{\text{aromatic}}$
AND $C^\beta, C^{\text{aromatic}}$ PLUSH TACSU EXPERIMENTS

Residue	C^δ ^a	C^ϵ ^a	PLUSH TACSU ^b
Phe³⁴			
H^δ	59.2	—	73.7
H^ϵ	—	19.3	56.0
H^ζ	—	—	49.9
Tyr²³			
H^δ	167.1	—	192.2
H^ϵ	—	123.9	142.1
Tyr⁶⁵			
H^δ	78.4	—	90.5
H^ϵ	—	74.1	55.2
Tyr₁⁷⁶			
H^δ	24.8	—	25.5
H^ϵ	—	16.0	21.4
Tyr₂⁷⁶			
H^δ	11.1	—	19.1
H^ϵ	—	12.2	20.6

The histidine residues have been disregarded.

^a Experiments proposed by Yamazaki et al. (1993).

^b On the example of the aromatic side chains of rhodniin.

TACSU-type experiment. A comparison of the signal-to-noise values extracted for various residues is presented in Table 1. The results show that the PLUSH TACSU experiment has a considerably better signal-to-noise ratio than the pulsed versions. This is especially true for phenylalanine, probably due to strong coupling between the aromatic carbons which is detrimental for Relay-type transfer experiments as opposed to TOCSY or TACSU transfer (Grzesiek and Bax, 1995). The big improvement of signal to noise observed in the HBCB(CC_{ar})H experiment for the two Tyr⁷⁶ residues can be attributed to the effective enhancement of the lifetime of exchange-broadened resonances during the mixing time of TOCSY or TACSU (Krishnan and Rance, 1995), as opposed to Relay transfer. It should be noted that the lack of distinction between the C^δ , C^ϵ , and C^ζ resonances in the PLUSH TACSU compared to the pulsed experiments can be of disadvantage for phenylalanines. No problems arise for tyrosine or histidine residues.

Conclusions

We have shown that the C^α, C' correlations employed

in many of the three-dimensional experiments can be substituted with advantage by a PLUSH TACSYS mixing element which allows the implementation of a planar coupling mixing operator between two spectral bands. The signal to noise is better in this sequence than in the corresponding pulsed version, among other reasons due to the possibility to restrict the coherence transfer to the two-spin system consisting of the C^α and the C^β nucleus. The C^β is effectively decoupled from C^α. Less sensitivity to B₁ inhomogeneities and better offset behaviour also contribute to the enhanced performance of the PLUSH TACSYS sequence compared to the pulsed analogs.

We have extended the sequence also to the C^β, C^{aromatic} correlation by concatenating a PLUSH TACSYS with isotropic mixing (DIPSI-2). The sequence again shows improved signal to noise compared to the pulsed counterparts. However, the information about the assignment of the resonances (relayed as opposed to double-relayed) is lost in this experiment. We observe the PLUSH TACSYS version of the HBCB(CC^{aromatic})H experiment to be superior compared to the pulsed counterparts, probably due to the following three reasons: the TOCSY transfer within the aromatic carbon system (i) is twice as fast (Braunschweiler and Ernst, 1983); (ii) is less prone to strong coupling between the carbon resonances (Grzesiek and Bax, 1995); and (iii) induces longer T_{1ρ} of exchanging carbon resonances (Krishnan and Rance, 1995) than the pulsed counterpart.

Finally, the proposed sequence also constitutes a novel band-selective heteronuclear Hartmann-Hahn sequence (Gardner and Coleman, 1994).

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