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A Pure-Phase Homonuclear *J*-Modulated HMQC Experiment with Tilted Cross-Peak Patterns for an Accurate Determination of Homonuclear Coupling Constants

Wiktor Koźmiński

Department of Chemistry, Warsaw University, ul. Pasteura 1, 02-093 Warszawa, Poland

E-mail: kozmin@chem.uw.edu.pl

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A new HMQC-based experiment is presented which allows for an efficient determination of accurate homonuclear coupling-constant values. Pure absorption lineshapes with tilted cross-peak patterns are obtained by a combination of the active-coupling-pattern tilting (ACT) scheme with *J*-scaling. Characteristic features include separate heteronuclear echo and antiecho acquisition with a BIRD$_y$ pulse positioned before or after the t_1 period, respectively, to refocus *I*-spin homonuclear coupling evolution. Additionally, due to the incorporation of *J*-scaling the relative spacing of the *S*-spin chemical-shift differences and *I*-spin homonuclear coupling splittings in the F_1 domain is largely under experimental control. The most important advantage of the proposed method is that the *I*-spin homonuclear coupling evolution occurs simultaneously with the evolution of the heteronuclear zero and double-quantum coherences, which exhibit a slower transverse relaxation than *I*-spin single-quantum coherences. The effectiveness of the new sequence is demonstrated by a determination of the $^3J_{HN,H\alpha}$ couplings in a peptide sample. Additionally, the broadband property of the new sequence is verified with a sucrose sample.

Key Words: **HMQC; *J*-couplings; *J*-scaling; active-coupling-pattern tilting, ACT.**

In a recent Communication (*1*) the ACT-ct-COSY experiment (active-coupling-pattern tilting constant-time COSY), for the determination of homonuclear coupling constants, was proposed. There, tilted cross-peak patterns were obtained by proper co-addition of in- and antiphase signals in both dimensions of the 2D spectra, using techniques previously introduced for heteronuclear experiments (*2*). An important feature of homonuclear ACT is that it provides the advantage of tilted cross-peak patterns for two-spin systems which are unsuitable for E.COSY (*3*) and P.E.COSY (*4*) methods. Additionally, the ACT-ct-COSY experiment is one of the sequences that provide pure-phase homonuclear *J* spectra as was demonstrated with a determination of $^3J_{HN,H\alpha}$ coupling constants in a peptide at natural isotope abundance. It was pointed out that due to the versatility of the homonuclear ACT scheme it could be combined with a different chemical-shift labeling block, e.g., a HSQC experiment. However, the main drawback of such an approach would be an evolution of the homonuclear couplings on the same coherence level in both time domains which would limit the applicability to cases where the homogeneous linewidths are smaller than the coupling magnitudes. Moreover, the transverse relaxation of a single-quantum ^{1}H coherence, which would have to be selected for the coupling evolution, is usually fastest in the case of biomolecules.

In this Communication a new ACT scheme is presented and combined with *J* scaling to obtain pure-phase homonuclear *J*-modulated HMQC spectra without the disadvantages outlined above. Heteronuclear multiple-quantum coherences are selected in the evolution period of the experiment to achieve simultaneous *S*-spin chemical-shift labeling and homonuclear *I*-spin coupling evolution. Thus, the advantage of slower transverse relaxation rate of zero- and double-quantum heteronuclear coherence is taken (*5–10*). Additionally, since there is only one incremented period, the new sequence is shorter in time in comparison to the previous implementation (*1*). Figure 1 displays the pulse-sequence scheme for the new experiment. Two subspectra, a heteronuclear echo (Fig. 1a) and antiecho (Fig. 1b), are acquired for each t_1 increment. The most obvious difference in these experiments is the position of the BIRD$_y$ (*11, 12*) pulse, which acts as a 0 and $(\pi)_I$ pulse for *I* nuclei with and without a $^1J(S,I)$ coupling, respectively. Hence, it refocuses the evolution of couplings between an *I* spin bonded to a magnetically active *S* spin and other *I* spins. In analogy to the ACT-ct-COSY sequence (*1*) the sense of the homonuclear coupling evolution needs to be inverted in one experiment. However, here, this is not achieved by application of either a 0 or π selective pulse but by the BIRD$_y$ pulse placed either before or after the t_1 evolution period. The combination of the echo and antiecho data yields pure phase *J*-modulated spectra with tilted cross-peak patterns. The two additional $(\pi)_I$ pulses ensure a complete refocusing of the *I*-spin chemical-shift evolution over the entire experiment up to $t_2 = 0$. Signal modulation due to homonuclear coupling evolution during the constant delays is eliminated by the BIRD$_y$ pulse. To achieve *J*

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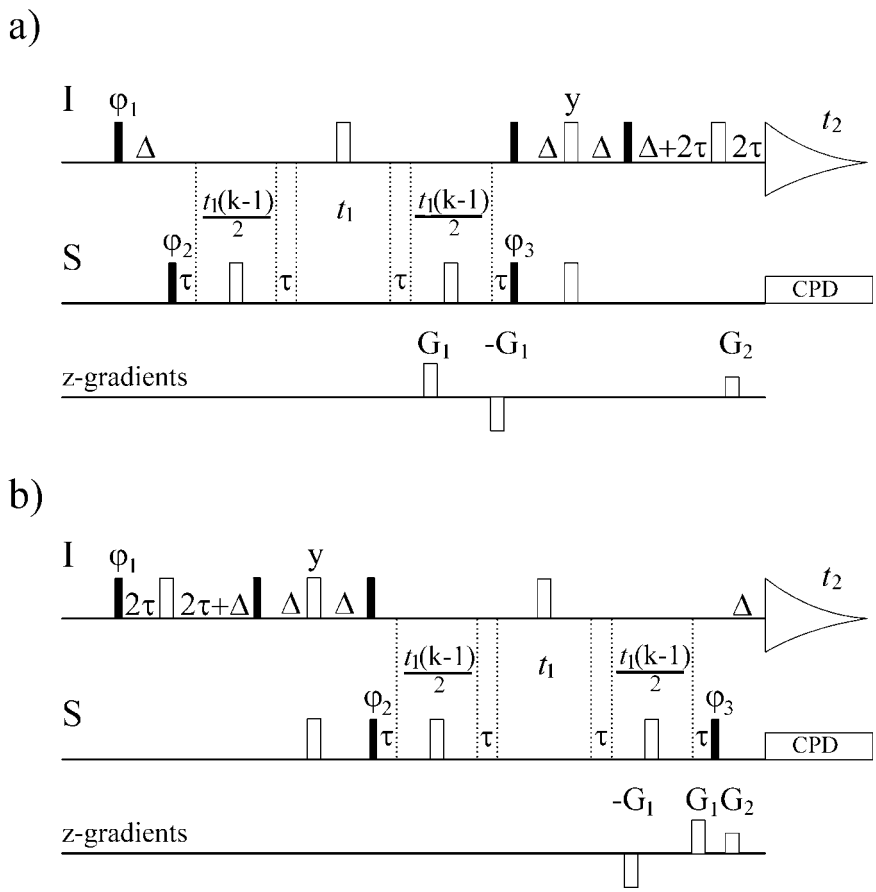

FIG. 1. Pulse sequence for the PPJ-HMQC experiment, heteronuclear echo (a) and antiecho (b). Dark-filled and open bars represent $\pi/2$ and π pulses, respectively. All pulses are applied along the x axis, unless indicated differently. The delay Δ should be tuned to $0.5/{}^1J_{IS}$. Delay τ includes the rectangular-shape gradient pulse and a 100 μs recovery delay. Gradients G_1 and G_2 had a duration of 2.5 and 1 ms, and an amplitude of 10 and 5 G/cm, respectively, in the ^{1}H–^{15}N experiments. For ^{1}H–^{13}C experiments G_1 and G_2 with equal length and amplitudes of 10 and 5 G/cm, respectively, were used. The echo and antiecho data sets per each t_1 increment were acquired separately and combined using standard VNMR software. The basic phase cycle of $\varphi_1 = 4x, 4y, 4-x, 4-y$; $\varphi_2 = x,-x$; $\varphi_3 = x,x,-x,-x$; and receiver $= (x,-x,-x,x) + \varphi_1$ was used. The scaling of I-spin homonuclear coupling constant and S-spin chemical shift evolution is achieved by selection of constant k.

scaling (*13*) a factor $k > 1$ must be selected. The two $(\pi)_S$ pulses refocus S-spin chemical-shift evolution during the gradient pulses and the additional homonuclear coupling evolution periods if $k > 1$. The different separation in time of defocusing and refocusing gradient pulses, G_1 and G_2, respectively, could result in unequal amplitudes of the echo and antiecho signals, which in turn would lead to quadrature ghosts in the F_1 dimension. However, in our experiments with water solutions and relatively weak gradients, up to 10 G/cm, such artifacts could not be observed since the diffusion coefficients are not large enough.

As an application example, the proposed sequence, pure-phase J-resolved HMQC (PPJ-HMQC) was used for the measurement of the vicinal $^3J_{HN,H\alpha}$ coupling constants in the [Me, Ala^7]-AVP-vasopressin analog at natural isotopic abundance. The sample was a 25 mM solution in 9/1 H_2O/D_2O. The established methods for determination of these important couplings are evaluation of extrema separations in absorptive and dispersive signals from phase-sensitive COSY spectra (*14*), fitting in- and antiphase signals acquired in different spectra (*15, 16*), quantitative 3D J correlations (*8, 9*), and J-modulated HSQC experiments (*6, 17*). In case of ^{15}N, ^{13}C isotopically enriched samples HNCA-E.COSY and for ^{15}N-labeled samples J_{HH}-TOCSY experiments (*18*) are valuable alternatives producing E.COSY-type cross-peak structure. Other approaches include evaluation of couplings from a comparison of DQ and ZQ spectra of ^{13}C- and ^{15}N-labeled molecules (*19*) and spin-state-selective excitation (S^3E) or spin-state-selective coherence transfer (S^3CT) methods for $^nJ_{HH}$ (*20–23*) determination. The latter require the acquisition of the two separate data sets displaying a single doublet component for the evaluation of the couplings and with the exception of Ref. (*23*) are also designed for isotopically labeled samples. Recently another technique, a reduced-dimensionality version of the 3D J-resolved HSQC experiment (*24*), was proposed for the measurement of $^3J_{HN,H\alpha}$ coupling constants (*25*). This method, J-multiplied HSQC (MJ-HSQC), is based on homonuclear J-resolved period followed by simultanously incremented sensitivity enhanced

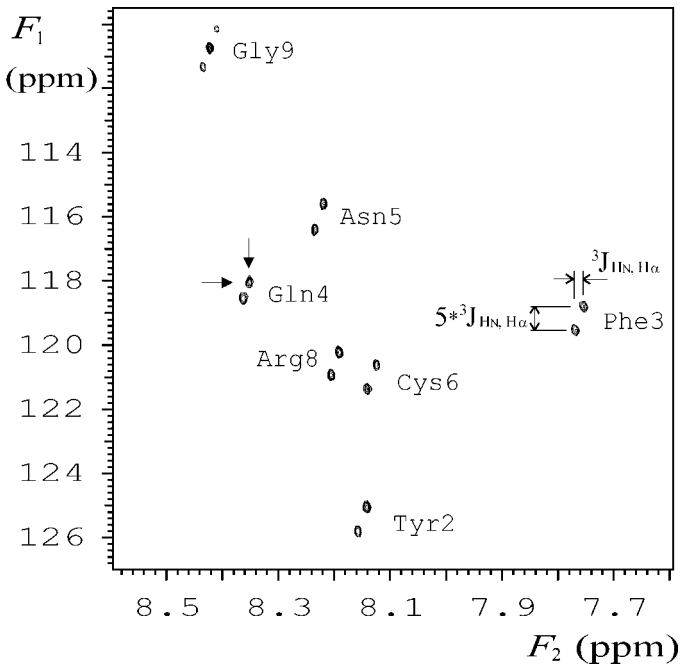

FIG. 2. Amide proton region from the PPJ-HMQC spectrum of the [Me, Ala^7]-AVP-vasopressin analog. Contour levels for the major isomer only are shown. The spectrum was acquired with a 25 mM 90% H_2O/D_2O solution. 16 scans were coherently added for each data set for 96 t_1 increments. The maximum t_1 and t_2 times were 64 and 280 ms, respectively. A k constant of 5 was chosen. A relaxation delay of 1.2 s was used. The delay Δ was tuned to a coupling of 90 Hz. The data matrix containing 96 $\times$ 1400 complex points in t_1 and t_2 respectively, was zero-filled to 512 $\times$ 16384 complex points; a cosine function was applied prior to Fourier transformation in both time domains.

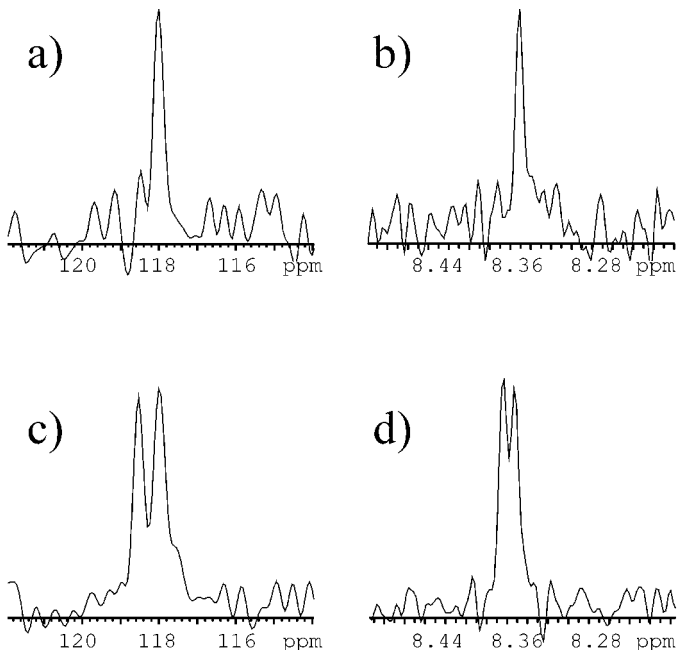

FIG. 3. Comparison of the cross section through the Gln4 correlation signal at the point marked by arrows in Fig. 2. Traces (a) and (b) are obtained from the F_1 and F_2 domain of PPJ-HMQC spectra from Fig. 2. Traces (c) and (d) are taken from a sensitivity enhanced MJ-HSQC spectrum, acquired accordingly to Ref. (*25*), under identical conditions (experiment time, scaling constant, and number of points in both time and frequency domains). The PPJ-HMQC spectra reveal good separation of both doublet components and similar signal to noise ratio.

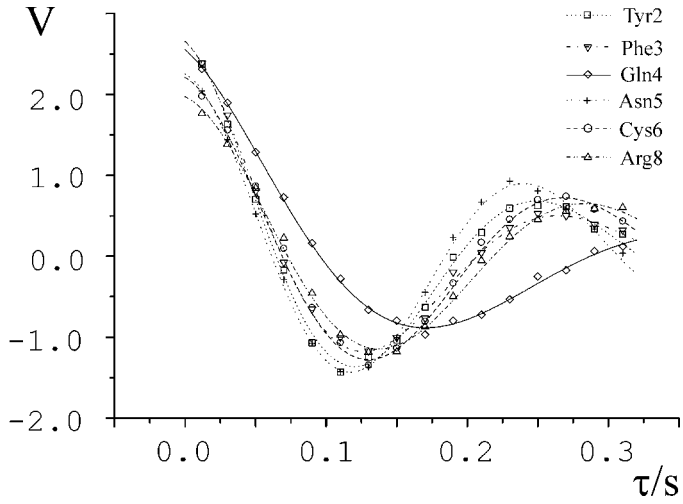

FIG. 4. Plot of measured cross-peak volume against dephasing delay t obtained by application of the J-modulated [^{15}N,^{1}H]-COSY method from Ref. (*6*). The curves are obtained by the nonlinear least-square fitting of the function $V(\tau) = V_0\cos(\pi J\tau)\exp(-\tau/T_2)$, to the experimental data. V_0, J, and T_2 are, respectively, peak volume for $\tau = 0$, actual coupling constants, and the effective transverse relaxation time. Extracted values of coupling constants are collected in Table 1. In comparison to the original pulse-sequence from Ref. (*6*), the first INEPT step was modified by the omission of spin-lock pulse, and application instead, of the 2 ms 10 G/cm gradient, placed between the $\pi/2$ I- and S-spin pulses, which rejects all transverse magnetization components and leaves unchanged the longitudinal two-spin order ($2I_zS_z$). 16 independent experiments with τ chosen from the range 12–310 ms were performed. 48 scans were coherently added for the two States-TPPI data sets for 64 t_1 increments. The maximum t_1 and t_2 times were 43 and 280 ms, respectively. A relaxation delay of 1.4 s was used. The experiment was tuned to a $^1J_{N,H}$ coupling of 90 Hz. The data matrix containing 64 $\times$ 1400 complex points in t_1 and t_2 respectively, was zero-filled to 256 $\times$ 2048 complex points; a cosine function was applied prior to Fourier transformation in both time domains. The cross-peak volume integration was achieved using the standard VNMR software.

HSQC. However, it does not separate the doublet components and is more prone to transverse relaxation losses due to the two evolution periods at the single-quantum ^{1}H and ^{15}N coherence levels. Additionally the effect of sensitivity enhancement is lost due to retention of only cosine modulated part of homonuclear coupling evolution.

Figure 2 displays a contour plot of a spectrum acquired with the new sequence (^{1}H–^{15}N) PPJ-HMQC spectrum, that reveals all vicinal $^3J_{HN,H\alpha}$ coupling constants of the major isomer of the [Me, Ala^7]-AVP-vasopressin analog; however, for the glycine residue (Gly9) only a sum of coupling constants with two H_α protons could be obtained in this case. The solution contains two isomers in an approximate 4:1 ratio. The coupling constants can be accurately measured in the F_2 as well as in the F_1

TABLE 1
Comparison of Coupling Constants $^3J_{HN,H\alpha}$ Obtained by the Different Methods

	Tyr2	Phe3	Gln4	Asn5	Cys6	Arg8	Gly9
J-modulated [^{15}N,^{1}H]-COSY (*6*)	7,75	7,26	5,23	8,14	7,26	6,87	—
PPJ-HMQC	7,7	7,3	5,2	8,2	7,3	7,0	12,1[a]
ACT-ct-COSY (*1*)	7.7[b]	7.3	5.3	8.1	7.4[b]	7.0	12.0[a]
MJ-HSQC (*25*)	7,6	7,1	5,0	8,0	7,1	6,8	11,9[a]

Note. The accuracy of coupling constant magnitudes obtained by ACT-ct-COSY, PPJ-HMQC, MJ-HSQC, and *J*-modulated [^{15}N,^{1}H]-COSY methods is estimated to 0.1, 0.15, 0.15, and 0.05 Hz, respectively.

[a] The sum of both possible $^3J_{HN,H\alpha}$ coupling constants only could be obtained due to its similar magnitude.

[b] The assignments of Tyr2 and Cys6 were reversed in Ref. (*1*).

dimension, independently of the linewidth. In contrast to ACT-ct-COSY (*1*) all signals are in-phase. Figure 3 shows a comparison on the same absolute intensity scale of cross sections through the Gln4 cross-peak at the points marked by arrows in Fig. 2. The spectra were obtained with the PPJ-HMQC and MJ-HSQC (*25*) methods, respectively, under identical conditions (number of scans, number of points in both time domains, and the scaling constant) and after identical post-processing. In contrast to the MJ-HSQC spectra the sequence proposed in this Communication produces clearly separated lines in both di-

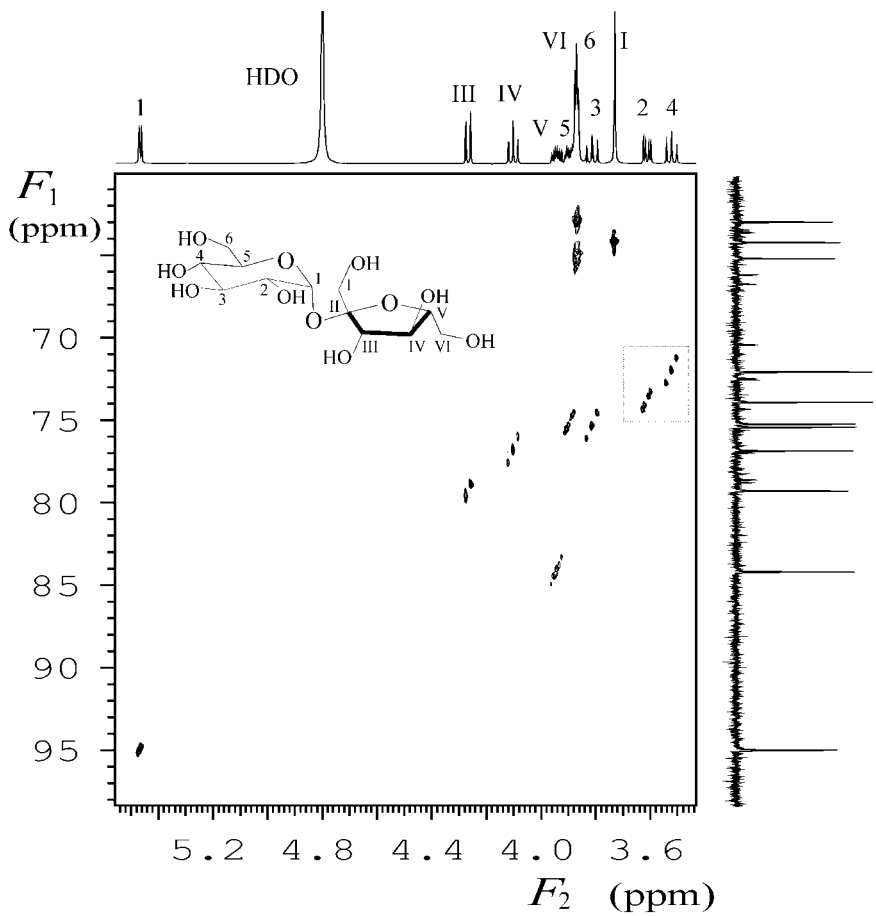

FIG. 5. PPJ-HMQC spectrum of 0.05 M sucrose D_2O solution. All of the CH correlation signals reveal pure-phase tilted cross-peak patterns. Eight scans were coherently added for each data set for 256 t_1 increments. The maximum t_1 and t_2 times were 35 and 300 ms, respectively. A relaxation delay of 1.5 s was used. The delay Δ was tuned to couplings of 150 Hz. The data matrix containing 256 $\times$ 512 complex points in t_1 and t_2, respectively, was zero-filled to 1024 $\times$ 2048 complex points; a cosine function multiplication was applied prior to Fourier transformation in both time domains. A scaling constant of 10 was chosen. The region marked by the dashed box is expanded in Fig. 6.

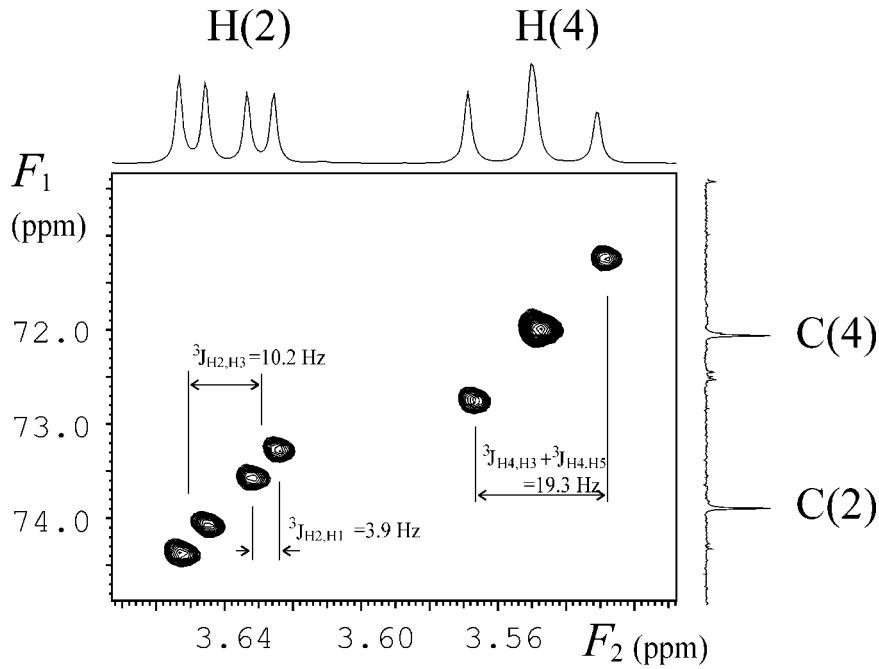

FIG. 6. Expansion of the H(2)/C(2) and H(4)/C(4) correlation peaks from the PPJ-HMQC 0.05 M sucrose spectrum.

mensions while retaining the same signal-to-noise ratio. In case of ^{15}N-labeled compounds the PFG coherence selection in the PPJ-HMQC sequence might be omitted resulting in a theoretical S/N gain of $\sqrt{2}$.

Additionally the $^3J_{HN,H\alpha}$ coupling constants were measured by the quantitative *J*-modulated [^{15}N,^{1}H]-COSY method described in Ref. (*6*). The measured cross-peak volumes and curves obtained by a nonlinear least-square fitting procedure are plotted in Fig. 4 as a function of dephasing delay τ. All coupling constant values obtained by different methods are collected for comparison in Table 1. The values of the $^3J_{HN,H\alpha}$ coupling constants observed by the proposed PPJ-HMQC method along both dimensions agree mutually with those measured previously by the ACT-ct-COSY experiment, as well as those obtained from *J*-modulated [^{15}N,^{1}H]-COSY (*6*) within the error limits. However, the MJ-HSQC method (*25*) produces systematically underestimated results due to no separation of the doublet components. The accuracy of the proposed PPJ-HMQC approach could be increased by improved digitization or by application of the lineshape fitting procedure. Due to the faster relaxation of antiphase terms, also known as scalar relaxation of the second kind (*26*), the couplings observed by the proposed method could tend to be systematically smaller than its actual value. However, this effect is not relevant in the present application, it must be considered in the case of larger proteins. In this case data extracted along the F_1 domain should be less prone to the systematic error due to superior relaxation behavior of the multiple-quantum coherences.

The broadband properties of the proposed PPJ-HMQC sequence are demonstrated in Fig. 5 displaying a ^{1}H–^{13}C PPJ-HMQC spectrum of a 0.05 M sucrose/D_2O solution. All CH correlation peaks exhibit the pure-phase and tilt characteristics which simplifies the evaluation of coupling constants. Again, for the measurement of, e.g., the vicinal $^3J_{HH}$ coupling constants between the carbohydrate –CH(OD) groups, the (P).E. COSY-type methods could not have been used, due to the requirement of at least three nuclei with resolvable mutual couplings. The expansion of the H(2)/C(2) and H(4)/C(4) correlation region is plotted in Fig. 6. In the case of H(2)/C(2) cross-peak all four doublet components are clearly separated and both coupling constants can be extracted; however, for the H(4)/C(4) correlation only the sum of two couplings is readable. Similar problems might occur for the complicated multiplet patterns of H(5) and H(V) protons coupled to exocyclic CH_2 groups; however, couplings of H(5)–H(6) and H(V)–H(VI) are obtainable from (P.)E.COSY-type experiments. The evolution of the coupling constant between geminal protons cannot be refocused by a BIRD pulse, and thus it is not possible to obtain pure-phase cross-peaks for CH_2 groups with nonequivalent protons by application of the proposed experiment.

All spectra presented were acquired at 300 K on a Varian Unity Plus 500 spectrometer, equipped with a Performa I z-PFG unit, and using standard 5 mm ID_PFG probehead. High power ^{1}H, ^{13}C, and ^{15}N $\pi/2$ pulses (8, 12 and 27 μs, respectively) were used. For the heteronuclear decoupling during data acquisition the GARP-1 (*27*) scheme was applied with $\gamma B_1/2\pi$ of 1.4 and 3.3 kHz for ^{15}N and ^{13}C experiments, respectively.

In conclusion, the new sequence presented allows for an accurate and relatively sensitive determination of homonuclear coupling constants. Tilted cross-peak patterns are obtained for

all spin systems except I_2S groups with chemically nonequivalent I nuclei; however, this tilt does not contain information about the relative signs of the coupling constants. The proposed method seems to be particularly useful for two spin systems such as H_N–H_α in peptides at natural isotope abundance. The method is also applicable to the S-spin enriched compounds with isolated I_1S–I_2 groups (e.g., $^1H^{15}N$–$^1H_\alpha$ in peptides). Although, in the case of uniformly ^{13}C-labeled peptides the application of ^{13}C decoupling in both time domains would be necessary. The method could also be applied to a variety of other organic compounds, particularly those with spin systems inadequate for (P.)E.COSY-type experiments, at the natural abundance level.

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