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Measurement of ^{2}J(H,C)- and ^{3}J(H,C)-coupling constants by α/β selective HC(C)H-TOCSY

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Abstract

A new heteronuclear NMR pulse sequence for the measurement of ^{n}J(C,H) coupling constants, the α/β selective HC(C)H-TOCSY, is described. It is shown that the S^{3}E element (Meissner et al., 1997a,b) can be used to obtain spin state selective coherence transfer in molecules, in which adjacent CH moieties are labeled with ^{13}C. Application of the α/β selective HC(C)H-TOCSY to a 10nt RNA tetraloop 5′-CGC**UUUU**GCG-3′, in which the four uridine residues are ^{13}C labeled in the sugar moiety, allowed measurement of two bond and three bond J(C,H) coupling constants, which provide additional restraints to characterize the sugar ring conformation of RNA in cases of conformational averaging.

Introduction

The measurement of ^{3}J(C,H) coupling constants provides valuable information for the local conformational analysis by NMR. In conjunction with ^{3}J(H,H) coupling constants, they can be utilized to derive stereospecific assignments, e.g. for the diastereotopic methyl groups of valines (Schwalbe et al., 1993; Karimi-Nejad et al., 1994)) and leucines (Sattler et al., 1992) in proteins. For oligonucleotides, the measurement of ^{n}J(C,H) coupling constants is of interest to determine the conformational dynamics of the five membered furanose ring (Haasnoot et al., 1980, 1981). The local conformation of the furanose ring affects the conformation of the phosphodiester backbone (Sänger, 1988; Eschenmoser and Dobler, 1992). The site-specific characterization of non-canonical conformations or conformational equilibria found in RNA loops and bulges (Cheong et al., 1990; Heus et al., 1991; Varani et al., 1991) can be inferred from NMR-spectroscopic techniques, in principle. The pseudo rotation phase and amplitude can be determined from analysis of two ^{3}J(H,H) coupling constants (Van de Ven et al., 1989) or two dipole,dipole cross-correlated relaxation rates (Felli et al., 1999). Recently, we have introduced a method relying on the E. COSY-principle (Griesinger et al., 1985, 1987) for the determination of ^{3}J(H,H) coupling constants (Schwalbe et al., 1995; Marino et al., 1996; Glaser et al., 1996) and a quantitative Γ-HCCH experiment (Felli et al., 1999; Richter et al., 1999) to measure dipole,dipole cross-correlated relaxation rates in the sugar moiety of RNA.

However, additional experimental parameters for the description of conformational averaging in oligonucleotides are of interest. A number of stud-

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ies have provided empirical and theoretical values for ^{2}J(C,H) and ^{3}J(C,H) coupling constants in mononucleosides (Podlasek et al., 1995, 1996; Bandyopadhyay et al., 1997) and small oligonucleotide model compounds (Wijmenga and van Buuren, 1998; Ippel et al., 1996). Experiments applied to oligonucleotides proposed so far have focused on the measurement of selected sets of ^{2}J(C,H) (Marino et al., 1996) and ^{3}J(C,H) coupling constants (Hines et al., 1993, 1994). Experiments that would allow the determination of a large number of ^{2}J(C,H) and ^{3}J(C,H) coupling constants in sizeable RNA in a single experiment have not yet been proposed.

In principle, ^{n}J(H,X) coupling constants can be determined using E. COSY-type experiments for molecules with a single X label as for example in ^{15}N labeled proteins, in peptides with ^{13}C in natural abundance or in oligonucleotides to measure ^{n}J(H,P) coupling constants. In these I_1-S-I_2 spin systems, an E. COSY-pattern will be observed whenever I_1 and I_2 are correlated without changing the spin state of the S spin. However, in I_1-S_1-S_2-I_2 spin systems, i.e. in spin systems with more than just a single S spin label, E. COSY-type determination of ^{n}J(I,S) coupling constants is difficult since cross peaks show splittings due to passive ^{1}J(I,S) and additional long range heteronuclear couplings. In addition, even in isotope labeled RNA, the decreased resolution renders interpretation of coupling constants particularly difficult due to the limited chemical shift resolution.

In this communication, we introduce an α/β selective HC(C)H-TOCSY experiment (Bax et al., 1990; Fesik et al., 1990; Clore et al., 1990; Pardi and Nikonowicz, 1992) for the determination of ^{2}J(C,H)- and ^{3}J(C,H)-coupling constants in fully ^{13}C labeled RNA. In this new experiment, the multiplet arising from ^{1}J(CH) and ^{n}J(CH) couplings is resolved into four subspectra without loss of sensitivity. This improves the resolution and increases the accuracy of the determination of small coupling constants compared to standard E. COSY-type techniques. Furthermore, using a CC-TOCSY transfer to achieve the required correlations is among the most sensitive coherence transfer modules to correlate between different carbons in the H-C-$(C)_n$-C-H moiety.

Results and discussion

The proposed α/β selective HC(C)H-TOCSY relies on the E. COSY-principle in conjunction with the S_3E element (Meissner et al., 1997a,b). In an H_i-C_i–C_{i+n}-H_{i+n} spin system, the heteronuclear long range coupling $^{n+1}$J(H_{i+n},C_i) is measured on the resonance of H_{i+n} by recording four spectra, which allow spin state selective correlation of the different spin states of C_i and C_{i+n} (αα, αβ, βα and ββ), respectively. The coupling constants can be determined from the relative shifts of the H_{i+n} resonances in the four spectra.

The pulse sequence of the 3D α/β selective HC(C)H-TOCSY is given in Figure 1, the applied phase cycle and gradients are shown in Table 1. After H,C polarization transfer, the S_3E element creates C_i coherence with H_i in either the α or the β state at **a**. This is accomplished by partial refocusing and adding or subtracting the result from the experiment with $\phi_3 = \phi_4$ and $\phi_3 = \phi_4 + \pi$ (Willker and Leibfritz, 1992; Griesinger et al., 1994; Meissner et al., 1997a,b). After chemical shift evolution in t_2 and C,C-TOCSY-transfer, which conserves the carbon coherence order (Sattler et al., 1995; Glaser and Quant, 1996), C^-_{i+n}-coherence is created with H_i still in the α or β state ($H^\alpha_i C^-_{i+n}$ and $H^\beta_i C^-_{i+n}$). The pulse sandwich between **b** and **c** accomplishes the conversion of $H_i^{\alpha/\beta} C^-_{i+n}$ to $2H^-_{i+n} C_i^{\alpha/\beta} C_{i+n,z}$, in which the spin-state of $H_i^{\alpha/\beta}$ is transferred to $C_i^{\alpha/\beta}$ in a spin-state selective manner (Schulte-Herbrüggen et al., 1991). Simultaneously, by a coherence order selective transfer (COS), conversion of C^-_{i+n} to $2H^-_{i+n} C_i^{\alpha/\beta} C_{i+n,z}$ is achieved. In order to avoid the doublet structure in ω_2 due to ^{1}J(H_{i+n},C_{i+n}) and since carbon decoupling during detection would remove the interesting $^{n+1}$J(H_{i+n},C_i) coupling from the spectrum, a second S_3E element is applied between **c** and **d**, which creates the submultiplet coherences $2H^-_{i+n} C_i^{\alpha/\beta} C^\alpha_{i+n}$ (recombined spectra 1 and 2 in Figure 2, see below) and $2H^-_{i+n} C_i^{\alpha/\beta} C^\beta_{i+n}$ (recombined spectra 3 and 4) from antiphase coherence $2H^-_{i+n} C_i^{\alpha/\beta} C_{i+n,z}$ in an manner analogous to the first S_3E element. A total of eight experiments have to be carried out for t_2-sign discrimination via gradient coherence selection. For the antiecho part, the phases ϕ_7 and ϕ_8 have to be shifted by π together with the conventional sign change in the gradient G_5 that selects carbon coherence. Spectral separation of the four multiplett components is accomplished by addition or subtraction of the four spectra in which ϕ_4 and ϕ_6 are phase cycled for spin state separation.

The post-acquisitional recombination procedure is summarized in Figure 2 and Table 2. One dimensional

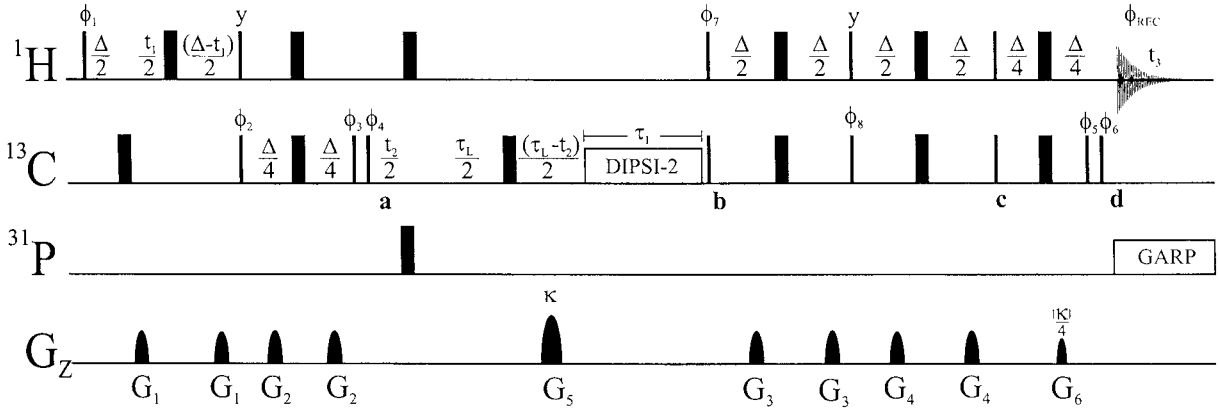

Figure 1. Pulse sequence for the 3D α/β selective HC(C)H-TOCSY experiment. Narrow and wide black bars denote 90° and 180° pulses, respectively. The default phase is x. t_1-sign discrimination is achieved by cycling ϕ_1 in a States-TTPI manner. For each t_2 value, echo- and antiecho coherences are recorded by changing the sign of κ on G_5 and $\phi_8 = \pm y$; $\phi_7 = \pm x$ (Kay et al., 1992; Schleucher et al., 1993, 1994). $\Delta = 3.2$ ms, $\tau_1 = 20$ ms, $\tau_I = 12.4$ ms. For practical reasons, these values deviate from the optimal ones for τ_1 and τ_I that have been obtained by simulations. DIPSI-2 (Shaka et al., 1988; Rucker and Shaka, 1988) with a spin lock field of 9.5 kHz was used for the C,C-TOCSY transfer. ^{31}P decoupling during acquisition is applied with a field strength of 1.7 kHz. The phase cycle is given in Table 1. Gradients (sine bell shaped) G_1-G_4 were applied for 300 μs 10 G/cm, G_5 = 300 μs, 40 = G/cm and G_6 = 300 μs, 10 G/cm. The gradient recovery delay was 300 μs for all gradients. Pulse sequences and C-program for the linear combination of the acquired data can be obtained upon request.

Table 1. Phase cycle [°] and sign of selected gradients for gradient coherence selection of the 2D-α/β-HC(C)H-TOCSY

	ϕ_2	ϕ_3	ϕ_4	ϕ_5	ϕ_6	ϕ_7	ϕ_8	G_5	
Exp. 1	45	0	0	0	0	0	90	+	Echo-spectra
Exp. 2	45	0	180	0	0	0	90	+	
Exp. 3	45	0	0	0	180	180	90	+	
Exp. 4	45	0	180	0	180	180	90	+	
Exp. 5	45	0	0	0	0	180	270	−	Anti-echo-spectra
Exp. 6	45	0	180	0	0	180	270	−	
Exp. 7	45	0	0	0	180	0	270	−	
Exp. 8	45	0	180	0	180	0	270	−	

Table 2. Post-acquisitional recombination procedure for the α/β selective HC(C)H-TOCSY. The resulting spectra are denoted I to IV (see Figure 2), the initial unprocessed eight experiments are termed 1 to 8, (re) and (im) denote the real and the imaginary part of each initial experiment, respectively

Recombined spectrum	Echo-spectrum (real part)	Echo-spectrum (imaginary part)	Antiecho-spectrum (Real Part)	Antiecho-spectrum (imaginary part)
I	−1(re)+2(re)+3(im)−4(im)	−1(im)+2(im)−3(re)+4(re)	−5(re)+6(re)+7(im)−8(im)	−5(im)+6(im)−7(re)+8(re)
II	1(im)−2(im)−3(re)+4(re)	−1(re)+2(re)−3(im)+4(im)	5(im)−6(im)−7(re)+8(re)	−5(re)+6(re)−7(im)+8(im)
III	1(im)+2(im)+3(re)+4(re)	−1(re)−2(re)+3(im)+4(im)	5(im)+6(im)+7(re)+8(re)	−5(re)−6(re)+7(im)+8(im)
IV	1(re)+2(re)+3(im)+4(im)	1(im)+2(im)−3(re)−4(re)	5(re)+6(re)+7(im)+8(im)	5(im)+6(im)−7(re)−8(re)

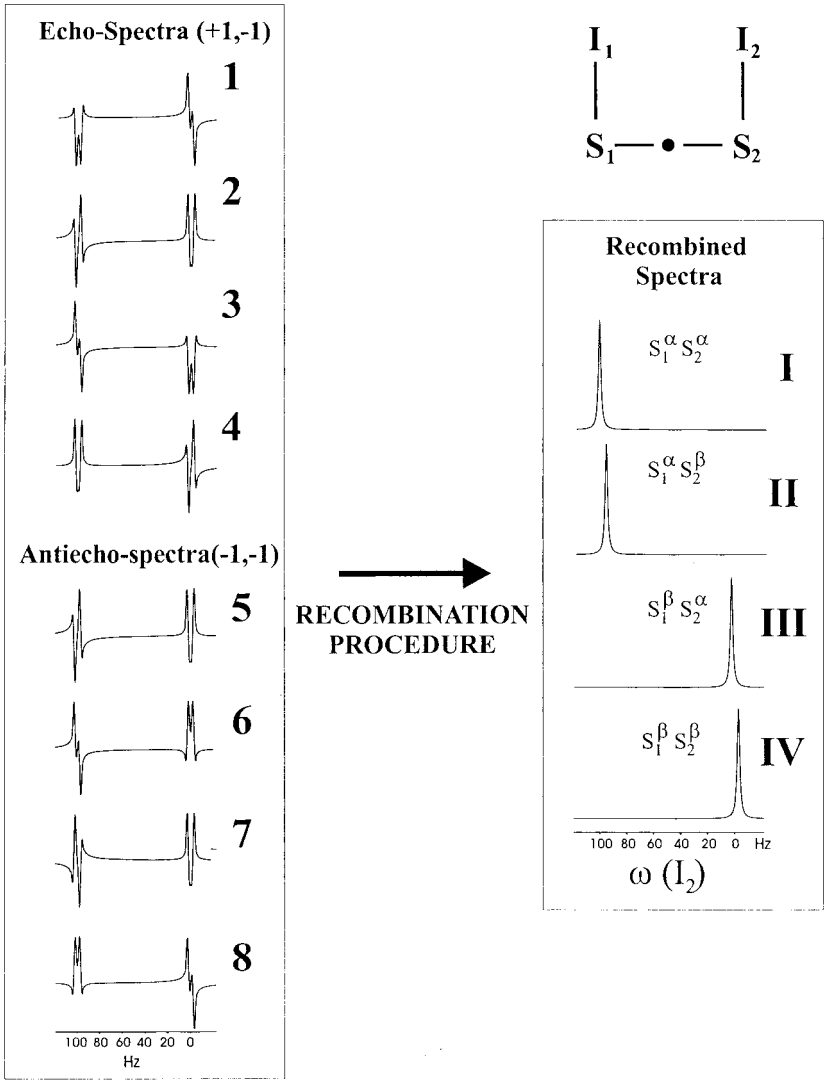

Figure 2. Wtest (Mádi and Ernst) simulations of the α/β-HC(C)H-TOCSY pulse sequence for an I-S-S-I spin system. Relaxation and H-H homonuclear coupling are not taken into account. The eight subspectra with the phase cycle given in Table 1 are simulated separately and combined as reported in the Table 2, yielding multiplet separated spectra in pure absorption.

spectra simulated in wtest (Z.L. Mádi and R.R. Ernst, personal communication) for an H-C-C-H-spin system are shown before and after the recombination. The recombined spectra contain only one of the four multiplet components. In the model compound diacetone glucose, cross peaks in C-C-H moieties cannot be phased to pure inphase (Figure 3). As simulations show, this antiphase dispersive lineshape originates from evolution of J(H,H) as well as J(C,C) couplings during the period between the marks **a** and **c** in the pulse sequence (see Figure 1). The dispersive lineshape, however, does not affect the relative displacement of the multiplet components in the different subspectra and ^{2}J(C,H) coupling constants can still be extracted. For spectra recorded on the 10nt RNA (see Figure 5), the dispersive lineshape is not apparent for C_iH_{i+1}-cross peaks due to increased overall linewidth. The lineshape in cross peaks originating from C-C-C-H correlations, in contrast, is unaffected by homonuclear long-range couplings and consequently purely absorptive as shown by simulations as well as experiments (Figure 5).

The CC-coherence transfer during the constant time period (τ_1) and the TOCSY transfer time (τ_I)

Table 3. Homo- and heteronuclear coupling constants [Hz] in the ribose moieties of the four labeled U residues in the 10nt RNA: ^{3}J(H,H) coupling constants have been extracted from HC-TOCSY-CCH-E. COSY (Schwalbe et al., 1995), $\Gamma^{DD,DD}_{CH,CH}$ have been derived as described earlier (Felli et al., 1999). ^{3}J(H,C) coupling constants have been determined from 3D-α/β selective HC(C)H-TOCSY. In addition, ^{n}J(H,C) coupling constants involving C1′ have been extracted from an 2D-C1′-selective, α/β selective HC(C)H-TOCSY. (a) Sum of ^{3}J and ^{4}J, (b) Average of values derived from 3D-α/β selective HC(C)H-TOCSY and 2D-C1′-selective, α/β selective HC(C)H-TOCSY, (c) Values from the 2D-C1′-selective, α/β selective HC(C)H-TOCSY. ^{2}J(H1′,C2′) coupling constants have been also determined using an E. COSY-type H1′,C1′-selective HSQC (values are given in brackets). Also given are coupling constants for pure N-type and S-type conformation. ^{3}J(H,H) have been derived using the generalized Karplus equation (Haasnot et al., 1981), heteronuclear coupling constants have been taken from Ippel et al. (1996). All spectra have been recorded at 600 MHz and 20 °C using the parameters given in the caption of Figure 1. In the 3D-α/β selective HC(C)H-TOCSY, eight scans per t_1 (40 complex points, spectral width: 1800Hz), t_2 (40 complex points, spectral width: 5882Hz) experiment were recorded with 512 complex points in t_3 (spectral width: 4000 Hz). A repetition delay of 1.1 s was used, total measurement time for all four experiments was 38 h at 600 MHz using a H,C,F,P probe with z-gradients

	U4	U5	U6	U7	N-type	S-type	
H1′	$\Gamma^{DD,DD}_{C1H1,C2H2}$	-1.8 ± 0.5	7.5 ± 0.5	5.8 ± 0.5	6.4 ± 0.3	–	–
	^{3}J(H1′,H2′)	2.6 ± 0.3	8.7 ± 0.1	6.8 ± 0.1	8.1	1.6	10.2
	^{2}J(H1′,C2′)	-1.7 ± 0.1	-3.1 ± 0.3	-2.6 ± 0.2	-3.1 ± 0.4	−2.5	−2.6
		(−1.9)	(−3.1)	(−2.7)	(−3.1)		
	^{3}J(H1′,C3′)	1.1 ± 0.1	0.4 ± 0.04	0.6 ± 0.06	0.7 ± 0.04	2.8	0.7
H2′	^{3}J(H2′,H3′)	5.2 ± 0.3	5.5 ± 0.1	5.4 ± 0.1	5.4 ± 0.3	4.7	5.1
	^{2}J(H2′,C1′)	0.9 ± 0.4[b]	-4.5 ± 0.7[b]	-1.8 ± 0.2	−4.0	1.2	−4.3
	^{2}J(H2′,C3′)	−0.4	n.d.	−1.8	n.d.	−1.5	2.9
H3′	$\Gamma^{DD,DD}_{C3H3,C4H4}$	13.9 ± 0.3	-2.0 ± 0.6	5.0 ± 0.3	-2.5 ± 0.1	–	–
	^{3}J(H3′,H4′)	8.9 ± 0.2	1.6 ± 0.2	4.7 ± 0.1	3.1 ± 0.2	8.5	1.5
	^{3}J(H3′,C1′)	1.1 ± 0.1[b]	4.9 ± 0.4[b]	3.6 ± 0.1[b]	4.6 ± 0.4[b]	1.7	6.5
	^{2}J(H3′,C2′)	−0.3	−2.6	−1.5	n.d.	2.3	−2.0
H4′	^{3}J(H4′,H5′^{proS})	2.6	2.7	degenerate	3.3	–	–
	^{3}J(H4′,H5′^{proR})	6.6	6.2	degenerate	2.7	–	–
	^{3}J(H4′,C1′)[a]	1.6 ± 0.4[b]	0.8 ± 0.3	-3.7 ± 0.1[c]	1.4 ± 0.06	n.d.	1.2
	^{3}J(H4′,C2′)	-0.3 ± 0.1	1.2	−0.7	−0.5	1.0	1.1
	^{2}J(H4′,C3′)	n.d.	−3.2	n.d.	−2.8	−5.5	−5.2

has been optimized by calculating the CC-coherence transfer amplitudes in dependence of the two delays using the program wtest (Z.L. Mádi and R.R. Ernst, personal communication) (Figure 4). During τ_1, carbon transversal coherence evolves under the longitudinal Hamiltonian H_L, whereas it evolves with the isotropic Hamiltonian H_I during the TOCSY transfer (Glaser and Quant, 1996). Overall coherence transfer is optimal at a τ_1 of 11–12 ms and a τ_I of 12–14 ms. The delays, however, have to be optimized according to the required resolution in τ_2 and to the relaxation properties of the individual sample. A τ_1 of 12 ms yields a spectral resolution of 90 Hz. While this proved to be sufficient for the 10 nt RNA with only 4 labeled nucleotides, the constant time period may have to be extended in order to increase the resolution for larger oligonucleotides. It should be noted that an alternative method for spinstate selection in t_1 has been proposed (Meissner et al., 2000).

During the constant time delay τ_L and the CC-TOCSY-transfer τ_I (see Figure 1, period between the marks **a** and **b**), the spin state of the passive proton H_i needs to be conserved in order to determine the heteronuclear coupling constants accurately. Relaxation of the proton during this time leads eventually to a mixture of the $H^-_{i+n}C^\alpha_i$ and $H^-_{i+n}C^\beta_i$ submultiplets. The percentage of spin flips for H1′ has been calculated with the assumption of an overall correlation time of 1 ns, 10 ns, and 20 ns, respectively. The surrounding protons are modeled as a pseudoatom at a distance of 2.2 Å (assuming a C3′-endo conformation in a regu-

Table 4. N-type and S-type populations (P_N and P_S) for the residues U6 and U7 in the 10nt RNA. Populations derived from the generalized Karplus equation of ^{3}J(H1′,H2′) and ^{3}J(H3′,H4′) and **R** = $\Gamma^{DD,DD}_{C1'H1',C2'H2'}/\Gamma^{DD,DD}_{C3'H3',C4'H4'}$ are given as a reference. $J^{U4,U5}$: Populations calculated with the respective coupling constant of U4 as pure S-type and U5 as pure N-type value. $J^{N,S}$: Populations calculated with pure S-type and N-type coupling constants from Ippel et al. (1996). *Populations could not be determined

	U6				U7			
	P_N (%)		P_S (%)		P_N (%)		P_S (%)	
^{3}J(H1′,H2′)	40		60		24		76	
^{3}J(H3′,H4′)	46		54		23		77	
$\Gamma^{DD,DD}_{C1'H1',C2'H2'}/\Gamma^{DD,DD}_{C3'H3',C4'H4'}$	35		65		19		81	
	$J^{U4,U5}$	$J^{N,S}$	$J^{U4,U5}$	$J^{N,S}$	$J^{U4,U5}$	$J^{N,S}$	$J^{U4,U5}$	$J^{N,S}$
^{2}J(H1′,C2′)	36	*	64	*	0	*	100	*
^{3}J(H1′,C3′)	29	0	71	100	29	0	71	100
^{2}J(H2′,C1′)	50	45	50	55	9	5	91	95
^{3}J(H3′,C1′)	33	53	67	47	10	36	90	64
^{2}J(H3′,C2′)	48	12	52	88	n.d.	0	n.d.	100
$^{3+4}$J(H4′,C1′)	*	n.d.	*	n.d.	*	n.d.	*	n.d.
^{3}J(H4′,C2′)	*	*	*	*	*	*	*	*

lar RNA duplex) (Schmidt, 1993). For a duration of 40 ms between **a** and **b** a percentage of spin flips of 2%, 15% and 30% has been found for the indicated correlation times. Furthermore, the effects due to H_i T_1-relaxation on the precision of coupling constants determination can be eliminated from the spectrum as described (Schwalbe et al., 1994). Adverse effects due to scalar relaxation of the second kind are expected to be small due to the long T_1 of ^{13}C for larger correlation times (Harbison, 1993).

The α/β selective HC(C)H-TOCSY has been applied to a 1.5 mM sample of a 10 nt RNA hairpin loop (5′-CGC**UUUU**GCG-3′) in which the four loop residues (in bold) have been uniformly ^{13}C labeled in the ribose moiety by chemical synthesis (Quant et al., 1994) and the oligonucleotides have been prepared by phosphoamidite chemistry. Figure 5 shows 1D-slices through selected cross peaks of the four HC(C)H-TOCSY subspectra. All ^{2}J(C,H) and ^{3}J(C,H) coupling constants determined with the α/β selective HC(C)H-TOCSY method are given in Table 3 together with ^{3}J(H,H) measured in an HCC-TOCSY-CCH-E. COSY and $\Gamma^{DD,DD}_{CH,CH}$ from a quantitative Γ-HCCH-experiment (Felli et al., 1999). The heteronuclear long-range coupling constants are in good agreement with those measured in a H1′,C1′ selective HSQC also carried out on the 10 nt RNA hairpin (Table 3, values for ^{2}J(H1′,C2′) given in parenthesis). Based on the generalized Karplus equation derived for ribose moieties (Haasnot et al., 1981), ^{3}J(H,H) coupling constants for the 10nt RNA (Richter et al., 1994) are in good agreement with a two state conformational average between the N-and the S-type sugar pucker mode. While U4 shows pure N-type puckering ($P_N = 97\%$), U5 adopts the S-type conformation ($P_S = 90\%$). U6 ($P_S = 57\%$), and U7 ($P_S = 76\%$) are more averaged. Cross-correlated relaxation rates $\Gamma^{DD,DD}_{CH,CH}$ (Felli et al., 1999; Richter et al., 1999) are in agreement with these results (U6: $P_S = 65\%$, U7: $P_S = 81\%$). The heteronuclear coupling constants observed for U4 in the N-conformation and for U5 in the S-conformation are in general in agreement both in sign and in relative size with the values derived by Ippel et al. (1996) (see Table 3). However, deviations in the absolute size of specific coupling constants are observed. In order to investigate the significance of these deviations, the N-type and S-type populations for the more averaged residues U6 and U7 have been calculated for all heteronuclear coupling constants using J^{U4} and J^{U5} as reference values (Table 4) for the N- and S-conformation, respectively. The agreement of the populations using the new values reported here with the ones derived from the analysis of ^{3}J(H,H) coupling constants and cross-correlated relaxation rates $\Gamma^{DD,DD}_{CH,CH}$ has been compared to populations calculated using the J^N and J^S values by Ippel et al. (Table 4).

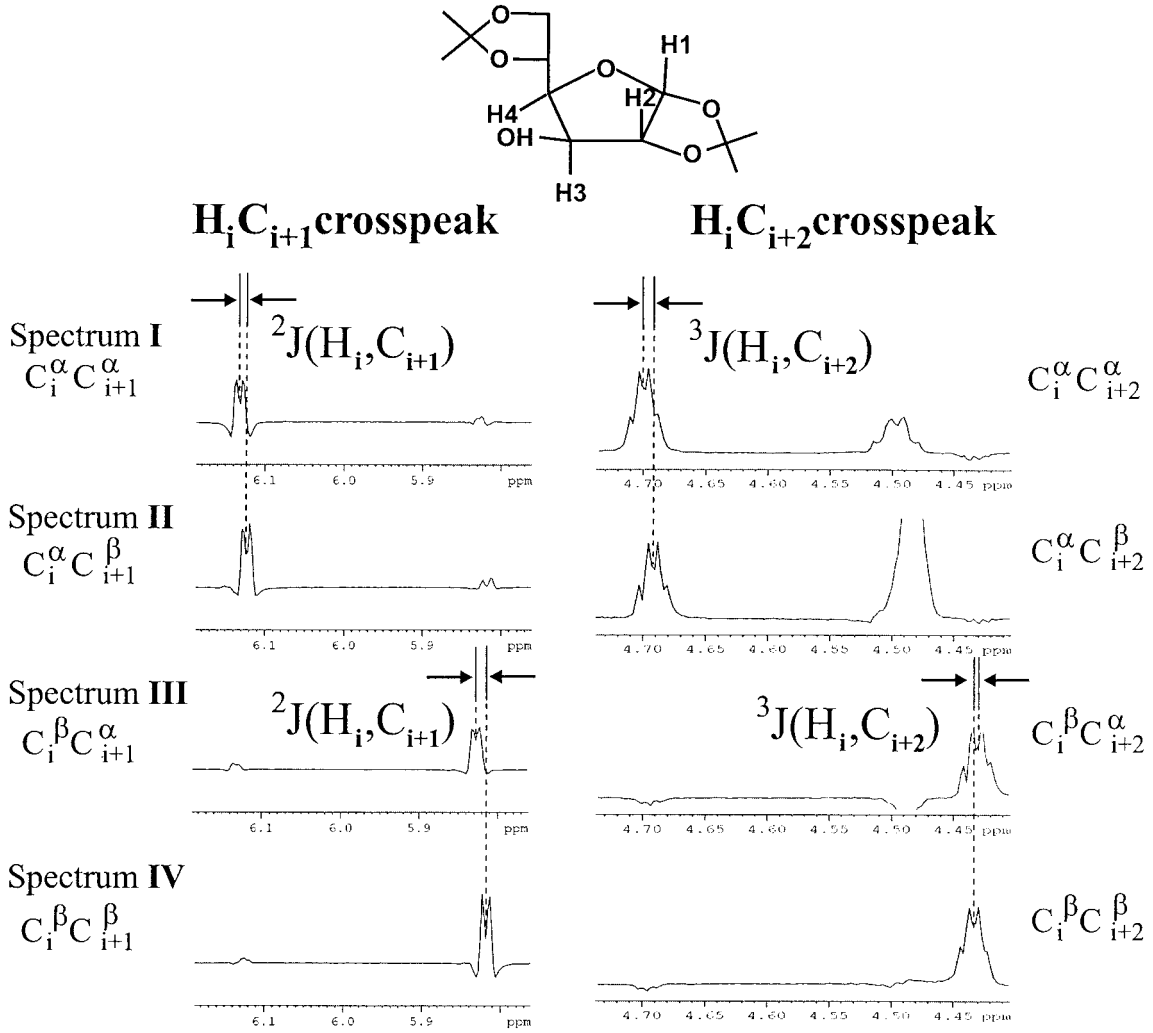

Figure 3. One-dimensional rows through selected cross peaks of the four subspectra of an α/β-selective HC(C)H-TOCSY of diacetone glucose. The relative displacements of the resonances due to CH-long range couplings are indicated. While the i,i+2-cross peaks are absorptive in lineshape, the i,i+1-cross peaks are dispersive as discussed in the text.

For $^2J(H1',C2')$,$^3J(H1',C3')$ and $^2J(H3',C2')$, the populations calculated for U6 and U7 from J^{U4} and J^{U5} are in better agreement with the populations derived from $^3J(H,H)$- and $\Gamma_{CH,CH}^{DD,DD}$ analysis than the values using J^N and J^S. In contrast, the fit is slightly improved for $^3J(H3',C1')$ and $^2J(H2',C1')$ when using J^N and J^S by Ippel et al. (1996). In this analysis, it has to be taken into account that the coupling constants from U5 do not represent full S-type puckering. Hence, the calculations carried out here tend to overestimate the content of the conformation. This and the error in the coupling constant determination could account for the larger deviation of the populations of U7, whose conformational average is closer to the one of U5 than the one of U6. $^{3+4}J(H4',C1')$ and $^3J(H4',C2')$ cannot be fit by either set of reference values. While for $^{3+4}J(H4',C1')$ this result can be due to the summation of two coupling constants, the reason for the deviation of $^2J(H2',C1')$ is unclear.

Conclusions

In conclusion, the new proposed experiment provides further experimental data to determine the averaged conformation of nucleotides. It allows experimental verification of $^nJ(C,H)$ coupling constant prediction derived from model compounds. For the 10nt RNA under investigation, an almost complete set of heteronu-

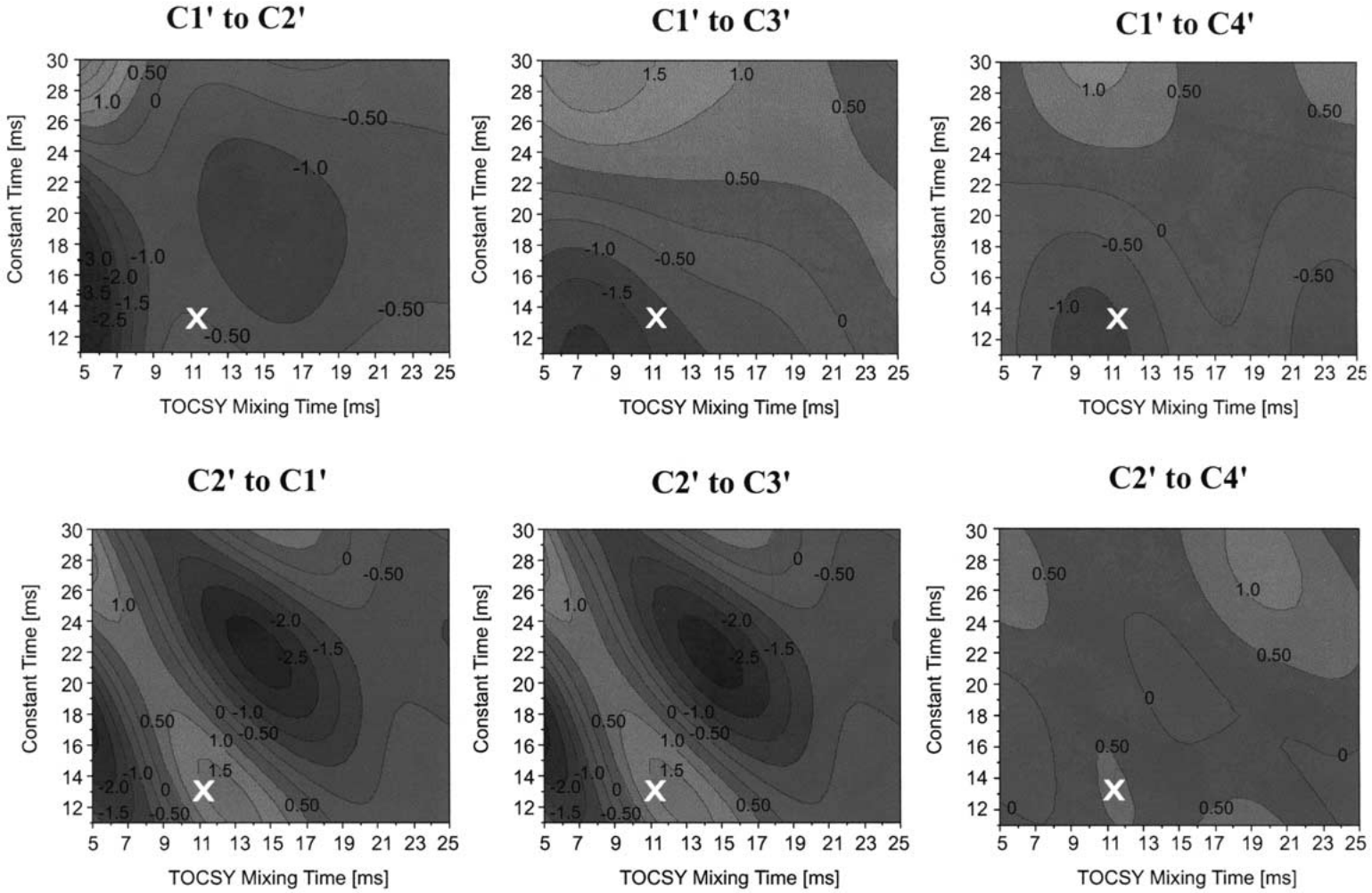

Figure 4. Wtest simulations of the CC-magnetization transfer during the constant-time (τ_1) and the CC-TOCSY transfer (τ_I) for the relevant one-bond to three-bond transfers. Relaxation is not taken into account. The overall optimal transfer times are indicated with a cross ($\tau_1 = 13$ ms, $\tau_I = 11$–12 ms).

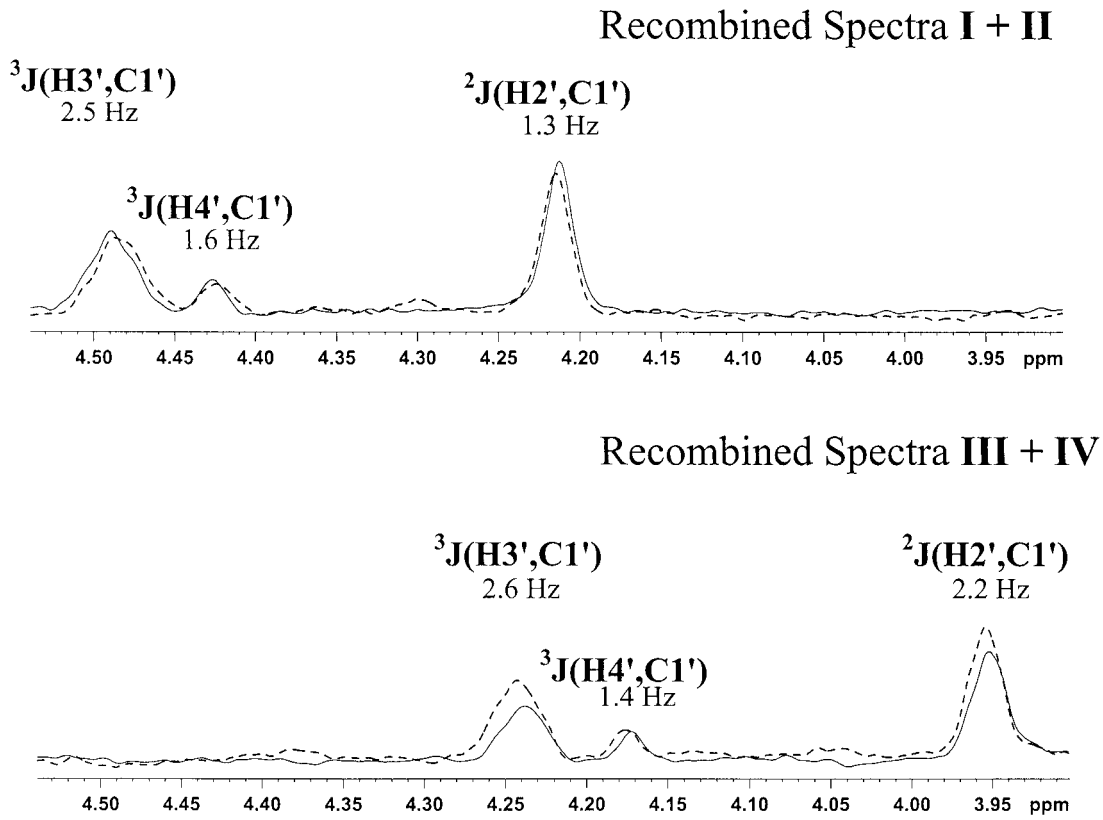

Figure 5. One dimensional rows through C1′H2′-, C1′H3′- and C1′H4′-crosspeaks of the four subspectra of an α/β-selective 2DHC(C)H-TOCSY of U4 of the 10 nt hairpin RNA. Spectra separated by the long-range H,C-couplings are overlaid and extracted coupling constants are given. The spectrum was recorded at 600MHz and 20 °C, using the parameters given in Figure 1. Total measurement time for all four spectra was 24 h with 256 scans per t_1-increment, 256 t_1-increments and a relaxation delay of 1.25 s.

clear coupling constants could be acquired in a single 3D-Experiment. The proposed novel experiment, the α/β selective H(C)CH-TOCSY has the same multiplet structure as a 3D HCCH-TOCSY. It therefore is applicable whenever the required correlation peaks are observed and resolved in a 3D HCCH-TOCSY.

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