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Determination of $^3J(C,P)$ and $^3J(H,P)$ coupling constants in nucleotide oligomers with FIDS-HSQC

H. Schwalbe[a], W. Samstag[a], J.W. Engels[a], W. Bermel[b] and C. Griesinger[a,*]

[a]*Institut für Organische Chemie, Johann Wolfgang Goethe Universität, Frankfurt/Main, Marie-Curie-Str. 11, D-60439 Frankfurt, Germany*

[b]*Bruker Analytische Meßtechnik GmbH, Silberstreifen, D-76287 Rheinstetten, Germany*

Dedicated to Prof. R.R. Ernst on the occasion of his 60th birthday.

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SUMMARY

A method for measuring J(C,P) and J(H,P) coupling constants is presented, based on fitting a target multiplet containing the heteronuclear coupling to a reference multiplet that lacks the heteronuclear coupling. In DNA and RNA oligonucleotides, information on backbone torsion angles can be obtained from these couplings. Experimental multiplets are obtained from ^{31}P-coupled and ^{31}P-decoupled $^1H,^{13}C$ HSQC spectra of Rp-cyclic methylphosphonate. The accuracy to which the heteronuclear coupling constants can be determined depends on the signal-to-noise ratio of the experimental data and is analyzed in detail.

The precise knowledge of the conformation of the phosphodiester backbone is a major concern for the structure determination of RNA and DNA. The dihedral angles α, β, γ, δ, ε and ζ determine the overall conformation between monomeric units (Saenger, 1988; Van de Ven et al., 1988; Majumdar and Hosur, 1992).

$^3J_{HH}$-couplings provide information about the backbone dihedral angles γ and δ, whereas the heteronuclear $^3J(H3'_{(n)},P_{(n+1)})$, $^3J(C2'_{(n)},P_{(n+1)})$ and $^3J(C4'_{(n)},P_{(n+1)})$ coupling constants are related with the dihedral angle ε. The dihedral angle β can be determined by measuring $^3J(C4'_{(n)},P_{(n)})$, $^3J(H5'_{(n)},P_{(n)})$ and $^3J(H5''_{(n)},P_{(n)})$ couplings. $^3J(C,P)$ coupling constants have previously been measured in 1D ^{31}P-coupled, ^1H-decoupled ^{13}C spectra (Davies, 1978; Jarowski et al., 1978; Niemczura and Hruska, 1980) and $^3J(H,P)$ couplings inter alia in 3D J-resolved spectra (Majumdar and Hosur, 1991). In spite of the importance of these coupling constants, a general method has not been proposed that does not depend on linewidth, that uses proton excitation and detection, and can therefore be applied on macromolecules.

*To whom correspondence should be addressed.

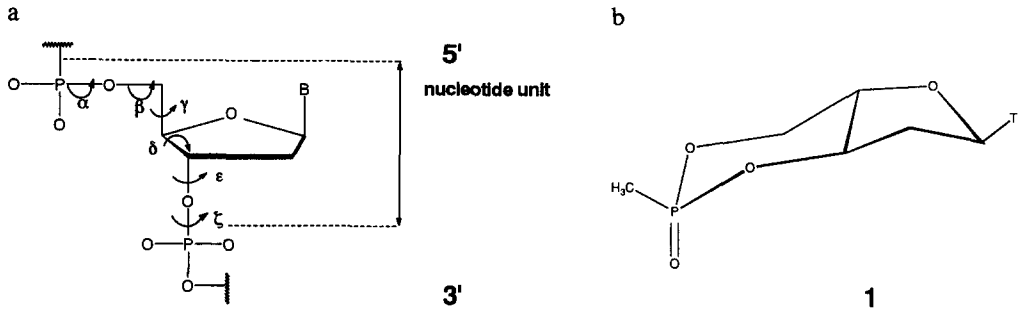
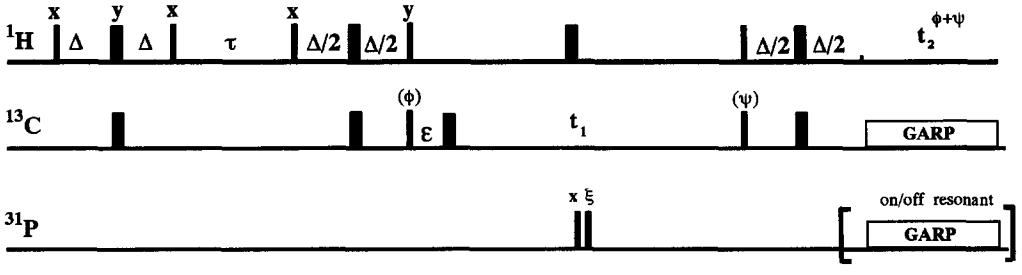


Fig. 1. (a) Definition of backbone torsion angles in oligonucleotides. (b) The experiments were carried out on a sample of Rp-cyclic methylphosphonate **1** (35 mg in 0.4 ml DMSO) (Lesnikowski, 1987).

In this communication we present a method for measuring heteronuclear J(C,P) and J(H,P) coupling constants and give an evaluation of the accuracy to which these coupling constants can be determined depending on the signal-to-noise ratio of the spectrum. The method proposed is related to the Keeler-Neuhaus-Titman approach (Keeler et al., 1988; Titman et al., 1989; Richardson et al., 1990) for the determination of $^3J(X,H)$ coupling constants from ω_2-coupled HMBC-spectra, in which a target multiplet with the desired heteronuclear coupling in antiphase is simulated from a reference multiplet lacking the heteronuclear coupling. The reference spec-

$^1H,^{13}C$-FIDS-HSQC with and without ^{31}P decoupling in t_1 and t_2



Experiment 1: $\xi = +x$; **on resonance decoupling**
Experiment 2: $\xi = -x$; **off resonance decoupling**
Experiment 3: $\xi = -x$; **on resonance decoupling**
Experiment 4: $\xi = +x$; **off resonance decoupling**

Fig. 2. Pulse sequences for the triple resonance [^1H, ^{13}C]-(^{31}P)-coupled/decoupled-BIRD-HSQC-experiment. Four scans per t_1 (1024 experiments, t_1^{max} = 256 ms) were recorded with 2048 points in t_2 (4400 Hz spectral width). The spectrum was Fourier-transformed using squared cosine weighting functions in both dimensions to 1024*2048 real points. The experiments were carried out on an AMX 400 equipped with a triple $^1H,^{13}C,^{31}P$ resonance probe. BIRD presaturation (Bax, 1986) of ^{12}C-bound protons was carried out with a recycle delay of 0.8 s and a recovery delay τ of 0.435 s. Δ = 0.0034 s, $\varepsilon = (\tau_p\,(180°\ ^1H) + \tau_p\,(180°\ ^{31}P) + 2*t_1(0))$. Off-resonance decoupling 30 kHz from the phosphorus resonance was applied for experiments without ^{31}P decoupling to achieve identical sample heating in all experiments. ϕ = x,−x; ψ = x, x,−x,−x; receiver as indicated. TPPI (Marion et al., 1983) was used for frequency sign discrimination in ω_1.

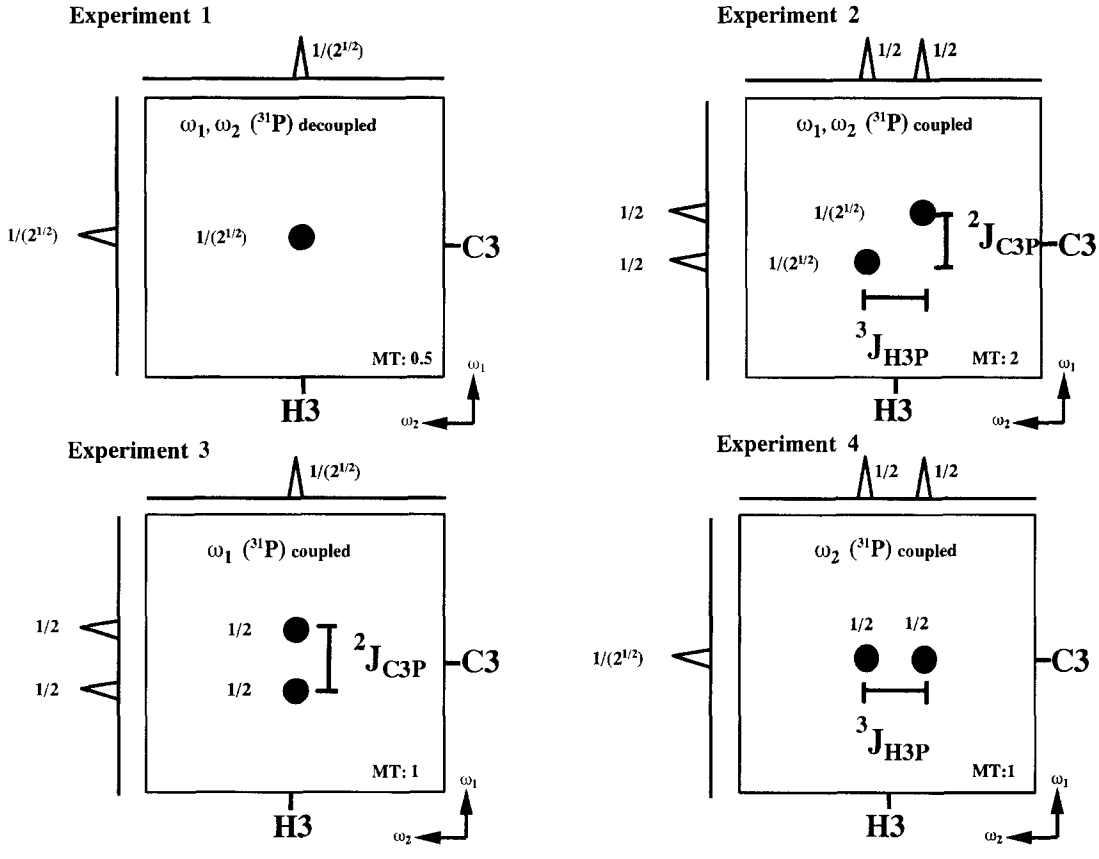


Fig. 3. Schematic multiplet pattern in Experiments 1–4; the signal-to-noise ratio of the experiments after units measurement time (MT) as indicated in the text are given at the summed projections in each experiment.

trum has an intensity that is in an unknown ratio to the HMBC spectrum and that must therefore be fitted together with the heteronuclear coupling. In contrast to this method the relative intensities of spectra recorded with and without ^{31}P-decoupling are known and need not be fitted, since ^{31}P has 100% natural abundance. Only the heteronuclear coupling has to be fitted.

The coupled and decoupled multiplets can be obtained from ^{1}H,^{13}C HSQC-experiments (Fig. 2). In principle, there are two possibilities to record ^{31}P-coupled and ^{31}P-decoupled ^{1}H,^{13}C HSQC-spectra as indicated in the schematic multiplet patterns in Fig. 3 and the experimental pattern in Fig. 4.

One can compare a fully ^{31}P-decoupled spectrum (Experiment 1 in Figs. 2 and 3) with a fully ^{31}P-coupled spectrum (Experiment 2). The fully coupled spectrum gives rise to an E.COSY-type pattern, because ^{31}P is left untouched during the experiment. If the associated coupling is large enough to resolve the desired coupling, it can be measured directly (Schmieder et al., 1992). The associated couplings for the structurally interesting coupling constants ^{3}J(H,P) and ^{3}J(C,P) would be ^{2}J(C,P) and ^{4}J(H,P), respectively. Both these associated couplings have been reported to be not larger than 10 Hz (Van de Ven et al., 1988).

The other possibility is to record two spectra with an alternating decoupling scheme: a ^{1}H,^{13}C HSQC-experiment with ^{31}P-decoupling in ω_1 only (Experiment 3) or in ω_2 only (Experiment 4). In

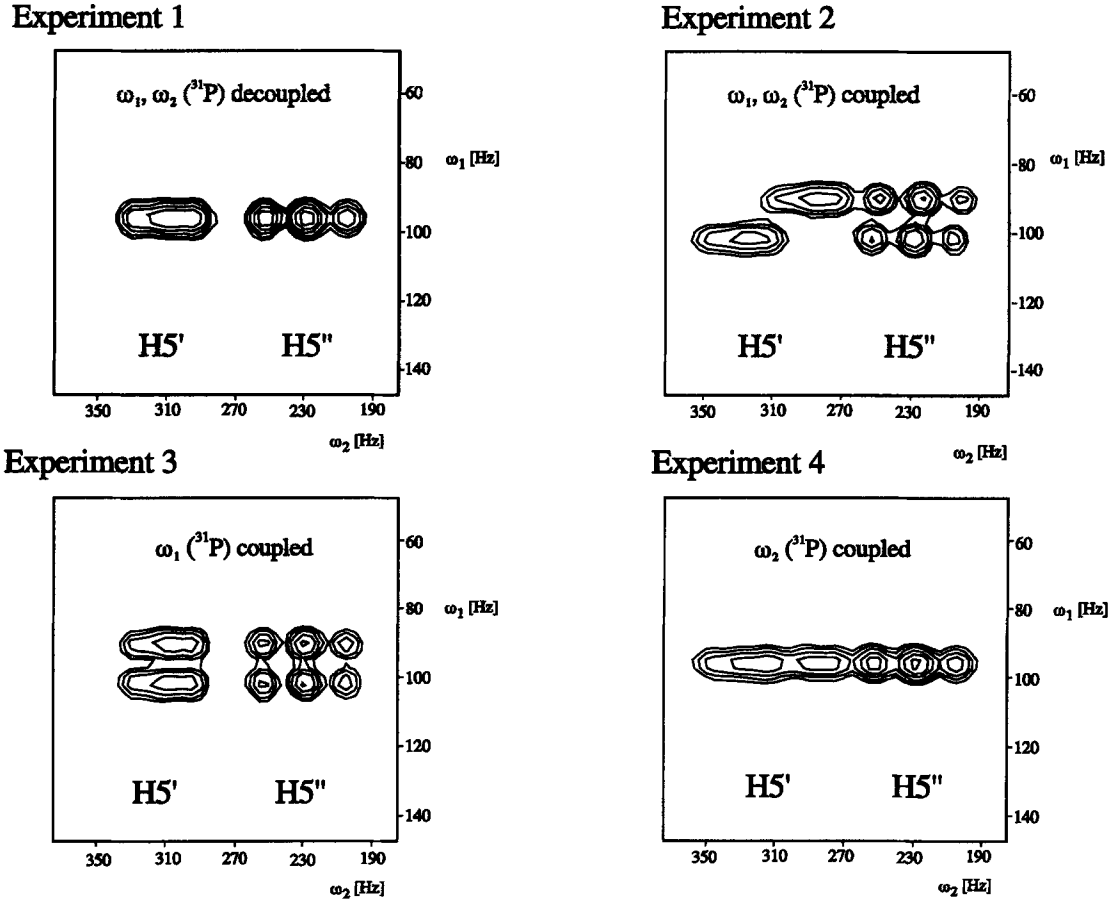


Fig. 4. Experimental multiplet patterns for H5',H5"-C5' cross peaks in Experiments 1–4.

Experiment 3 evolution of the heteronuclear $^{13}C,^{31}P$-coupling during t_1 leads to a multiplet with an in-phase splitting in ω_1. In Experiment 4 the in-phase splitting due to $^3J(H,P)$ is observed in ω_2. Experiment 3 now may serve as the reference spectrum for Experiment 4 and vice versa. For example, the summation over the doublet along ω_1 in Experiment 3 causes the CP-coupling to collapse. The singlet both in ω_1 and ω_2 can serve as the reference singlet to fit the HP-coupling in Experiment 4. Summation over the multiplet in ω_2 in Experiment 4 yields a singlet to fit the CP-coupling in Experiment 3. We call this procedure FIDS-HSQC (**F**itting of coupling constants from **d**oublets and **s**inglets).

If the signal-to-noise ratio in the fully decoupled spectrum from Experiment 1 is calibrated to be $1/\sqrt{2}$ in half a unit measurement time (MT) (see Fig. 3), then in each of the partly decoupled experiments (3 and 4) recorded with one unit MT the signal-to-noise ratio is $1/\sqrt{2}$ after summation over the doublet (ω_1-summation in Experiment 3 and ω_2-summation in Experiment 4) and 1/2 after summation over the singlet (ω_2-summation in Experiment 3 and ω_1-summation in Experiment 4). In the fully ^{31}P-coupled Experiment 2 the signal-to-noise ratio is $1/\sqrt{2}$ in two units MT and 1/2 after summation along either frequency axis, since the peaks are displaced in both dimensions.

Comparing the two experimental approaches, one finds that the total MT for Experiments 1 and 2 is 25% larger than for Experiments 3 and 4. At the same time the signal-to-noise ratio of the summed doublets is 1/2 and of the singlets $1/\sqrt{2}$ in both approaches. Therefore the approach with Experiments 3 and 4 is advantageous from signal-to-noise arguments.

In contrast to the measurement of coupling constants of low-abundance heteronuclei by the Keeler-Neuhaus-Titman-approach, the amplitude factor in the FIDS-HSQC experiment between coupled and decoupled signal does not have to be fitted. Since only the trial coupling constant J^t has to be fitted, the precision of the coupling constant is expected to be high even for linewidths considerably larger than the desired coupling constant. The accuracy of the coupling constant depends on the signal-to-noise ratio of the spectrum. Assuming Lorentzian lineshapes $L(\omega)$ with linewidth T_2^*, the coupled spectrum with the desired coupling constant J_o has the form: $S^{coup}(\omega) = L(\omega + \pi J_o) + L(\omega - \pi J_o)$. For the fitting we use the following procedure. Two copies of the decoupled spectrum shifted with respect to each other by $2\pi J^t$ are subtracted from the coupled spectrum. The penalty function $P(J^t)$ is the integral of the power difference spectrum as a function of J^t.

$$P(J^t) = \int_{-\Delta\Omega/2}^{+\Delta\Omega/2} [L(\omega + \pi J^t) + L(\omega - \pi J^t) - L(\omega + \pi J_o) - L(\omega - \pi J_o)]^2 \, d\omega = \tag{1}$$

$$\pi/T_2^* \, [2 + 1/(1 + (\pi J^t T_2^*)^2) + 1/(1 + (\pi J_o T_2^*)^2) - 2/(1 + (\pi(J^t + J_o)T_2^*)^2) - 2/(1 + (\pi(J^t - J_o)T_2^*)^2)]$$

In order to estimate the error for the coupling constant J_o we write the signal-to-noise ratio in the ^{31}P-decoupled trace after summation over the doublet as:

$$S/N = 1/\sigma_{dec} \tag{2}$$

Assuming identical measurement times for Experiments 3 and 4, the noise in the trace showing the doublet after summation over the singlet in the orthogonal dimension is:

TABLE 1
MEASURED COUPLING CONSTANTS IN CYCLIC METHYLPHOSPHONATE. FOR EACH MULTIPLET, THE SIGNAL-TO-NOISE RATIO AND THE NOISE OF THE INTEGRAL ARE GIVEN

	J [Hz]	S/N	σ_I	Remarks
^{3}J(C2′,P)	7.1 ± 0.2	32 : 1	0.24	Det. in H2′
^{3}J(C2′,P)	7.4 ± 0.2	32 : 1	0.24	Det. in H2″
^{2}J(C3′,P)	3.5 ± 0.2	46 : 1	0.18	
^{3}J(C4′,P)	8.1 ± 0.2	40 : 1	0.20	
^{2}J(C5′,P)	8.9 ± 0.2	43 : 1	0.18	Det. in H5′
^{2}J(C5′,P)	8.9 ± 0.2	43 : 1	0.18	Det. in H5″
^{5}J(H2′,P)	0.4 ± 0.4	13 : 1	0.60	
^{3}J(H3′,P)	1.55 ± 0.3	18 : 1	0.44	
^{3}J(H4′,P)	2.8 ± 0.3	20 : 1	0.40	
^{3}J(H5′$^{pro\text{-}R}$,P)	17.5 ± 0.3	18 : 1	0.44	
^{3}J(H5″$^{pro\text{-}S}$,P)	3.35 ± 0.3	18 : 1	0.44	

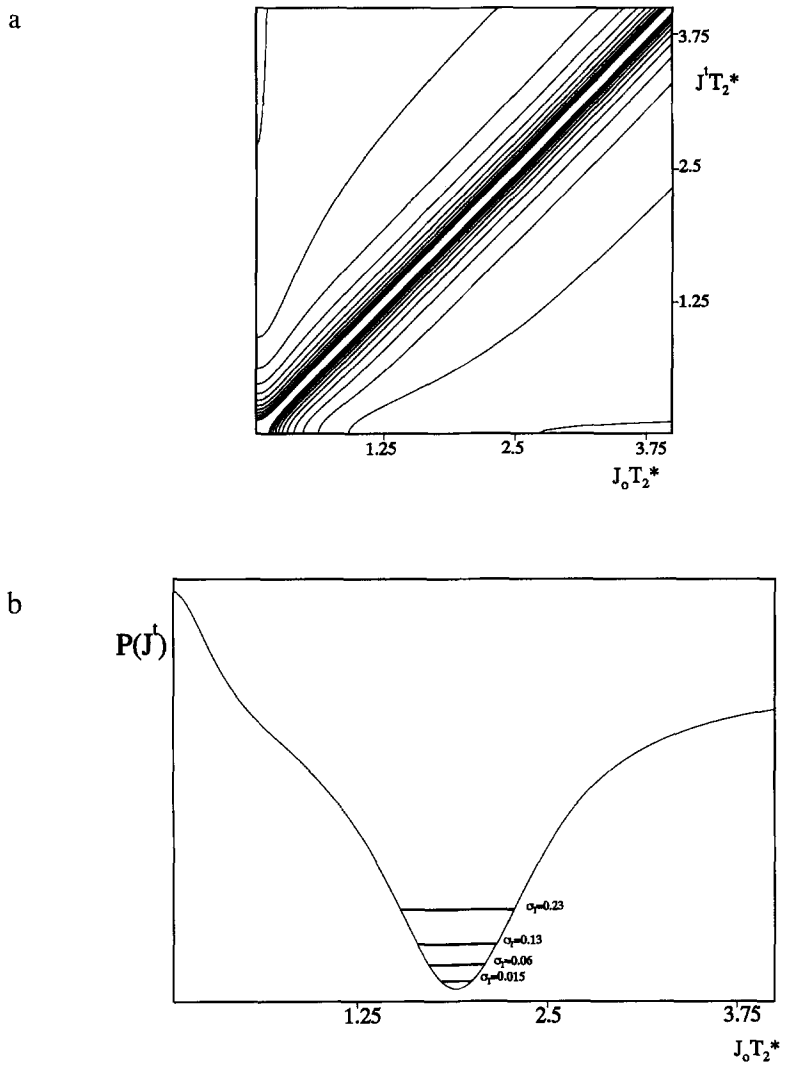


Fig. 5. (a) Simulation of penalty function P versus the product of coupling constants times the linewidth $J'T_2^*$ and $J_oT_2^*$ as explained in the text. (b) Trace along the horizontal axis through Fig. 5a at $J'T_2^* = 1.875$. The expected error for the coupling constant depends on σ_I. The accuracy of the determination of the coupling is widely independent of $J_o\tau T_2^*$ since the error function is broadened only for $J_oT_2^* < 0.15$. There is no systematic error in the determination of the coupling since the minimum of the error function is always found at $J_o = J'$.

$$\sigma_{coup} = (1/\sqrt{2})\ \sigma_{dec} \tag{2a}$$

The difference spectrum is obtained from the coupled spectrum scaled by a factor 2 and two decoupled spectra: therefore the noise in the difference spectrum equals:

$$\sigma^2_{diff} = (2\sigma_{coup})^2 + 2(\sigma_{dec})^2 \tag{3}$$

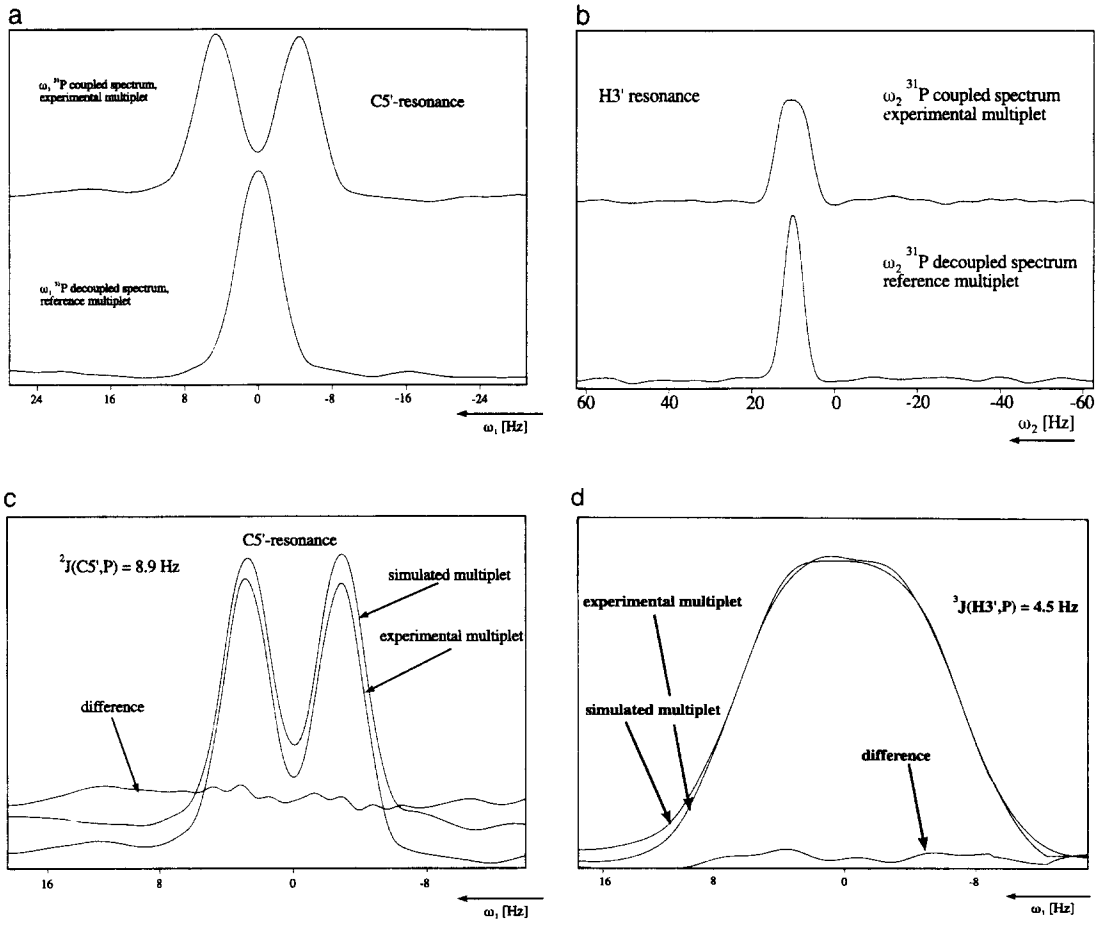


Fig. 6. (a) C5′,H5′ and (b) C3′,H3′ cross peak without decoupling (top) and with decoupling (bottom). The linewidth is 4.7 Hz. (c) and (d) Experimental coupled multiplet due to the ^{3}J(C4′,P) and ^{3}J(H3′,P)-coupling, respectively (bottom), the simulated multiplet (middle) with a coupling of 8.9 Hz and 4.5 Hz, respectively, and the difference between the two (top).

$$\sigma_{diff} = 2\sigma_{dec} \tag{4}$$

Integration of this noise over the region of integration $\Delta\Omega$ yields the noise of the integral σ_I:

$$\sigma_I = 2(\Delta\Omega)^{1/2}\,\sigma_{dec} = (\Delta\Omega)^{1/2}\,2/(S/N) \tag{5}$$

The latter equation makes use of Eq. 2. The ratio penalty function-to-integral noise is then:

$$P/\sigma_I = (P*S/N)/\,2(\Delta\Omega)^{1/2} \tag{6}$$

Figure 5a shows the dependence of the theoretical penalty function $P(J')$ on the products $J'T_2$* and J_oT_2*. Figure 5b shows a horizontal slice through Fig. 5a at $J'T_2$* = 1.875 (J = 8.7 Hz and T_2*

= 213 ms). The dependence of the accuracy of the coupling constant J_o on the S/N and the integration region is indicated by horizontal lines.

Table 1 lists the measured coupling constants of cyclic methylphosphonate **1** (see Fig. 1b). Figures 6a,c show representative 1D columns of the coupled and decoupled spectrum for the ^{2}J(C5′,P) and the ^{3}J(H3′,P) couplings. In Figs. 6b,d the 1D column with the coupled trace (lowest) and the simulated (middle) trace obtained by shifting copies of the decoupled spectrum with respect to each other by J^t = 8.9 Hz and 4.5 Hz, respectively, as well as the difference between both, are shown.

In oligonucleotides, the following difficulties arise: each C4′ couples to two ^{31}P spins. In Experiment 3, one would therefore observe a doublet of doublets on each C4′ peak. Distinguishing which coupling arises from which ^{31}P spin is not trivial but can be achieved in uniformly labeled oligonucleotides using 3D experiments.

In conclusion, we have introduced a new sensitive method for the measurement of structurally interesting ^{3}J-coupling constants along the phosphodiester backbone in oligonucleotide structures. A detailed analysis is given of how accurate a coupling can be determined depending on the signal-to-noise ratio of the spectrum. This technique can be used to improve the structure elucidation of RNA- or DNA-structures and in case of unusual β angles it provides the stereospecific assignment of H5′, H5″-protons.

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