Stereospecific Measurements of the Vicinal $^1H-^{31}P$ Coupling Constants for the Diastereotopic C5′ Methylene Protons in a DNA Dodecamer with a $^{13}C/^2H$ Doubly Labeled Residue. Conformational Analysis of the Torsion Angle β

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The dihedral angle dependence of the $^1H-^{31}P$ vicinal coupling constants, $^3J_{HP}$, has been used for many years in the conformational analysis of the phosphodiester linkages in nucleotides.[1] This approach, however, has been severely hampered by the difficulties in measuring accurate $^3J_{HP}$ values and also in discriminating the diastereotopic methylene protons at the 5′ carbons (C5′) of oligonucleotides. Even with recently developed 2D-NMR methods, such as ^1H J-resolved spectroscopy,[2] simulation of the cross-peak patterns for $^1H-^1H$ COSY spectra,[3,4] and high-resolution $^1H-^{13}C$ HSQC spectra at natural isotopic composition,[5] the $^3J_{HP}$ values for the C5′ methylene protons in oligonucleotides, which can be correlated with the β angles, are still difficult to measure in most cases, due to the incidental spectral overlap, line broadening, and small chemical shift difference between diastereotopic proton pairs. A stereospecific assignment of the C5′ methylene protons has recently been made by the relative sign of $^2J_{CH}$, which can be determined for uniformly ^{13}C labeled nucleotides at a low ^{13}C enrichment level.[6] Even with isotopically labeled nucleotides, recently published heteronuclear NMR methods to measure $^3J_{HP}$, FIDS-HSQC,[7] do not seem to be appropriate for the tightly scalar and dipolar coupled methylene protons, such as those at C5′. This method, which depends on the observation of the actual J_{HP} splitting of the cross peaks, is difficult to use for much smaller coupling constants than the line widths. We describe here a more robust method to measure the $^3J_{HP}$ by spin-echo difference spectroscopy,[8,9] which is applicable to DNA/RNA oligomers with $^{13}C/^2H$ labels at C5′.

The stereoselective deuteration of the C5′ protons[10] was applied to the thymidine with a uniformly ^{13}C labeled (98 atom % ^{13}C) deoxyribose ring.[11] By estimating the residual signal intensities of the ^1H-NMR spectrum of the $^{13}C/^2H$ doubly labeled thymidine, the deuteration of either one of the C5′ methylene protons was found to take place with an efficiency of 95 atom % ^2H, and thus only 5% of the thymidine has two protons at the C5′. It was further shown that the residual signal intensity for the *pro-S* proton, which corresponds to *H5′* in the conventional notation, was half of the relative intensity of the residual signal of the *pro-R* proton, which corresponds to *H5″*. This result indicates that the deuteration at C5′ takes place stereoselectively, with an approximately 2-fold excess of the (5′*S*)-^2H- over (5′*R*)-^2H-labeled diastereomers, as described previously.[10] The configuration of each diastereomer was confirmed by the $^3J_{HH}$ values among H5′, H5″, and H4′, with the $^3J_{HC}$ values among H5′, H5″, and C3′.[12] The *stereoselectively* $^{13}C/^2H$ doubly labeled thymidine was then incorporated into a DNA dodecamer, d(CGCGAAT*TCGCG)$_2$, where the residue with the asterisk denotes the location of the labeled thymidine (T7). By virtue of the different deuteration levels, we could easily assign the two $^1H-^{13}C$ cross peaks at δ 4.33 and 4.16 in the ^1H, ^{13}C HSQC spectrum to be H5′ (*pro-S*) and H5″ (*pro-R*) of the T7, respectively, by their peak intensities.

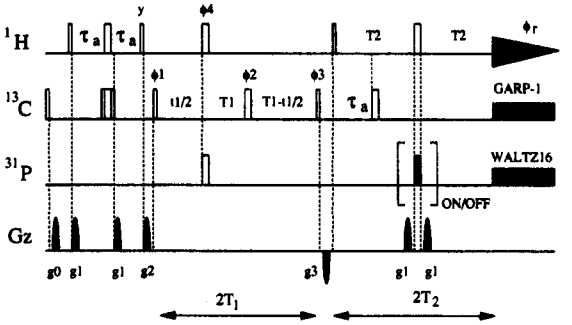

Figure 1. Pulse scheme for the {^{31}P} spin-echo differences $^1H-^{13}C$ dual CT-HSQC experiment. The durations were the following: τ_a = 1.5, T_1 = 3.3, and T_2 = 17.9 ms. Narrow and wide pulses denote 90° and 180° flip angles, respectively. The $^1H-^{13}C$ HSQC spectra with (ON) or without (OFF) the ^{31}P 180° pulse were recorded in an interleaved manner, with spectral widths of 4504.5 and 8000 Hz for ^1H and ^{13}C, respectively. All of the ^{13}C pulses with an rf field strength of 14.7 kHz were set at 61.7 ppm, the center of the ^{13}C resonances of the deoxyribose ring. GARP1 decoupling was applied from the ^{13}C channel during acquisition, using a 1.4 kHz rF field. All rectangular ^1H pulses were applied with a field strength of 31.3 kHz. A 15.6 kHz field for the ^{31}P 180° pulse and 0.5 kHz WALTZ16 decoupling on the ^{31}P channel were used during acquisition. The ^{31}P frequency was set at −4.2 ppm, which was around the spectral center of the ^{31}P resonances of the dodecamer. Unless otherwise indicated, all pulses were applied along the x-axis. Phase cycling was $\phi_1 = y, -y$; $\phi_2 = 2(x), 2(y), 2(-x), 2(-y)$; $\phi_3 = 8(x), 8(-x)$; $\phi_4 = 16(x), 16(-x)$; receiver $(\phi_r) = 2(x, -x, -x, x), 2(-x, x, x, -x)$. Quadrature detection in t_1 was obtained with the States-TPPI by incrementing ϕ_1. Pulsed field gradient durations were g_0 = 1.0, g_1 = 0.4, g_2 = 2.3, and g_3 = 1.5 ms. All of the field gradient pulses were sine-bell shaped, with a maximum gradient amplitude of 10 G/cm.

Figure 1 depicts the {^{31}P} spin-echo difference dual constant time $^1H-^{13}C$ HSQC pulse scheme used to measure the $^1H-^{31}P$ spin couplings, which was derived from the standard $^1H-^{13}C$ CT-HSQC experiment.[13] The second INEPT transfer step has been modified so as to implement the amplitude modulation to the observed $^1H-^{13}C$ correlation signals, according to the $^1H-^{31}P$ spin-coupling interactions. Namely, by applying the ^{31}P

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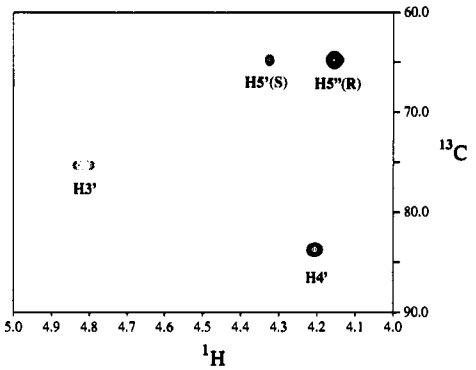

Figure 2. The {^{31}P} spin-echo difference ^{1}H−^{13}C dual CT-HSQC spectra of d(CGCGAAT*TCGCG)$_2$ with the J_{HP} modulation (^{31}P 180° pulse "ON" during $2T_2$). The spectrum was measured at 30° using 240 μL of a 1 mM solution containing 0.1 M NaCl, 0.1 mM EDTA, and 0.01 M sodium phosphate in D_2O, at pD 7.0, in a Shigemi microcell (Shigemi Co. Ltd., Tokyo). The acquired data sizes were (2 × 45) (t_1) × 512 (t_2), and the Fourier transformed data for the "ON" and "OFF" states were 256 × 1024 real points, after zero filling in both dimensions. For the present data acquisition, we used 128 transients per t_1 increment, giving rise to a total experimental time of 8 h for a complete data set. All spectra were recorded on a Bruker AMX500 spectrometer equipped with a 5 mm ^{1}H−{^{13}C,X} triple resonance gradient probe, with the X-channel tuned for the ^{31}P frequency.

180° pulse during the second constant time duration, the ^{1}H magnetization would be attenuated by $\cos(2\pi J_{HP}T_2)$, but no such attenuation would occur without this ^{31}P pulse. With these two complementary experiments executed in an interleaved manner, the two J_{HP} values can be measured by the relative intensity differences of the two cross peaks calculated from the two data sets, S_a (^{31}P-decoupled) and S_b (^{31}P-coupled), with $(S_a-S_b)/S_a$, which equals 2 $\sin^2(\pi J_{HP}T_2)$.[8,9] With this method, one has to use a relatively long T_2 duration, in order to measure the small J_{HP} value accurately. For example, a T_2 longer than 18 ms would be necessary to measure a J_{HP} as small as 2 Hz, which ensures the amplitude modulation difference, $S_a - S_b$, to be at least 4 times larger than the noise level. If large ^{1}H homonuclear coupling exists, such as the geminal coupling for the C5′ methylene protons, −14 Hz, the ^{1}H magnetization would be substantially lost during such a long T_2 by the additional passive coupling modulation, $\cos(2\pi J_{HH}T_2)$. On the other hand, a shorter T_2, which is required to observe the signals due to the coupled methylene protons, would result in a poor signal to noise ratio for the modulation difference signals and thus yield less accurate J_{HP} values. The problem due to these contradictory factors can be solved by replacing either one of the C5′ methylene protons with deuterium. Using the selectively ^{2}H/^{13}C doubly labeled nucleotide, one can use a longer delay time, T_2, to achieve larger amplitude modulation due to J_{HP} without signal attenuation, as long as the proton transverse relaxation times of the residual protons would allow the use of such a T_2. In practice, we have selected 17.9 ms for the T_2, in order to completely eliminate the possible signal overlap from the residual methylene protons attached at the ^{13}C-labeled site, due to incomplete deuteration (95 atom % ^{2}H).

To calculate $^{3}J_{HP}$ from the amplitude modulation of the cross peaks, we have used the signal intensities in each spectral data set, estimated by the 2D parabolic interpolation in the program PIPP[14] on the NMR data processed by nmrPipe.[15] The vicinal ^{1}H−^{31}P coupling constants for the C5′ methylene protons of the T7 residue were 2.5 ± 0.2 Hz ($^{3}J_{H5'P}$) and 3.6 ± 0.3 Hz ($^{3}J_{H5''P}$), respectively. The errors of the coupling constants were evaluated by the equation 2 $\sin^2(\pi J_{HP}T_2) = (S_a - S_b \pm E)/S_a$, where the error E was estimated on the basis of the random noise level, E_a and E_b, in each spectrum by $E^2 = E_a^2 + E_b^2$. The systematic errors due to the difference in the relaxation rates between the anti-phase and in-phase ^{1}H magnetizations[16] and to the ^{31}P pulse imperfection have been estimated to be less than 4% of the observed values and should not affect the validity of the conformational analysis of the phosphodiester bonds.[17]

With the observed values for $^{3}J_{H5'P}$ and $^{3}J_{H5''P}$, the β angle of the T7 residue was uniquely determined as 176.5 ± 0.7°, by applying a Karplus-type equation, $J_{HCOP} = 15.3 \cos^2\phi - 6.2 \cos\phi + 1.5$.[18] The obtained β angle for the T7 in solution is actually very similar to the corresponding value in the crystal, 177.5 ± 3.5°, which represents the average β angle for the T7 and T19 residues in a single crystal of the dodecamer duplex.[19] We also obtained the $^{4}J_{H4'P}$ value, 2.8 ± 0.3 Hz, and the $^{3}J_{H3'P}$, value, 2.9 ± 0.5 Hz, from the same experiment. Accurate values of the $^{3}J_{H3'P}$ together with the $^{3}J_{C2'P}$ values, which also can be obtained by spin-echo difference spectroscopy, can be used to uniquely determine the torsion angle ϵ. The method described here can be applied to larger DNA/RNA oligomers or their complexes with proteins, since the deuterium substitution of either of the two diastereotopic methylene protons at C5′ causes the transverse relaxation times of the residual H5′ or H5″, as well as that of the ^{13}C of the C5′ with deuterium label, to be much longer. In the case of our DNA dodecamer, for example, the transverse relaxation times of the residual proteins of the T7 were 170 ms, which were approximately twice as long as the value for the nondeuterated counterpart, which was 80 ms. The effect of deuterium decoupling on the present and related pulse sequences is currently under investigation.

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Supporting Information Available: The 1D spectra used for the stereospecific assignment of the C5′ protons of [1′,2′,3′,4′,5′-$^{13}C_5$;5′/5″-$^{2}H_1$]-5′-*O*-(dimethoxytrityl)thymidine (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be download from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(17) One of the major sources of the systematic error in the estimated J_{HP} values can be attributed to the faster relaxation rate of the anti-phase ^{1}H magnetization, with respect to the ^{31}P component.[30] The imperfection of the ^{31}P 180° pulse may also contribute to the systematic error. The former factor has been estimated to reduce the observed spin-coupling constants by as much as 3%, by assuming the overall correlation time of 1.5 ns and a ^{31}P T_1 relaxation time of 560 ms. Taking the latter term into consideration as well, the scaling factor is no more than 4%, which is negligible. For example, the corrected $^{3}J_{H5'P}$ and $^{3}J_{H5''P}$ values for this scaling factor will be increased only by 0.2 and 0.1 Hz, respectively, which will in turn result in a 0.4° increase of the T7 β angle.

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