Assignment of the $^{31}$P and $^1$H resonances in oligonucleotides by two-dimensional NMR spectroscopy

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The use of new $^1$H-detected heteronuclear $^1$H-$^{31}$P shift correlation experiments is demonstrated for oligonucleotides of 12 and 40 base pairs. The methods give unambiguous assignments of the $^{31}$P resonances and also permit identification of the C4' and C5' sugar protons. Use of the new methods enables one to make sequence-specific resonance assignments without reference to a known or assumed conformation of the DNA fragment.

Oligonucleotide $^{31}$P-NMR 2D NMR Shift correlation Poly(dA-dT)
Fig. 1. Pulse schemes for generating 2D $^1$H-$^3$P correlation spectra. During the delay period between consecutive scans, both schemes use a series of 40 180° pulses, spaced by 50 ms, to presaturate $^1$H resonances. In both schemes, the phase $\phi$ of the first $^3$P pulse is cycled $x, y, -x, -y$ and the receiver is cycled $x, -x, x, -x$. In addition, the phase $\psi$ of the 180° pulses in scheme b is incremented by 90° every 32 scans; furthermore, each time $\psi$ is incremented, the phase of the receiver is inverted. Data in odd- and even-numbered scans are stored separately and processed in the standard manner to separate positive and negative modulation frequencies. Scheme a results in a 2D pure absorption spectrum whereas scheme b requires an absolute value mode calculation in the $t_2$ dimension. Dispersive data in the $t_2$ dimension are discarded after the first Fourier transformation and the final spectrum is absorptive in this dimension.

$^1$H-$^3$P multiple quantum coherence is not an effective process and in practice scheme 1a appears to be more efficient. The detected $^1$H magnetization transferred from $^3$P at $t_2 = 0$ is in antiphase with respect to $^3$P but in phase with respect to all other protons. Absorption mode 2D spectra can therefore be recorded readily without the need for $z$ filters or purge pulses [2]. Signals that do not originate from transfer from $^3$P are suppressed by phase cycling and by presaturation of the $^1$H signals using a series of 180° pulses in between experiments.

Fig. 2 shows the 2D correlation spectrum of the dodecamer. In addition to the expected three-bond connectivities to H3' and H5', H5' also all of the four-bond H4'-C4'-C5'-O-P connectivities are observed. Since most of the C4' protons are resolved and assignable by means of a 2D NOE experiment, sequential assignment information follows directly from this type of spectrum. We have found the presence of P-H4' connectivity in all other oligonucleotides studied with this method so far. It may therefore be expected that this important connectivity is a general feature observed in this type of spectrum.

In scheme 1a, the amount of $^3$P magnetization transferred to $^1$H is a function of the length of the evolution period, $t_1$. The $t_1$ duration for which maximum transfer occurs depends on the sizes of the $J_{HP}$ couplings and on the $^3$P transverse relaxation time. In practice, a maximum is reached for a $t_1$ duration of about 25 ms. Scheme 1b exploits this fact by keeping the length of the evolution period fixed to a time, $T$ (~25 ms), and moving the pair of $^1$H/$^3$P 180° pulses stepwise through this period. This makes it appear as if the evolution period is varied from $-T$ to $+T$, i.e. it gives an effective acquisition time of $2T$ in the $t_2$ dimension. Note that the detected $^1$H signals are modulated as a function of $t_1$ only by the $^3$P chemical shifts and not by scalar coupling, providing the highest possible resolution in the $t_1$ dimension. Unfortunately, the overall duration of the apparent length of the evolution period is limited to $2T$, which limits the final resolution obtainable. However, use of the modern maximum entropy type processing methods may significantly alleviate this problem [9]. As an example, fig. 3 shows the $^1$H-$^3$P correlation spectrum of the synthetic DNA oligomer d(TA)$_{20}$. Although both the $^1$H and the $^3$P linewidths are relatively broad for this large fragment, a clear correlation for both $^3$P resonances is observed. This spectrum confirms the previously made assignment of the $^3$P resonances, made by comparison with two phosphorothioate analogues [10].

3. DISCUSSION

We have shown that the $^1$H-$^3$P correlation method can be applied successfully to the study of DNA fragments of a significant size, requiring only moderate NMR sample quantities. Not only does this type of experiment make it possible to obtain an unambiguous assignment of the $^3$P spectrum, important for structural studies, but it also
Fig. 2. 2D absorption mode $^1$H-$^{31}$P correlation spectrum of the dodecamer d(CATGGATm'CCATG), recorded with the scheme of fig.1a. $^1$H and $^{31}$P chemical shifts are relative to TSP and TMP, respectively. The spectrum results from a $2 \times 64 \times 1024$ data matrix with 80 scans per $t_1$ value. Acquisition times in the $t_1$ and $t_2$ dimension are 0.128 and 1.02 s, respectively. The total measuring time is 8 h. Both negative and positive contours are shown and resolution-enhanced 1D $^1$H and $^{31}$P spectra are shown along the two axes of the 2D spectrum. 8 Hz Gaussian broadening is used in the $t_2$ dimension and 9 Hz exponential narrowing followed by 12 Hz Gaussian broadening is used in the $t_1$ dimension. The centers of the correlation multiplets are marked + and only correlations with H3' and H4' protons are labeled.

provides a means to make sequence-specific resonance assignments independent of an assumed structure of the DNA fragment. The high intensity of the H4'-P correlations suggests a coupling constant larger than the 2 Hz previously estimated \[11\]. The relatively long transverse relaxation time of the C4' protons also contributes favorably to the intensity of these cross-peaks.

4. EXPERIMENTAL

The dodecamer was synthesized manually by the solid-phase phosphite triester method using the O-β-cyanoethylphosphoramidites. 600 $A_{260}$ units were dissolved in 0.4 ml D$_2$O, containing 0.1 M NaCl, 10 mM sodium phosphate, pH 7.4. The spectrum of fig.2 was recorded at 35°C.

The 5'-dimethoxytrityl (DMT) derivative of the self-complementary 40-mer, d(TA)$_{20}$, was synthesized on a $2 \times 1\mu$mol scale using a previously described \[12\] automated (Applied Biosystems model 380B) version of the phosphoramidite coupling method, with the exceptions that O-β-cyanoethylamidites were employed, and deprotec-
Fig. 3. 2D correlation spectrum of the 40-mer d(TA)$_{20}$, recorded with the scheme of fig. 1b. The spectrum is absorptive in the $^1$H dimension but in the absolute value mode in the $^{31}$P dimension. $^1$H and $^{31}$P chemical shifts are relative to TSP and sodium phosphate, respectively. The spectrum results from a $2 \times 50 \times 512$ data matrix, with the effective $t_1$ acquisition period ranging from $-25$ to $+25$ ms and the $t_2$ acquisition period from $0$ to $108$ ms. The $^{31}$P $T_1$ (1.4 s) was shorter than the $^1$H $T_1$ ($\approx 2$ s), and a 2-s delay between scans was used. The total measuring time was 12 h. The resolution-enhanced $^1$H spectrum and the projection of the 2D spectrum on the $^{31}$P axis are shown along the sides of the 2D spectrum.

The crude 5'-DMT material obtained from the parallel syntheses was pooled and dissolved in 12 ml of 0.1 M triethylammonium acetate (pH 7, TEAA). Four equal-volume portions of the resultant solution were each eluted from a reversed-phase HPLC column (Hamilton PRP-1, 7 x 305 mm) with a 1%/min gradient of acetonitrile in 0.1 M TEAA (pH 7) that began and ended at acetonitrile-TEAA ratios of 20:80 and 30:70, respectively [13]. The center-cut fractions which were collected at 10.5-11.5 min were pooled, lyophilized, detritylated, lyophilized, dissolved in 1 ml of 0.2 M NaCl, and then eluted with water from a size-exclusion column (Sephadex G-25M PD-10) to yield the sodium form of d(TA)$_{20}$ (105 $A_{260}$ units, 12.5% yield based on support-bound nucleoside). The sample was dissolved in 0.4 ml D$_2$O, containing 0.1 M NaCl, 10 mM sodium phosphate, p$^5$H 7.2. The NMR spectrum was recorded at 45°C.

NMR spectra were recorded on a modified Nicolet NT-500 spectrometer equipped with a Cryomagnet Systems 5-mm $^1$H probe that has a broad-band ($^{15}$N-$^{31}$P) decoupling coil for irradiation of low-gamma nuclei.
REFERENCES