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Anomalous Temperature Dependence of the Phosphorus-31 Nuclear Magnetic Resonance Chemical Shift in d(CCGG) and d(CCTAGG) at the Junction of the Pyrimidine Stack Followed by the Purine Stack

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Two self-complementary deoxyribo-oligonucleotides, d(CCGG) and d(CCTAGG), were synthesized. Thier ^{31}P and ribose ^{1}H nuclear magnetic resonance (NMR) assignments were achieved by the use of ^{31}P–^{1}H and ^{1}H–^{1}H shift correlated two-dimensional NMR techniques in low salt solution. One of the phosphorus atoms in each of these two oligomers showed an anomalous behavior: its resonance was shifted to lower field instead of higher field on lowering the temperature in the range below the melting temperature. This "anomalous" ^{31}P was assigned to CpG for d(CCGG), and to TpA for d(CCTAGG). Among the four self-complementary G.C tetramers [d(CCGG), d(CGCG), d(GGCC), d(GCGC)], this anomalous behavior of ^{31}P was found only in d(CCGG) [D. J. Patel, *Biopolymers*, **15**, 533 (1976); *ibid.*, **16**, 1635 (1977); *ibid.*, **18**, 553 (1979)]. It was suggested from the results that a sequence 5′···(Py)p(Py)p(Pu)p(Pu)···3′ always shows a conformational peculiarity at the (Py)p(Pu) portion, where Py=pyrimidine and Pu=purine nucleosides, and that the peculiarity involves the unusually low twist angle, t_g, (*i.e.* the local residual rotation around the helix axis). The anomalous ^{31}P behavior can be explained by postulating an intermediate form in the coil to helix transition.

Keywords——NMR; DNA; local structure; sequence-dependent conformational peculiarity

The sequence-dependent conformation of a deoxyribonucleic acid (DNA) double helix probably plays an essential role in every specific protein–DNA interaction. To establish such a sequence-dependence of the conformation, it would be effective to accumulate examples of the conformational analyses of oligonucleotides with known sequences. A number of single crystal X-ray analyses have been made along this line.[1–5] On the other hand, Sato *et al.*[6] demonstrated a relationship between the mode of recognition of nucleotide sequences by restriction endonucleases and the circular dichroism (CD) spectra of the oligonucleotides having a C:G terminus. For studies of the conformation in solution, nuclear magnetic resonance (NMR) is widely applied. Recent developments in two-dimensional (2D) NMR spectroscopy have made the assignment of the resonances in oligonucleotides possible, providing important information about the structure in solution. Pardi *et al.*[7] first reported

the backbone assignment involving phosphorus atoms of a tetranucleotide by the use of ^{31}P–^{1}H and ^{1}H–^{1}H shift correlated (COSY) spectroscopy.

Usually the chemical shift of phosphorus atoms in an oligonucleotide is moved towards higher field on lowering the temperature. However one of the three phosphorus signals in d(CCGG) shifts towards lower field on lowering the temperature.[8] Among the four self-complementary G.C tetramers examined by Patel such an anomalous ^{31}P behavior is found only in d(CCGG).[8-10] We have succeeded in assigning the phosphorus atoms by the method of Pardi *et al.*[7] The anomalous ^{31}P was located at the CpG portion of d(CCGG). Such an anomalous ^{31}P was also found in d(CCTAGG) at the TpA portion. On the basis of these data, we discuss in this paper a possible conformational peculiarity at the pyrimidine–purine junction which comes after a pyrimidine stack and before a purine stack. The temperature effect on the ^{31}P chemical shift of an oligonucleotide in solution is also discussed. In contrast to what Gorenstein *et al.*[11] considered, we propose here to take into account the ring current effect of the base residues.

Materials and Methods

Materials——Deoxytetranucleoside triphosphate, d(CCGG), and deoxyhexanucleoside pentaphosphate, d(CCTAGG) were synthesized by the solid-phase phosphotriester method.[12] The 3′-terminal nucleoside, the 3′-terminal of which was bound to polystyrene resin, was used as the starting material. By repeating activation and capping of the pertinent nucleotides alternately, a stepwise condensation was carried out from the 3′-terminal in the 5′-direction. The protected tetramer or hexamer product was released from the polystyrene resin by treatment with tetramethylguanidine and *syn*-pyridinealdoxime. This was also effective for removing the chlorophenyl group from phosphates. The protecting group of the base moiety was removed by alkaline treatment. The desired tetramer or hexamer was subjected to a preliminary purification on a C-18 open column with 0.1 M triethylammonium acetate buffer, pH 7.0, with a CH_3CN 5 to 40% gradient. The detritylation at the 5′-terminal was done with acetic acid (80%), and after ethyl acetate extraction, the product was purified by C-18 reversed phase high-performance liquid chromatography (HPLC). Here the buffer used was 0.1 M trimethylammonium acetate, pH 7.0+9% CH_3CN. Ionic exchange was done by the use of AG 50W (×8) resin, in its pyridine form and then in its Na-form. The product was lyophilized twice from 99.95% D_2O. For NMR observation the samples were dissolved in 0.5 ml of 99.95% D_2O containing 0.1 M NaCl at pH 7.8 (direct reading of a pH meter).

Methods——NMR spectra were obtained on a JEOL GX-400 spectrometer operating at 399.95 MHz for ^{1}H and 161.70 MHz for ^{31}P. 2,2-Dimethyl-2-silapentane-5-sulfonate (DSS) for ^{1}H and trimethyl phosphate (TMP) for ^{31}P were used as external references. COSY[13] spectra were collected with quadrature detection (2048 data points for 512 t_1 values). A recycle delay of 1.2 s was used. Spectra were acquired with a spectral width of 3000 Hz. ^{31}P–^{1}H shift-correlated 2D-NMR spectra[7] was collected into 2048 points for 128 t_1 values with a recycle delay of 2.2 s. Spectra were acquired with a spectral width of 800 Hz for ^{31}P, and 600 Hz for ^{1}H. A pulse delay of 31.25 ms was used before and after the data acquisition.

Results

Figure 1 shows a contour plot of a ^{31}P–^{1}H shift correlated 2D-NMR spectrum for d(CCGG) at 21 °C. Figure 2 shows a contour plot of a COSY spectrum for d(CCGG) at 25 °C. An enlarged plot of a portion of Fig. 2 is shown in Fig. 3. From a combination of the two 2D-NMR spectra, the assignment of phosphorus and ribose proton resonances was completed; Firstly from a COSY spectrum, the connectivity from H3′ through H4′ to H5′ in one ribose unit can be traced. Secondly from a ^{31}P–^{1}H shift-correlated spectrum, the H3′ and H5′ coupled to the phosphate can also be followed. Lastly, an in-turn combination of the two 2D-spectra makes the assignments of the phosphorus and ribose resonances possible. Table I lists the chemical shifts of the ribose protons in d(CCGG). From the data the anomalous phosphorus mentioned in the introduction was assigned to CpG (Fig. 4 insert). The phosphorus shifts of d(CCTAGG) as a function of the temperature are shown in Fig. 4. One of the five ^{31}P resonances shows an anomalous temperature effect. A contour plot of a ^{31}P–^{1}H shift correlated spectrum for d(CCTAGG) is shown in Fig. 5. In Fig. 6 an enlarged plot of a

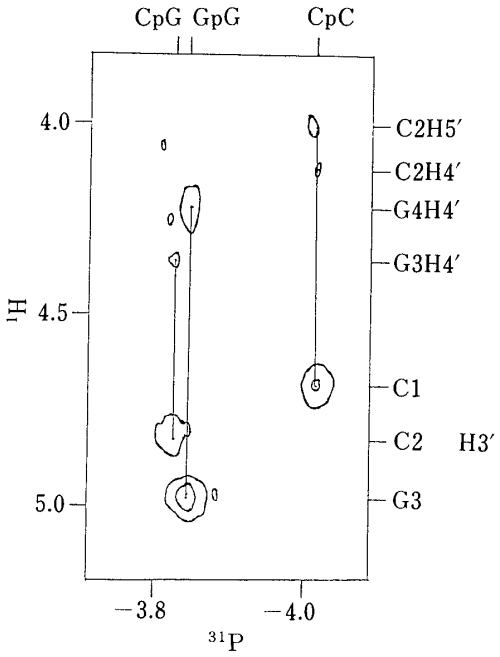

Fig. 1. A Contour Plot of a ^{31}P–^{1}H Shift Correlated 2D-NMR Spectrum of d(CCGG) (440 OD/ml) at 21 °C

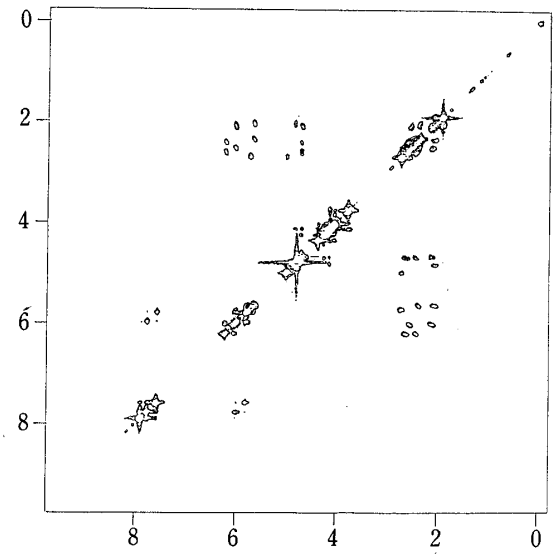

Fig. 2. A Contour Plot of a COSY Spectrum of d(CCGG) (440 OD/ml) at 25 °C

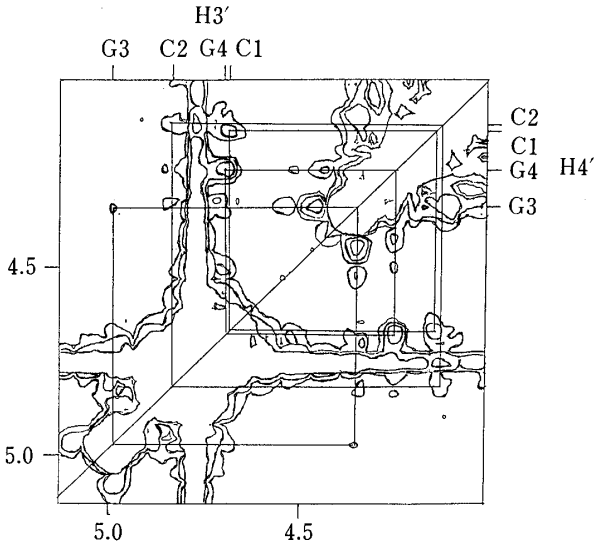

Fig. 3. Expansion of Fig. 2 Indicating the Connectivity between H3′ and H4′

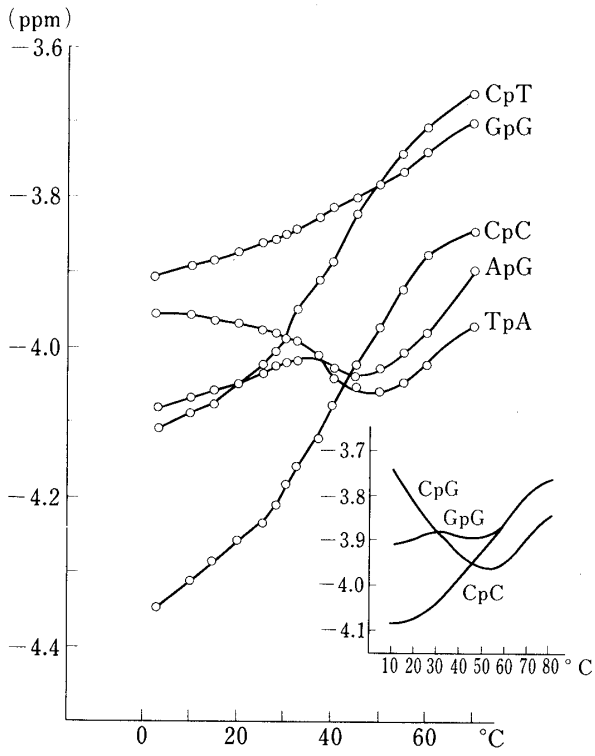

Fig. 4. ^{31}P Chemical Shifts of d(CCTAGG) as a Function of Temperature and Their Assignments

Insert: ^{31}P chemical shifts of d(CCGG) as a function of temperature from ref. 8 with the assignments made in the present work.

Table I. ^{1}H Chemical Shifts (ppm) of d(CCGG) Riboses at 25 °C

	H1′	H2′		H3′	H4′	H5′
C1	6.07	2.11	2.53	4.68	4.15	3.76 3.78
C2	5.67	2.36	2.03	4.83	4.12	4.02
G3	5.74	2.70	2.70	4.98	4.35	4.10
G4	6.22	2.62	2.42	4.72	4.23	4.15

Table II. ^{1}H Chemical Shifts (ppm) of d(CCTAGG) Riboses at 25 °C

	H1′	H2′		H3′	H4′	H5′
C1	6.11	2.22	2.36	4.85	4.31	3.79
C2	6.12	2.36	2.48	4.62	4.21	4.10
T3	6.03	2.28	2.56	4.70	4.29	4.02
A4	5.98	2.72	2.82	5.03	4.39	4.11
G5	5.64	2.46	2.60	4.95	4.36	4.22
G6	6.06	2.20	2.48	4.85	4.22	4.12

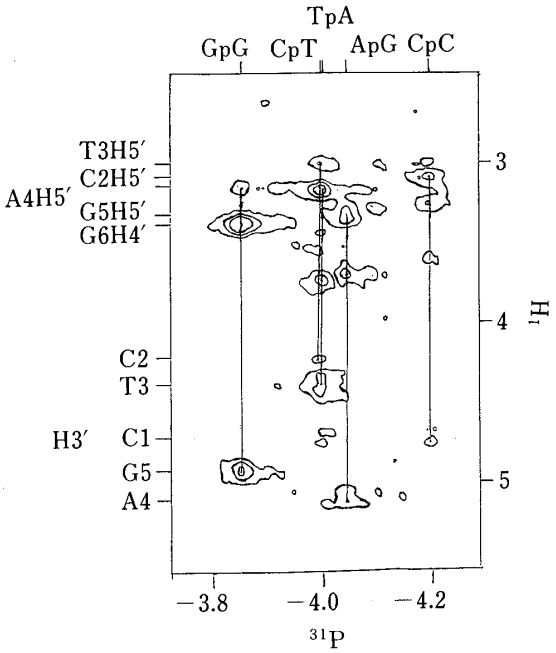

Fig. 5. A Contour Plot of a ^{31}P–^{1}H Shift Correlated 2D-NMR Spectrum of d(CCTAGG) (1400 OD/ml) at 31 °C

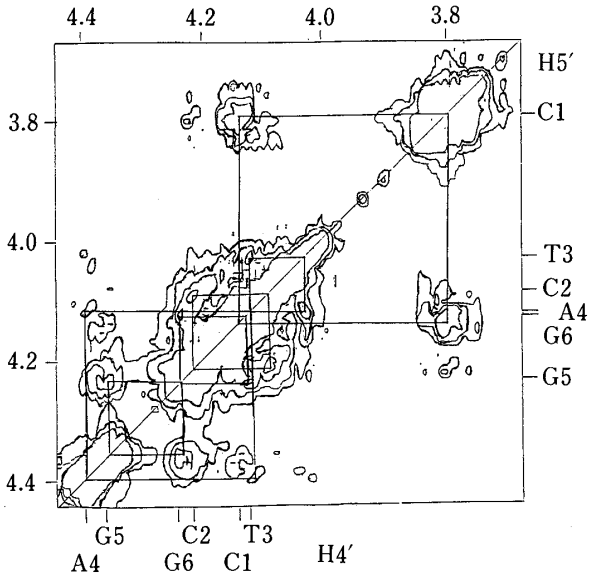

Fig. 6. An Enlarged Plot of a Portion of a COSY Spectrum of d(CCTAGG) (1400 OD/ml) at 25 °C, Indicating the Connectivity between H4′ and H5′

portion of the COSY spectrum for d(CCTAGG) is shown. Marison and Lancelot[14] successfully assigned the NMR signals of a hexanucleotide using the H3′–P coupling and the long range H4′–P coupling. They did not use the H5′–P coupling because many of the H5′ signals were found to overlap H4′. In the present study, however, we could use both H5′–P and H4′–P couplings to assign the resonances of d(CCTAGG). The results are summarized in Table II. The anomalous phosphorus in d(CCTAGG) is now assignable to TpA (Fig. 4).

Discussion

Characterization of the Anomalous ^{31}P

a) Location——The insert of Fig. 4 shows the phosphorus chemical shifts of d(CCGG) as a function of the temperature given by Patel,[8] with the assignments newly made in this work. In both d(CCGG) and d(CCTAGG), the anomalous ^{31}P, which shifts towards lower field on lowering the temperature, is located at the (Py)p(Pu) portion in the sequence 5′···(Py)p(Py)p(Pu)p(Pu)···3′; where Py=pyrimidine nucleoside and Pu=purine nucleoside. Such an unusual phosphorus was not found in any of d(CGCG), d(GCGC), d(GGCC),[8-10] d(AGCT),[15] d(CGCGCG),[9] d(GGAATTCC),[16] d(CGCGAATTCG-

CG),[17] and d(GACGATATCGTC).[18] None of these have (Py)p(Py)p(Pu)p(Pu) portions.

(b) Behavior in the Course of Duplex Formation——On lowering the temperature from 70 to 40 °C, d(CCGG) or d(CCTAGG) is considered to change from a single-stranded form into a duplex form.[19] During this duplex formation, all of the ^{31}P resonances including the anomalous one shift towards higher field (see Fig. 4). For d(CCGG) or d(CCTAGG), however, a second transition seems to take place, on lowering the temperature from 40 to 0 °C, from an intermediate duplex form (designated as H_i) into a final stable duplex form (H_f). In this H_i–H_f transition the particular ^{31}P located at the (Py)p(Pu) portion shows a downfield shift, whereas most of the other ^{31}P resonances continue to shift towards higher field. It should be pointed out here, however, that one of the neighboring phosphorus nuclei shows a slight but appreciable tendency of anomaly. For both d(CCGG) and d(CCTAGG) the adjacent ^{31}P on the down-stream side, namely at the (Pu)p(Pu) portion, shows a small down field shift around 40 °C before it again follows the general trend (upfield shift) in the 20 to 0 °C range.

A Sequence-Dependent Conformational Peculiarity

It appears that an anomalous conformation arises in a DNA duplex at the junction of a pyrimidine stack followed by a purine stack. Taking into account the Calladine–Dickerson proposal,[20,21] we suggest that in the (Py)p(Pu) portion in question a marked purine–purine clash must take place in the minor groove side of the DNA double helix, while the (Py)p(Py) and (Pu)p(Pu) portions form a stable base stacking piles without such a clash. To avoid the clash at the (Py)p(Pu) portion, some of the local helix parameters should change from the standard values; this in turn should cause the phosphorus chemical shift anomaly. We consider that the "normal" chemical shift changes from 70 to 40 °C are caused by the overall coil-to-helix transition with the formation of stable stacking of the (Py)p(Py) and (Pu)p(Pu) stacks, and that the anomalous one from 40 to 0 °C is caused by a change in a local helix parameter at the (Py)p(Pu) portion.

As one of such local helix parameters, the twist angle (t_g) is plausible. This is the local residual rotation around the helix axis, and it is considered to be unusually low at the (Py)p(Pu) portion.[21] This idea is supported by the following findings:

(i) The twist angle (t_g) in the CpG portion of d(CCGG) was actually found to be extremely low in the crystalline state by X-ray analysis.[1] According to the Calladine-Dickerson rule,[20,21] the values in this portion were expected to be the lowest among the twelve phosphorus atoms of the four self-complementary G.C tetramers[22] (see Fig. 7).

(ii) Ott and Eckstein[17] reported that the NMR signals of the third and ninth CpG phosphorus atoms in d(CGCGAATTCGCG) were found at lower field strength than they had expected on the basis of their locations in the sequence, and for these two portions Dickerson and Drew[23] found unusually low t_g values (27.4° and 30.3°, respectively) in the

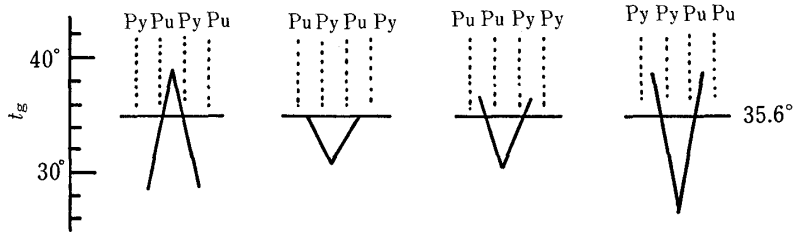

Fig. 7. The Local Helix Twist Angle (t_g), for Each Portion of the B-Form DNA Fragments, Calculated on the Basis of the Calladine–Dickerson Method[20,21]

t_g is the change in orientation of the C1′–C1′ vectors in two successive base-pairs viewed down the helix axis. The standard B-structure is assumed to have a value of $t_g = 35.6°$.

crystal of this dodecamer duplex.

(iii) Wang *et al.*[5] found that the central CpG portion of d(GGCCGGCC)$_2$ has an unusually low t_g with unusually short inter-base distance; from their crystallographic analysis, the t_g values were found to be 35°, 28°, 45°, and 16°, respectively for the first, second, third, and fourth (central) phosphodiester bridges from the 5′ terminal.

We first considered that it would be more appropriate to correlate the anomaly in ^{31}P shift with a peculiality in the backbone torsion angles. However, no unusually low or high torsional angles at the portion where the t_g values are unusually low in the above three oligonucleotides; d(CCGG), d(CGCGAATTCGCG), and d(GGCCGGCC), are found in their single crystal X-ray data. Thus we prefer to propose here that the unusually low value of the local helix parameter, t_g, itself causes the phosphorus chemical shift anomaly.

Some Speculations on the Mechanism of the Temperature Dependence of the Phosphorus Shifts in DNA——The following questions remain to be answered: why does the DNA ^{31}P resonance usually shift towards higher field on going from single strand to double helix, and does the anomalous ^{31}P shift take place on going from H_i to H_f. Unfortunately, the answers are not yet clear.

It was once proposed by Gorenstein *et al.*[11] that the factor which determines the phosphorus chemical shift change in the helix-coil transition in oligonucleotides is the set of torsional angles around the P–O ester bond which is correlated with the O–P–O bond angle. They suggested that the conformations with respect to the internal rotation angles, O3′–P–O5′–C5′ (α) and C3′–O3′–P–O5′ (ζ), are *gauche*, *gauche* for the double helical form but *gauche*, *trans* for the single-stranded form, and that this difference is responsible for the phosphorus shift change in the helix-coil transition. Ott and Eckstein,[17] however, reported that the phosphorus shifts observed in the NMR spectrum cannot be explained by the α and ζ values found in d(CGCGAATTCGCG) by X-ray crystallographic analysis. This theory does not seem suitable for explaining the anomalous behavior of the phosphorus we found; it is improbable that the drastic *gauche* to *trans* change occurs in the H_i–H_f transition in the lower temperature range.

Let us tentatively examine here the possibility that the ring current of the base residues may influence the phosphorus shift in oligonucleotides. We can cite here a suggestion from a study of the nuclear Overhauser effect that guanosine monophosphate[24] and pyrimidine nucleoside[25] spend a considerable proportion of time in the *syn* conformation around the glycosidic bond in aqueous solution. Following this suggestion, we can speculate that the single stranded DNA molecule may be so flexible that the *syn*$\rightleftharpoons$*anti* fluctuation may take place around the glycosyl bond. In the *syn* state the phosphorus nucleus is placed close to the base plane located at its 3′-side (down-stream), thus experiencing a large deshielding effect. On going to the double stranded state the deshielding effect at the position of the phosphorus is reduced because the glycosidic torsional angle is fixed in the *anti* position, which results in a smaller ring current effect on the phosphorus NMR. We can estimate the amount of the shift here from the Johnson–Bovey map[26]: it would be 1.0—1.5 ppm in the *anti*-to-*syn* change. On the other hand, the actually found shift changes from the single- to the double stranded state in the oligonucleotides were less than 0.5 ppm. These values therefore can be explained if 30—50% of the single-stranded molecule is in the *syn* conformer.

In d(CCGG) and d(CCTAGG) there must be an additional transition H_i–H_f as indicated by the anomalous low-field phosphorus shifts at the (Py)p(Pu) portion. In H_f, an unusually low t_g is to be postulated at the position in question. We suggest that at this position a shortening of the inter-base distance may take place together with lowering of the t_g value in the H_i–H_f transition,[5] and that this shortening may in turn result in the downfield shift of the phosphorus signal through the effect of the ring current of the bases. However, factors other

than the ring current might also influence the anomalous ^{31}P shift; for example the shortening of the O–P–O bond angle at this site.[11)]

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