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Complete Assignment of the Deoxyribose 5′/5″ Proton Resonances of the *Eco*RI DNA Sequence Using Isotropic Mixing†

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ABSTRACT: Using two-dimensional isotropic mixing spectroscopy all 5′/5″ proton resonances of the *Eco*RI restriction site DNA dodecamer $[d(CGCGAATTCGCG)]_2$ have been assigned. This completes the previous assignments of 1′H to 4′H resonances of the deoxyribose spin systems (Hare et al., 1983). With mixing times of up to 500 ms, many of these resonances showed connectivities of 5′/5″ protons in the two-dimensional isotropic mixing spectrum. Relying only on through-bond connectivities makes these assignments independent of assumptions about the conformation of the DNA oligonucleotide. The assignment of the 5′H/5″H resonances will allow the interpretation of intra- and interresidue NOEs to these protons, providing information about the DNA backbone conformation.

Nuclear magnetic resonance is the only method that is able to yield detailed information about the conformation of biopolymers in solution. Structural information is obtained by measuring the distance-dependent nuclear Overhauser effect (NOE) between assigned resonances (Wüthrich, 1986). As only distances smaller than 5 Å can be determined, the accumulation of small errors can potentially result in a distorted overall structure obtained from these data. This problem is especially severe for molecules with extended, nonglobular structures like DNA. Therefore, it is essential to take as many distance constraints into account as possible, and this requires

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as many assignments of the resonance lines in a NMR spectrum to individual protons in the biopolymer as possible. The assignment strategy of oligonucleotide duplexes developed independently by Feigon et al. (1983), Hare et al. (1983), and Scheek et al. (1983) includes two steps. First the ^1H resonances of the individual deoxyribose and base spin systems are identified by using two-dimensional correlation spectroscopy (COSY) (Aue et al., 1976). These spin systems are then arranged in sequential order by using interresidue connectivities obtained from two-dimensional NOE spectroscopy (NOESY) (States et al., 1982). In practice, only that part of the deoxyribose spin system formed by the 1′, 2′, 2″, and 3′ protons can be delineated by using the COSY experiment. Although the 1′H and 2′H/2″H resonances are usually well dispersed and have unique chemical shift ranges in the NMR spectrum, the 3′H, 4′H, and especially the 5′H/5″H resonances have much less dispersion and fall within a narrow range of chemical shifts between 4 and 5 ppm. Thus, the cross peaks between these resonances are located close to the diagonal of the two-dimensional spectrum and are often difficult to resolve. Furthermore, in B-form DNA the vicinal coupling constants $J(3'-4')$, $J(4'-5')$, and $J(4'-5'')$ are often quite small (<2 Hz), resulting in low-intensity cross peaks in the COSY spectrum. This latter effect occurs because a transfer of anti-phase coherence between coupled spins results in an anti-phase multiplet structure of the cross peaks. If the active J coupling constant is comparable to or smaller than the resonance line width, cross-peak multiplet components with intensities of opposite signs cancel partially, limiting the sensitivity of the experiment. This in general also prevents the observation of double- or multiple-step coherence transfers between these protons in the RELAY experiment (Eich et al., 1982; Bax et al., 1985a), and as a result most of the 4′H resonance assignments depend on NOE connectivities that imply structural assumptions and the 5′H/5″H resonances are usually not even assigned.

Recently two-dimensional TOCSY or HOHAHA isotropic mixing experiments (Braunschweiler et al., 1983a; Bax et al., 1985b) with mixing times of up to 250 ms have been successfully applied to the assignment of the 1′H to 4′H resonances of a small hairpin forming dodecamer d-(CGCGTTTTCGCG) and a self-complementary duplex DNA, 14 base pairs in length $[d(GCCGTGGCCACGGC)]_2$ (Flynn et al., 1988). In the TOCSY spectra of the 14-mer no multiple-step coherence transfers involving the 5′H/5″H resonances were observed with the exception of the 3′H to 5′H/5″H cross peak of residue G1. The hairpin dodecamer showed several cross peaks between 1′H or 2′H/2″H and 5′H/5″H resonances resulting in the assignment of the 5′H/5″H resonances for 5 of the 12 deoxyribose spin systems.

In this paper we report the systematic application of the TOCSY experiment with mixing times of up to 500 ms to the *Eco*RI restriction site dodecamer $[d(CGCGAATTCGCG)]_2$, resulting in the complete assignment of all 5′H/5″H resonances. Recently a distance geometry structure of the dodecamer has been calculated, derived from NOEs between base and deoxyribose 1′H to 4′H resonances (Nerdal et al., 1989). It is expected that NOEs involving the now assigned 5′H/5″H resonances can provide additional distance constraints for the DNA backbone.

Materials and Methods

The DNA dodecamer $d(CGCGAATTCGCG)_2$ was synthesized by using solid-phase phosphoramidite techniques (Hare et al., 1983) and purified by chromatography on Sephadex G-25 (Kintanar et al., 1987). The purified and repeatedly lyophilized duplex was finally dissolved in 0.4 mL of 99.996% D_2O, giving a 12 mM solution.

The two-dimensional NMR experiments were performed on a homebuilt 500-MHz NMR spectrometer (Gladden and Drobny, unpublished results). The isotropic mixing spectra were acquired in the phase-sensitive mode using TPPI (Drobny et al., 1979; Bodenhausen et al., 1980). The mixing times of 150, 300, and 500 ms corresponded to 170, 340, and 600 MLEV-16 cycles with a radio frequency field strength of 18.5 kHz, corresponding to a radio frequency power of about 10 W. The sample heating during the longer mixing times resulted in slight shifts of the resonance lines on the order of 0.01 ppm. The number of scans for each t_1 value was 64, 160, and 196, respectively. The water resonance was presaturated by selective irradiation for 0.5 s, and a total relaxation delay of 2 s was allowed. The sample temperature was regulated at 25 °C for the experiment with 150-ms mixing time and at 37 °C for the experiments with 300- and 500-ms mixing periods.

The data were processed with software written by Dennis Hare (Hare Research, Woodinville, WA). The spectra were apodized in both frequency dimensions by using skewed sine-bell functions shifted by 45°, skew value of 0.8. The first t_1 experiment was multiplied by 0.5 to suppress t_1 ridges in the spectra (Otting et al., 1985).

Results and Discussion

TOCSY spectra have a number of favorable properties that help overcome the above-mentioned difficulties in assigning 5′H/5″H resonances.

First, the experiment effects a net coherence transfer between coupled spins that results in an in-phase multiplet structure of the cross peaks. Thus, there are no cancellation effects as all subpeaks have the same signs, and even cross peaks between very weakly coupled spins can be observed, provided the mixing time (and the transverse relaxation time of the involved spins) is long enough [ca. $1/(2J)$ for a 2-spin system]. In spectra with anti-phase multiplet structures like COSY (Aue et al., 1976), RELAY (Wagner, 1983), or multiple-quantum spectroscopy (Braunschweiler et al., 1983b), cancellation effects limit the sensitivity if the active coupling constant is smaller than or on the order of the resonance line widths, which is typically the case for couplings involving 5′H/5″H protons in B-form DNA.

Second, the narrow diagonal of the TOCSY spectra (cf. Figure 1) allows the identification of cross peaks that are located very close to the diagonal like the cross peaks between 4′H and 5′H/5″H resonances that typically have very similar chemical shifts.

Third, double- and multiple-step coherence transfers during the isotropic mixing period can give rise to cross peaks between the 5′H/5″H resonances and the 3′H, 2′H/2″H, and 1′H resonances. These cross peaks often fall in less populated parts of the spectrum and resolve ambiguities due to overlapping resonances.

The complete TOCSY spectrum of the *Eco*RI restriction site dodecamer with 300-ms mixing time is shown in Figure 1. The previous 1′H, 2′H/2″H, 3′H, and 4′H resonance assignments (Hare et al., 1983) were repeated, using only through-bond coupling information to assign the resonances of each deoxyribose spin system. This is illustrated with the 4′H resonances, which previously relied in part on NOE connectivities: In addition to the partially overlapping cross peaks between the 3′H and 4′H resonances (cf. Figure 2), multiple-step coherence transfers from 1′H and 2′H/2″H to the 4′H resonances were observed in the TOCSY spectrum.

Table I: TOCSY Connectivities Involving 4′H and 5′H/5″H Resonances[a]

	1′–4′	2′/2″–4′	3′–4′	1′–5′/5″	2′/2″–5′/5″	3′–5′/5″	4′–5′/5″
C1	ccc	ccc	ccc	ccc	ccc	ccc	ccc
G2	–wc	wcc	ccc	––w	–wc	wcc	ccc
C3	ccc	ccc	ccc	---	–ww	wwc	ccc
G4	–ww	–ww	ccc	---	–ww	–cc	ccc
A5	–wa	–ww	ccc	–ww	–wa	wcc	ccc
A6	wca	wcc	ccc	–cc	–wa	wca	ccc
T7	ccc	ccc	ccc	---	---	ooo	ccc
T8	wcc	wcc	ccc	–wa	––w	–oo	ccc
C9	ccc	ccc	ccc	---	---	ooo	ccc
G10	–wc	–oo	ccc	–oo	---	–oo	ccc
C11	ccc	ccc	ccc	–oo	–oo	www	ccc
G12	ccc	ccc	ccc	–cc	–cc	–wc	ccc

[a] Each entry consists of three letters, corresponding to the three mixing times of 150, 300, and 500 ms, respectively. Symbols: c, cross peak; w, weak cross peak; o, overlap with intense cross peak; a, overlap with experimental artifacts; –, no cross peak detected.

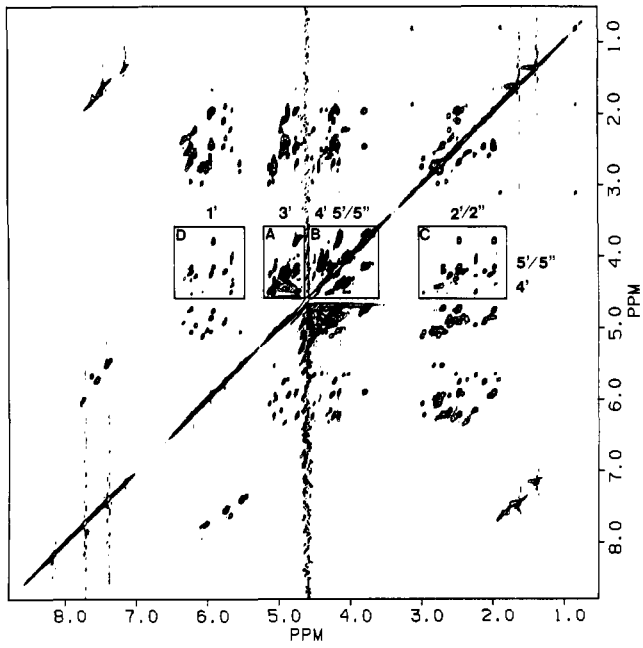

FIGURE 1: Contour plot of the 500-MHz TOCSY spectrum (300-ms mixing time) of d(CGCGAATTCGCG)$_2$ in D_2O at 37 °C. The boxed regions A–D correspond to expanded plots given in Figures 2–5, respectively.

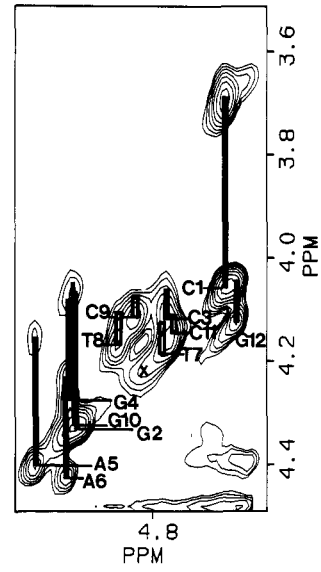

FIGURE 2: Expanded TOCSY contour plot of region A in Figure 1. (3′H,5′H/5″H) cross peaks due to two-step coherence transfer are connected to the labeled (3′H,4′H) cross peaks. Weak, overlapping (3′H,5′H/5″H) cross peaks are connected by open vertical lines to the corresponding (3′H,4′H) cross peaks. Cross peaks due to impurities in the sample are marked with an "x".

The experiments with longer mixing times of 300 and 500 ms showed virtually all of the (1′H,4′H) and (2′H/2″H,4′H) cross peaks (Figures 4 and 5). Table I summarizes the detected cross peaks involving 4′H and 5′H/5″H resonances in the spectra corresponding to 150-, 300-, and 500-ms mixing periods. The reliable assignment of the 4′H resonances forms the basis for the assignment of the 5′H/5″H resonances: The next step is the identification of cross peaks between the assigned 4′H and the resonances of the 5′/5″ protons. Figure 3 shows the region of the 300-ms TOCSY spectrum containing these cross peaks. Some of the (4′H,5′H/5″H) cross peaks (in Figure 3 identified as C3, T7, T8, C9, C11, and G12) form an overlapping cluster close to the diagonal, whereas others (C1, G2, G4, A5, A6, and G10) are reasonably well resolved. Although the overlapping cross peaks cannot be readily assigned at this stage, the possible range of chemical shift of the individual 5′H/5″H resonances is narrowed down to only several percent of a ppm. These cross peaks can be unambiguously assigned if in addition cross peaks due to multiple-step coherence transfers to the 5′H/5″H resonances can be observed. Two-step coherence transfer cross peaks from the 3′H to 5′H/5″H resonances are connected by vertical lines to the labeled (3′H,4′H) cross peaks in Figure 2. Some of these cross peaks (connected by open lines) are partially overlapping

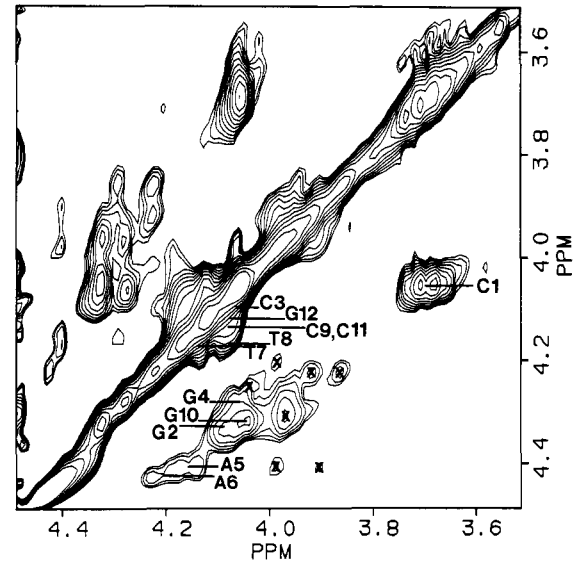

FIGURE 3: Expanded plot of region B in Figure 1 containing the (4′H,5′H/5″H) cross peaks.

with intense 3′H to 4′H cross peaks, but most are resolved well enough to confirm the (4′H,5′H/5″H) cross peak assignments in Figure 3. Furthermore, residues C1, G2, C3, G4, A5, A6,

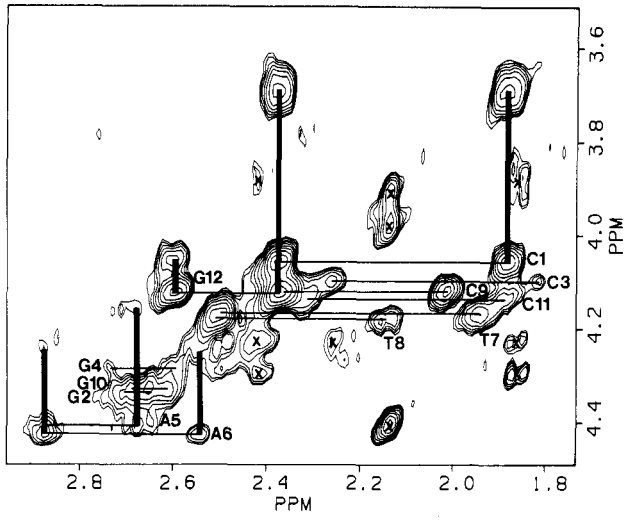

FIGURE 4: Expanded plot of region C in Figure 1. (2'H,4'H) and (2''H,4'H) cross peaks are connected by horizontal lines and are labeled. They are connected to (2'H/2''H,5'H/5''H) cross peaks by vertical lines.

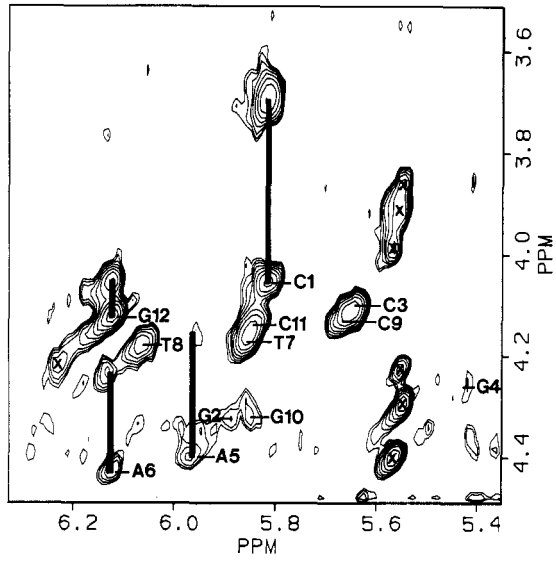

FIGURE 5: Expanded plot of region D in Figure 1. The (1'H,4'H) cross peaks are labeled and connected to (1'H,5'H/5''H) cross peaks.

T8, and G12 give rise to cross peaks due to 3- and 4-step coherence transfers during the 300- and/or 500-ms mixing time corresponding to coherence transfers from 1'H to 5'H/5''H, from 1'H to 4'H, and from 2'H/2''H to 5'H/5''H

Table II: Chemical Shifts of the Assigned Deoxyribose Protons at 37 °C[a]

	1'	2'/2''	3'	4'	5'/5''
C1	5.81	1.88/2.38	4.66	4.05	3.68/3.71
G2	5.89	2.65/2.71	4.95	4.33	4.08
C3	5.65	1.81/2.26	4.78	4.12	4.07
G4	5.43	2.59/2.74	4.97	4.28	4.06
A5	5.97	2.68/2.88	5.03	4.40	4.14/4.17
A6	6.13	2.54/2.88	4.97	4.43	4.19/4.24
T7	5.86	1.94/2.50	4.78	4.17	4.14
T8	6.07	2.14/2.51	4.86	4.18	4.08
C9	5.67	1.88/2.37	4.83	4.12	4.08
G10	5.85	2.61/2.68	4.95	4.33	4.05
C11	5.85	1.88/2.31	4.76	4.14	4.08
G12	6.13	2.38/2.60	4.64	4.13	4.08

[a] With the exception of C1, A5, and A6, the 5'H/5''H resonances are degenerate.

(cf. Table I). The region of the 300-ms TOCSY spectrum containing the (2'H/2''H,5'H/5''H) cross peaks that are connected to the labeled (2'H/2''H,4'H) cross peaks is depicted in Figure 4. Figure 5 shows the (1'H,4'H) and (1'H,5'H/5''H) cross-peak region of this spectrum. Additional cross peaks that were detected in the corresponding regions on the other side of the diagonal in the same spectrum are included in Table I. The abundance of consistent multiple-step coherence transfer cross peaks gives further confidence in the 5'H/5''H assignments indicated in Figure 3. The chemical shifts of the deoxyribose protons at 37 °C are presented in Table II.

In addition to the signals from the dodecamer, the spectrum contains a number of cross peaks labeled with an "x", which are due to impurities in the sample. According to their peak intensities in the 1D spectrum, the concentration of these impurities is less than 2% of the dodecamer concentration, and they do not give rise to observable cross peaks in NOESY spectra. Their intense cross peaks in the TOCSY spectrum suggest that they are due to shorter DNA sequences with a rapid overall tumbling or with mobile (end) pieces with short correlation times. Due to their slower T_2 relaxation during the extended mixing periods, their cross peaks are enhanced relative to the cross peaks originating from the 12-mer in the TOCSY experiment. On the other hand, the cross relaxation during the mixing time of the NOESY experiment favors longer correlation times, making these impurities negligible.

The most promising applications resulting from the 5'H/5''H resonance assignments are distance constraints for the DNA backbone conformation, which can be extracted from NOESY cross peaks to these resonances. Figure 6 shows a stereo plot of two sequential nucleotides in B-form DNA. In

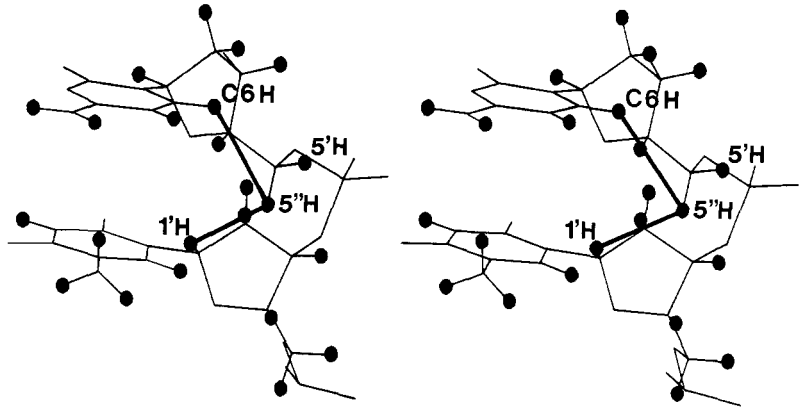

FIGURE 6: Stereo plot of two sequential nucleotides (T8, C9) in the crystal structure of the *Eco*RI restriction site DNA dodecamer in B-form conformation (Dickerson et al., 1981). Lines connect the 5''H proton to its own C6H base protons and to the 1'H proton of the preceding nucleotide, which are within NOE distance.

this conformation the 5″H of a pyrimidine (purine) nucleotide is close to its own C6H (C8H) base proton and to the 1′H proton of the preceding nucleotide in the sequence.

However, care has to be taken in deriving distance constraints from these NOEs. First, there is no through-bond mechanism for distinguishing between 5′H and 5″H resonances. In the preferred conformation about the C4′–C5′ bond (sc^+) the 4′H–5′H and 4′H–5″H couplings are nearly identical. Even if the coupling constants were different, the larger geminal 5′H/5″H coupling would lead to fast equilibration of the transferred coherence within the 5′H/5″H subsystem during the relatively long TOCSY mixing times, making the cross-peak intensities equal. A series of TOCSY experiments performed with varying lengths of mixing might clarify this point but would be time-consuming. Furthermore, the 5′H and 5″H resonances have nearly identical chemical shifts, which inevitably gives rise to overlapping NOESY cross peaks. Second, little is known about the dynamics of the backbone protons, which might have quite different correlation times, thus affecting the NOE intensities. Deuterium NMR studies of the DNA backbone dynamics are under way in this laboratory. However, being aware of these difficulties and taking them into account, it should nevertheless be possible to arrive at additional distance constraints for the backbone linker using the new 5′H/5″H resonance assignments.

The intensities of single- and multiple-step coherence transfer cross peaks in the TOCSY spectrum are markedly different for different nucleotides in the DNA sequence. The intensities of cross peaks to 5′H/5″H resonances depend critically on the 4′H–5′H and 4′H–5″H coupling constants, which in turn are a function of the torsion angle around the C4′–C5′ bond. However, differences in local dynamics along the DNA sequence could lead to differing relaxation times of the protons that affect the observed cross-peak intensities as well, allowing a qualitative interpretation of cross-peak intensities at best. For example, the strong intensity of all cross peaks involving the 5′H/5″H resonances of cytosine 1 is consistent with the unique dihedral angle about the C4′–C5′ bond of this residue as found in the crystal structure by Drew et al. (1981), but it might as well be a result of a higher mobility of the 5′-end in solution.

In summary, for the *Eco*RI restriction site we have demonstrated the feasibility of complete 5′H/5″H resonance assignment in a dodecamer using two-dimensional TOCSY experiments with mixing times of up to 500 ms. The small dispersion of 5′H/5″H chemical shifts in this dodecamer is rather typical, but there may be more favorable sequences in this respect. The extended mixing periods accentuated cross peaks due to small amounts of impurities with short correlation times that are completely negligible in NOESY experiments. For the assignment of longer or more complicated DNA sequences using isotropic mixing, further purification would be advantageous. Increasing the mixing time from 150 to 300 ms resulted in an increasing number of multiple-step coherence transfers involving 5′H/5″H resonances. The experiment with 500 ms yielded little additional information, mainly because some of the weaker cross peaks were obscured by experimental artifacts. We expect that the assignment of 5′H/5″H resonances will allow the inclusion of additional distance constraints in the calculation of DNA conformations.

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Registry No. d(CGCGAATTCGCG), 77889-82-8; deoxyribose, 533-67-5.

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