Structural studies of the 5'-phenazinium-tethered matched and G-A-mismatched DNA duplexes by NMR spectroscopy

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Summary

The mechanism through which modified oligo-DNA analogues act as antisense repressors at the transcriptional and translational level of gene expression is based on the information content in the nucleotide sequence which is determined by the specific base pairing. The efficiency of such action is largely determined by the stability of the duplex formed between the oligonucleotide reagent and the target sequence and also by the mismatched base pairing, such as G-A, that occurs during replication or recombination. We herein report that the phenazinium (Pzn)-tethered matched duplex p(d(TGTT-TGGC)):(Pzn)-p(d(CCAAACA)) (III) (T_m = 50°C) has a much larger stability than the parent matched duplex p(d(TGTTTGGC)):p(d(CCAAACA)) (I) (T_m = 30°C). On the other hand, the Pzn-tethered G-A-mismatched duplex p(d(TGTTTGGC)):(Pzn)-p(d(ACAAACA)) (IV) (T_m = 34°C) is only slightly more stable than its parent mismatched duplex p(d(TGTTTGGC)):p(d(ACAAACA)) (T_m = 25°C). A detailed 500 MHz NMR study and constrained MD refinements of NMR-derived structures have been undertaken for the DNA duplexes (I), (II), (III) and (IV) in order to understand the structural basis of stabilization of Pzn-tethered matched DNA duplex (ΔT_m = 20°C) compared to mismatched duplex (ΔT_m = 9°C). Assignment of the 1H-NMR (500 MHz) spectra of the duplexes has been carried out by 2D NOESY, HOHAHA and DQF-COSY experiments. The torsion angles have been extracted from the J-coupling constants obtained by simulation of most of the DQF-COSY cross-peaks using program SMART. The solution structure of the duplexes were assessed by an iterative hybrid relaxation matrix method (MORASS) combined with NOESY distances and torsion angles restrained molecular dynamics (MD) using program Amber 4.0. The standard Amber 4.0 force-field parameters were used for the

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oligonucleotide in conjunction with the new parameters for Pzn residue which was obtained by full geometry optimization using ab initio program (3-21G basis set). It has been shown that mismatched G-A bases are in the anti-anti conformation. The mismatched \(^7\)G-\(^1\)A form stable base pairs through inter-strand hydrogen bonds (N7(A)⋯HN2(G) (1.92 Å) with a subtended angle of 176° and N3(G)⋯HN6(A) (2.01 Å) with a subtended angle of 153° (the ‘amino-type’ hydrogen bond)) and a propeller twist of 36° for \(^7\)G-\(^1\)A residues. Observation of the nOe connectivities amongst the Pzn protons and Pzn-oligonucleotide protons in the spectrum of the duplex (III) has allowed the unambiguous determination of stereochemical orientation of the Pzn residue in the matched duplex (III). It has been found that the Pzn residue stacks with both residues of the neighbouring G-C base-pair in the matched duplex (III) and strongly stabilizes the matched G-C base-pair, while the Pzn residue in the mismatched duplex (IV) adopts at least three different conformations in the NMR time scale, and it only partly stacks with the neighbouring G-A base-pair in one of these three conformations. Excellent stacked geometry of the Pzn residue perpendicular to the main axis of matched DNA duplex (III) may be partly responsible for the additional stability of the B-type DNA structure.

Key words: \(^1\)H-NMR; 5'-Phenazinium; DNA duplex; Antisense oligonucleotide

Introduction

Design and structural investigations of the gene-directed biologically active compounds (antisense-oligonucleotides) is a promising approach for the construction of the specific therapeutic agents [1]. Furthermore, oligonucleotides that specifically recognize messenger RNA or double stranded DNA present unique opportunities for selectively inhibiting protein synthesis via the antisense approach or for modulation of gene expression, e.g., via triple helix formation. One of the ways to improve the efficiency and specificity of the antisense oligonucleotides is to increase the stability of the DNA duplex by introducing polyaromatic systems to the oligonucleotide chain through a covalent linker. The 2-methoxy-6-chloro-9-aminoacridine (Acr) [2,3] and \(N\)-(2-hydroxyethyl)phenazinium (Pzn) [4] have been shown by ultraviolet (UV) absorption studies to strongly stabilize the complexes (Table 1) by 10–20°C compared with DNA duplexes without dyes. NMR studies of the 2-methoxy-6-chloro-9-aminoacridine [5–8] derivatives have shown that these dyes promote stacking interactions with the base pairs in the duplex. In the process of transcription and translation of the genetic apparatus, mismatch G-A base-pair in the DNA occur inevitably as a consequence of either biosynthetic errors or mispairing during genetic recombination [9–11]. Moreover DNA mismatches have been found to occur as a result of the chemical modifications [12,13]. If these DNA mismatches are not corrected by cellular enzymatic repair system, they may lead to irreversible mutations [14]. The questions that we attempt to address in this paper are as follows: (1) can an oligo-DNA, bearing a polyaromatic residue at the 5’-end, discriminate between the complementary DNA sequence and a sequence having one mismatched G-A base-pair? (2) What are the differences in the structural characteristics of the matched DNA-dye duplex and mismatched DNA-dye duplex? In order to answer the first question we have investigated the stability of the four complementary DNA duplexes by UV absorption studies (Fig. 1). For this purpose we have used the Watson-Crick base-paired DNA matched duplex (I), the
TABLE 1
Melting points of the oligonucleotide duplexes bearing covalently attached polyaromatic system

<table>
<thead>
<tr>
<th>Intercalator</th>
<th>3' or 5'</th>
<th>Type of the duplex</th>
<th>T_m (°C)</th>
<th>Ref.</th>
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<tr>
<td>2-methoxy-6-chloro-9-aminoacridine (Acr)</td>
<td>3'</td>
<td>5'-d(TACG)-3'-(CH_2)_5-Acr + 5'-d(GATA)-3'</td>
<td>27</td>
<td>5</td>
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<td></td>
<td></td>
<td>5'-d(T*CTGT)-3'-(CH_2)_5-Acr + 5'-d(GCAGAA)-3', two isomers</td>
<td>42</td>
<td>6</td>
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<tr>
<td></td>
<td>5'</td>
<td>Acr-(CH_2)_5-α-5'-d(TCTAACTC)-3'</td>
<td>52</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>β-3'd(AGATTTGAG)5'</td>
<td>33</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α-5'-d(TCTAAACTC)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>β-3'd(AGATTTGAG)5' poly(A) with:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5' and 3'</td>
<td>Acr-(CH_2)_5-(pT)_4-p(CH_2)_5-Acr</td>
<td>39-42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'</td>
<td>Acr-(CH_2)_5-(pT)_8</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'</td>
<td>Acr-(CH_2)_5-p(Et)(CH_2)_5-(pT)_8</td>
<td>39-42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'</td>
<td>(Tp)_8(CH_2)_5-Acr</td>
<td>39-42</td>
<td></td>
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<tr>
<td></td>
<td>3'</td>
<td>(Tp)_8(CH_2)_5-Acr</td>
<td>42</td>
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<td></td>
<td></td>
<td>middle (Tp)_3Tp(CH_2)_5-AcrTp(pT)_3</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>3'</td>
<td>(Tp)_3Et</td>
<td>14</td>
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<tr>
<td>N-(2-hydroxyethyl)phenazinium chloride (Pzn)</td>
<td>5'</td>
<td>5'-p(dAACCTGTGGG)-3' + (Pzn)NH(CH_2)_nNH5'-p(d(CCAAAA)A)-3'</td>
<td>4</td>
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<td></td>
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<td>5'-p(dAACCTGTGGG)-3' + 5'-p(d(CCAAAA)A)-3'</td>
<td>28</td>
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<tr>
<td></td>
<td></td>
<td>5'-p(dAACCTGTGGG)-3' + NH_2(CH_2)_5NH5'-p(d(CCAAAA)A)-3'</td>
<td>28</td>
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</tbody>
</table>

Mismatched duplex having one G-A mismatch at the 5'-end (II), and the corresponding matched and mismatched DNA duplexes conjugated with 5'-PO_2-NH-(CH_2)_7-NH-Pzn moiety, (III) and (IV) respectively (Fig. 1). The reason to choose covalently linked N-(2-hydroxyethyl)phenazinium residue for stabilizing DNA duplexes is clear from the data presented in Table 1. It shows that the stability of the duplex having polyaromatic system depends upon the length of the methylene linker. For example, the T_m of a DNA duplex with a -(CH_2)_7- linker is 47°C while it decreases to 41°C with a -(CH_2)_2- linker. Clearly, an optimally tailored linker restricts the degrees of the stereochemical orientation of the polyaromatic system with respect to the duplex. In Fig. 1, the UV melting curves and the T_m of the duplexes (I), (II), (III) and (IV) are tabulated. It shows that the T_m of the Pzn-tethered matched duplex (III) increases by the 20°C with respect to its parent matched duplex (I) while the T_m of the Pzn-tethered mismatched duplex (IV) increases by only 9°C compared to its parent mismatched duplex (II). This paper deals with conformational studies on these duplexes (I)–(IV) in order to under-
Fig. 1. Comparison of melting curves of matched DNA duplex (I) and the 5'-Pzn-linked matched duplex (III) with those of the G-A-mismatched duplex (II) and the corresponding 5'-Pzn-linked mismatched duplex (IV) are shown along with their respective $T_m$. The comparison of $T_m$ of the duplex with the Pzn-linked heptamer (nitrogen of the Pzn ion linker is covalently linked with phosphorous of the 5'-phosphodiester ($\Theta$) of the heptamer) shows enhanced stability of the matched duplex (III) by at least 20° compared with the mismatched duplex (IV) which rises only by 9°. The numbering of the residues in the duplexes are shown by superscripts.

Understand the mechanism of stabilization of the mismatched vs. matched complementary DNA complexes by the 5'-tethered Pzn moiety using 500 MHz NMR spectroscopy (torsional angles, NOESY distance restrained hybrid matrix), molecular dynamics and mechanics calculations.
Materials and Methods

(I) Synthesis and sample preparation

Oligonucleotides 5'-p(d(TGTTTGGC))-3', 5'-p(d(CCAAACA))-3' and 5'-p(d(ACAAACA))-3' were synthesized by modified phosphotriester method [15] and isolated by ion-exchange and reverse-phase HPLC. N-(2-hydroxyethyl)phenazinium ion (Pzn) was covalently bonded through the -NH-(CH₂)₂-NH- linker attached to the 5'-phosphate of the oligonucleotides 5'-p(d(CCAAACA))-3' and 5'-p(d(ACAAACA))-3' using a published procedure [4]. The final products Pzn-NH-(CH₂)₂-NH-PO₂-S'(d(CCAAACA))-3' and Pzn-NH-(CH₂)₂-NH-PO₂-S'(d(ACAAACA))-3' were purified by reverse-phase HPLC chromatography and ion exchanged on a Dowex 50 × 4 (Na⁺) column, twice lyophilized to dryness from D₂O and dissolved in 0.4 ml of the buffer (0.1 M NaCl, 10 mM NaD₂PO₄, 10 mM EDTA (pH 7.4) in D₂O (99.96%)) for NMR measurements (10 mM).

(II) Nuclear magnetic resonance spectroscopy

All ¹H-NMR spectra were recorded on a Bruker AMX-500 NMR spectrometer (500 MHz ¹H). Experiments in aqueous solutions were performed by using the pulse sequence 45-τₘ-45 (τₘ = 195 ms, relaxation delay of 10 s) with a sweep width of 5050.5 Hz. NOe-spectra were obtained by subtracting the selectively irradiated spectrum from the nonirradiated spectrum. Irradiation was performed with low power on a corresponding resonance frequency, accumulated with 512 FIDs. The preirradiation period was 0.4-1.0 s. In order to study the chemical shift as a function of temperature, a series of spectra were recorded over the temperature range 283-343 K at 5-K intervals. Phase-sensitive NOESY experiments [16] were performed at 285 and 294 K using the following parameters: mixing time was varied 0.06, 0.08, 0.2, 0.4, 0.6, 0.8 s, 2000 complex data points in t₂, 256 complex data points in t₁, a relaxation delay of 2.0 s, a sweep width of 5050.5 Hz in both dimensions, acquisitions per FID was varied: 400 (τₘ = 0.06, 0.08 s), 112 (τₘ = 0.2, 0.4, 0.6 s), 64 (τₘ = 0.8 s); a Lorenz apodization function for t₂, and a shifted sine-bell apodization function for the t₁ dimension. The data were zero-filled in t₁ to give 2048 × 2048 complex data points. The residual water resonance was saturated during the relaxation delay. NOe cross peak volumes were measured using the program AURELIA [17] with segmentation level 0.5 and 100 iterations. Two-dimensional data sets for DQF-COSY [18] and HOHAHA [19,20] spectra were collected in the phase-sensitive mode with the time-proportional phase incrementation with and without phosphorus decoupling. Two-dimensional MLEV 17 HOHAHA experiments were recorded at mixing time of 96 ms. Typically 4096 or 8192 data points were collected for each of 512 t₁ values in DQF-COSY or HOHAHA experiments. The 512 × 4096 or 1024 × 8192 data points were resolution enhanced by a shifted squared sine-bell window function in both the t₁ and t₂ directions, then Fourier transformed and phase adjusted. A 90° pulse width of 10.25 ms and a relaxation delay of 2 s were used. The data were collected with the nonspinning sample to avoid t₁ noise. E.cosy [21–23] experiments were collected in the phase-sensitive mode at 294 K with and without ³¹P decoupling. These
experiments were run to help in extracting both $^1$H-$^1$H and $^1$H-$^31$P coupling constants that were not resolvable by DQF-COSY experiments. Typically 512 spectra of 4096 data points (128 scans) were collected using a sweep width of 5050.5 Hz. Quadrature detection in $t_1$ was achieved with TPPI mode. The $t_1$ domain was zero-filled to 2048 and a sinesquare ($\pi/4$) window was applied in both dimensions before Fourier transformation.

(III) NOESY distance restrained molecular mechanics/dynamics calculations of the duplexes

The initial Cartesian coordinates of the duplexes were generated by using the molecular mechanics/dynamics program Amber 4.0 [24] using Arnott right-handed B-DNA coordinates. The duplexes were then modified to accommodate the $^1$A adenosine base and $^7$G guanosine base either in an anti or syn form using the molecular modelling program QUANTA, operating on a Silicon Graphics 4D 35G workstation. The standard Amber 4.0 force-field parameters were used for the molecular mechanics/dynamics calculations. The force-field parameters and the geometry for N-(2-hydroxyethyl)phenazinium ion was added in to the Amber 4.0 force-field using a procedure described in the next section.

(IV) Parametrization and geometry definitions of the covalently linked phenazinium ion (Pzn) residue

The X-ray structure of N-methylphenazinium ion [25] was used as the starting geometry for the ab initio geometry optimization. To the X-ray structure was added the N2, HN2 and CC atoms (Fig. 2). Hydrogens for the two methyl groups were also manually added to the X-ray structure. This structure was completely (87 internal coordinates, ICs) optimized at RHF/STO-3G level by the Gaussian 90 ab initio program [26]. The final structure from the STO-3G optimization was then further optimized by the use of 3–21G basis set. To the structure was now added the CA and OA atoms as well as the CD and ND atoms (Fig. 2). Hydrogens were added to fill the valencies. The geometry of this new structure was again optimized with the internal coordinates of the tricyclic part held fixed (57 ICs) and only the new added part was optimized (57 ICs) using the 3–21G basis set. All constraints were removed and a full optimization was performed with the 3–21G basis set. The torsion C3-C2-N2-CC of the fully optimized structure was manually changed from $\sim 0^\circ$ to $180^\circ$ and all 114 internal coordinates of the new conformer were fully optimized again. The equilibrium values for the bond lengths and the bond angles to be used in the AMBER force-field were measured on the structure obtained after this multi-step geometry optimization procedure. The same structure was also used as input for the calculation of atomic charges for the electrostatic interactions in the AMBER force-field. The atom centered point charges used in the AMBER program package can be calculated with the GAUSSIAN 92 program. The electrostatic potential is computed at points in space close to the molecule, the points are however not allowed to be within the van der Waal radius of any of the atoms.
Atom centered point charges are then calculated which fit with the electrostatic potentials at the selected points in space. The Merz-Singh-Kollman approach [28,29] for the point selection with the dipole moment constrained to reproduce the Mulliken dipole (included in GAUSSIAN 92) was used with atomic radii as they are in the AMBER all atom force-field [30]. Since Singh and Kollman have employed [29] atomic charges based on STO-3G calculations, we have also used
the atomic charges based on the STO-3G basis set for AMBER calculations (vide infra).

It was realized that some of the atom types in the standard AMBER force-field could not be used for our Pzn residue since the standard equilibrium bond lengths

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TABLE 3
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and angles were too different from the ones found in the ab initio structure. The geometry of the tricyclic part was heavily distorted after energy minimization using the standard AMBER atom types and equilibrium bond lengths and angles. Therefore we introduced several new atom types and added our own equilibrium bond lengths and angles for these. All the necessary internal coordinates were taken from the final 3–21G ab initio structure since these are mutually consistent. The nonbonded parameters for our new atom types as well as the force constants were copied from the most similar atom types in the standard AMBER force-field. Force constants for the N-P bond and the bond angles involving these atoms were taken from the corresponding O-P bond. The equilibrium values for the N-P bond length and the related bond angles were, however, taken from a 3–21G geometry optimized structure (Me-O-PO$_2$-NH-Me). The force constants for torsional angles and improper torsions could not be copied from the most similar types in the standard force-field. In order to keep the phenazine rings in a plane it was necessary to increase the force constants for the improper dihedrals to 14.0 kcal.mol$^{-1}$ rad$^{-2}$ and to increase the dihedral force constants to 24.4 kcal.mol$^{-1}$ rad$^{-2}$ for the heavy atoms in the three rings to ensure that the tricyclic part of the Pzn residue is planar even if it is in an intercalated geometry in the DNA. All data are exhibited in Tables 2–7.

Results

Assignment of nonexchangeable protons

(A) Proton resonances of DNA duplexes (I) and (II) with and without mismatch. The assignment of the nonexchangeable protons of matched DNA duplex (I) was accomplished in a sequential manner [31–33]. The chemical shifts of protons of
TABLE 4
Added improper dihedral parameters for the Pzn residue in the AMBER calculations

<table>
<thead>
<tr>
<th>Atom types</th>
<th>Force constant, $V_n/2$ (kcal.mol$^{-1}$ rad$^{-2}$)</th>
<th>Phase, $\gamma$ (°)</th>
<th>Periodicity $(n)$</th>
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</thead>
<tbody>
<tr>
<td>X-X-NX-CT</td>
<td>14.0</td>
<td>180.0</td>
<td>2.0</td>
</tr>
<tr>
<td>NX-C9-CS-CO</td>
<td>14.0</td>
<td>180.0</td>
<td>2.0</td>
</tr>
<tr>
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<td>180.0</td>
<td>2.0</td>
</tr>
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<td>C6-NC-C5-CO</td>
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<td>180.0</td>
<td>2.0</td>
</tr>
<tr>
<td>CS-NC-C9-CO</td>
<td>14.0</td>
<td>180.0</td>
<td>2.0</td>
</tr>
<tr>
<td>C8-H2-N2-CT</td>
<td>1.0</td>
<td>180.0</td>
<td>2.0</td>
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<tr>
<td>CO-C7-C8-N2</td>
<td>14.0</td>
<td>180.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

this duplex (I) [34] at 294 K at different concentrations are shown in Table 8 which are based on our analysis of detailed NOESY and DQF-COSY spectra (not shown). The nonexchangeable base and sugar ring protons of the duplex with the G-A mismatch (II) have been assigned by using (a) Hartmann-Hahn spectroscopy

TABLE 5
Added bond parameters for the Pzn residue in the AMBER calculations

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<thead>
<tr>
<th>Atom types</th>
<th>Force constant (kcal.mol$^{-1}$ Å$^{-2}$)</th>
<th>Equilibrium bond length (Å)</th>
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<td>OS-P</td>
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TABLE 6
Added dihedral parameters for the Pzn residue in the AMBER calculations

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<th>Atom types</th>
<th>Multiplicity (number of bonds)</th>
<th>Force constant, $V_2/2$ (kcal.mol$^{-1}$ rad$^{-2}$)</th>
<th>Phase, $\gamma$ (°C)</th>
<th>Periodicity ($n$)</th>
<th>Force constant copied from these original AMBER atom types</th>
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<td>X-CT-NX-X</td>
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<td>2.0</td>
<td>X-CT-N*-X</td>
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<tr>
<td>X-CS-NX-X</td>
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<td>24.4</td>
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<tr>
<td>X-C6-NX-X</td>
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<td>24.4</td>
<td>180.0</td>
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<td>X-CO-CS-X</td>
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<td>180.0</td>
<td>2.0</td>
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<td>180.0</td>
<td>2.0</td>
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<td>X-C5-C9-X</td>
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<td>24.4</td>
<td>180.0</td>
<td>2.0</td>
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<tr>
<td>X-C5-C6-X</td>
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<td>2.0</td>
<td></td>
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<td>X-C1-CO-X</td>
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<td>2.0</td>
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</tr>
</tbody>
</table>

to demonstrate direct and relayed through-bond connectivities along the $H1' \leftrightarrow H2' \leftrightarrow H3' \leftrightarrow H4' \leftrightarrow H5'/H5''$ pathway within each sugar unit and (b) phase-sensitive NOESY spectroscopy to show through-space connectivities along the $H1'/H2'_{(i+1)} \leftrightarrow H8'/H6'_{(i)} \leftrightarrow H1'/H2'_{(i)}$ and the $H8'/H6'_{(i)} \leftrightarrow H5'/CH_{3(i+1)}$ pathway ($\tau_m = 0.06, 0.08, 0.2, 0.4, 0.6, 0.8$ s at temperature 294 K and $\tau_m = 0.1, 0.15, 0.2, 0.4$ s at 285 K). In this way, the set of proton resonances which belong to different residues of DNA duplexes have been unambiguously distinguished and assigned. Some examples of NOESY spectra with mixing time 0.8 s in D$_2$O are shown in Fig. 3. Expanded contour plot establishing nOe connectivities between the base protons (8.5–7.0 ppm) and the $H1'$, cytosine H5-H6 protons (Fig. 3a) (which were also established from the DQF-COSY spectra (6.5–5.0 ppm)), the base protons and $H2'/H2''$ (3.0–1.3 ppm) (Fig. 3b) and the $H1'$ and $H2'/H2''$ (Fig. 3c). Each base H8/H6 proton have been demonstrated to exhibit an nOe to its own and 5'-linked $H1'$. This general procedure helped us to trace from $^1A$ to $^7A$ and $^1T$ to $^8C$, respectively, including the $^7G$ and $^1A$ at the G-A mismatch site. However, the $^8C(H6)$ and $^2C(H6)$, $^1T(H6)$ and $^6G(H8)$, $^2G(H8)$ and $^7G(H8)$ overlap each other. In order to confirm our above assignments of the aromatic protons to the $H1'$, we have performed the NOESY experiments at 285 K. At 285 K, the cross-peak connecting H5 and H6 of one of the three cytosines absorbed more upfield than...
<table>
<thead>
<tr>
<th>Atom types</th>
<th>Force constant (kcal.mol(^{-1}) rad(^{-2}))</th>
<th>Equilibrium bond angle (°)</th>
<th>Force constant copied from these original AMBER atom types</th>
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<td>CA-CA-CA</td>
</tr>
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<td>CO-C1-HC</td>
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<td>CB-CB-N*</td>
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### TABLE 8

Proton chemical shifts (δ scale) of DNA duplexes (I), (II), (III) and (IV) at 294 K (0.1 M NaCl, 10 mM NaD₂PO₄, 10 mM EDTA (pH 7.4) in D₂O)

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<th>H5/CH₃</th>
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<th>H2'</th>
<th>H2''</th>
<th>H3'</th>
<th>H4'</th>
<th>H5'/H5''</th>
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(continued overleaf)
the other two cytosines at 7.20 and 5.25 ppm. They were unambiguously assigned from the nOe connectivities with $^5$A(H8) and $^5$A(H1') to the $^6$C(H5, H6). The cytosine H6 at 7.50 ppm shows an nOe connectivity with $^1$A(H1') resonance. By using these facts all the $^2$C(H5), $^8$C(H5), $^2$C(H1') and $^8$C(H1') were sequentially assigned as it is shown in Fig. 3a. It is noteworthy that $^2$C(H1') moves upfield by more than 0.7 ppm with respect to the corresponding protons in $^8$C and $^6$C. The same procedure was repeated to assign $^8$C(H1'). At 285 K it moves upfield by 0.1 ppm. The chemical shift of $^1$T(H6) has been established using nOe-connectivities of the 5'-neighbouring nucleotide protons: H8/H6 $\leftrightarrow$ CH3(i) $\leftrightarrow$ H6(i). In accordance with this approach, the sequence of assignment has been shown by dotted line in Fig. 3b: $^5$G(H8) $\leftrightarrow$ $^3$T(CH3) $\leftrightarrow$ $^3$ T(H6) $\leftrightarrow$ $^4$T(CH3) $\leftrightarrow$ $^4$T(H6) $\leftrightarrow$ $^5$T(CH3) $\leftrightarrow$ $^5$T(H6). In the NOESY spectra of duplex (II), the exchangeable cross-peak between $^6$G(H8) and $^6$G(H1') has disappeared. This allowed us to assign the downfield cross peak at 5.95, 7.65 ppm to $^1$T(H1', H6).

Fig. 3. The NOESY spectrum (800 ms) of the mismatched p(d(TG'TIT'FGGC)): p(d(ACAAACA)) duplex (II) in 0.1 M NaCl, 10 mM NaD2PO4, 10 mM EDTA (pH 7.4) in D2O at 294 K. Off-diagonal peaks in (a) show the sequential H1(i)- H8/H6(i) $\leftrightarrow$ H1'(i) connectivities for octamer (solid line) and heptamer (broken line) for H8/H6 (F1 axis) $\leftrightarrow$ H1'/H5 (F2 axis). Cross-peaks $^8$C(H5,H6), $^8$C(H5,H6), $^2$C(H5,H6) are marked by i, j, k, respectively. Cross-peaks between H8/H6 $\leftrightarrow$ H5(i+1) base protons are shown: (1) $^7$G(H8) $\leftrightarrow$ $^6$C(H5), (2) $^5$A(H8) $\leftrightarrow$ $^6$C(H5), (3) $^1$A(H8) $\leftrightarrow$ $^2$C(H5). Subspectra (b) and (c) show intraresidue (solid line) interactions between the H8/H6 $\leftrightarrow$ H2'/H2" and H1'/H2'/H2", respectively. H8/H6 (F1 axis) $\leftrightarrow$ H2'/H2"/CH3 (F2 axis) in subspectrum (b), H1'/H5 (F1 axis) $\leftrightarrow$ H2'/H2"/CH3 (F2 axis) in the subspectrum (c). The H8/H6(i-1) $\leftrightarrow$ CH3(i+1) connectivities are shown in (b) as dashed lines. Cross-peaks between H1'(i) $\leftrightarrow$ (CH3)i+1 are shown in (c) as: (1) $^4$T(H1') $\leftrightarrow$ $^5$T(CH3), (2) $^3$T(H1') $\leftrightarrow$ $^4$T(CH3), (3) $^2$G(H1') $\leftrightarrow$ $^3$T(CH3). Subspectrum (d) shows (H8/H6 (F1 axis) $\leftrightarrow$ H8/H6 (F2 axis)) cross-peaks between protons of neighbouring bases H8/H6(i) $\leftrightarrow$ H8/H6(i+1), H2(i) $\leftrightarrow$ H2(i+1), or H8/H6(i) $\leftrightarrow$ H5(i+1): (1) $^4$T(H6) $\leftrightarrow$ $^3$T(H6), (2) $^4$T(H6) $\leftrightarrow$ $^5$T(H6), (3) $^6$G(H8) $\leftrightarrow$ $^5$T(H6), (4) $^3$A(H2) $\leftrightarrow$ $^4$A(H2), (5) $^5$G(H8) $\leftrightarrow$ $^6$G(H8), (6) $^2$G(H8) $\leftrightarrow$ $^1$T(H6), (7) $^7$G(H8) $\leftrightarrow$ $^8$C(H6), (8) $^2$G(H8) $\leftrightarrow$ $^3$T(H6), (9) $^5$A(H8) $\leftrightarrow$ $^6$C(H6), (10) $^7$A(H8) $\leftrightarrow$ $^6$C(H6), (11) $^3$A(H8) $\leftrightarrow$ $^2$C(H6), (12) $^1$A(H8) $\leftrightarrow$ $^2$C(H6).

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The second independent assignment of the aromatic protons was carried out through the connectivities of H8/H6 and H2'/H2": 5'(H8/H6 ↔ H2'/H2")i, ↔ (H8/H6 ↔ H2'/H2")i−1, ↔ (H8/H6 ↔ H2'/H2")i−3'. Complete set of cross-peaks from fifteen H2'/H2" and aromatic resonances were indeed observed and are indicated by horizontal solid lines in Fig. 3b. To assign the signals of the H2'/H2" the ratio of intensities of these cross-peaks in the regions of H1', H2'/H2" and H8/H6, H2'/H2" were taken into consideration at a shorter mixing time. In the B-type DNA, the distance between H8/H6 and H2' is shorter than between H8/H6 and H2" [35,36]. The distance between H1' and H2" is not longer than between H1' and H2'. As seen from Figs. 3b and 3c, the off-diagonal peaks arising from H8/H6 and H2' are stronger than those from H8/H6 and H2" (Table 9). Also note that the cross-peaks from H1', H2" are more intense than those from H1', H2' (except for the terminal base 7A and 7G). The principal assignment pathway using the NOESY-COSY connectivity diagram are plotted in Fig. 4. At a mixing time of 80 ms, only (H8/H6 ↔ H2')i and (H2")i ↔ (H8/H6)i+1 connectivities are observed (Fig. 4). Thus there is one intraresidue and one sequential connectivity for each base. Following the arrows in Fig. 4, the assignments can be traced all the way to the last residue in the octanucleotide. The H2" protons are located more downfield than H2' which is in agreement with a B-type DNA structure [35,36]. But it can be seen from the NOESY/COSY connectivity (Fig. 4) that this is reversed for the corresponding resonances in 7A and 7G in which H2" is more upfield than H2'.

Discrimination between the H2' and H2" resonances have been made on the basis of proton-proton coupling constant considerations (vide infra, Table 13). The observation of relatively strong intraresidue nOes (H8/H6 ↔ H2')i (Table 9) in combination with equally strong interresidue (H2")i ↔ (H8/H6)i+1 (Table 10) suggests that the duplexes in the present study adopt predominantly a B-DNA-type helix in solution. Examination of the H8/H6 ↔ H2'/H2" region of the NOESY spectrum (τm = 200 ms) shows an unusually weak interaction between 1A(H8) and 2C(H2") and 7A(H8) and 6C(H2") in the heptamer strand of the duplex (II), suggesting a local distortion of the right-handed B-DNA-type structure. Note that for octamer strand in the same duplex (II), we however observe only expected intraresidue nOe cross-peaks (Table 10) which suggest that the mismatch in the heptameric strand do not seriously distort the stacked structure of the octamer.

To determine the stacking interactions amongst the base-pairs, we have examined the aromatic region of the NOESY spectrum (τm = 800 ms) of duplex (II) as shown in Fig. 3d. We observe cross-peaks arising from the core residues: 2G(H8) ↔ 3T(H6), 5A(H8) ↔ 6C(H6), 5A(H2) ↔ 4A(H2), 4T(H6) ↔ 5T(H6), 6G(H8) ↔ 5T(H6), 3A(H8) ↔ 2C(H6) and also from the terminal mismatched residues 1A(H8) ↔ 2C(H6), 7G(H8) ↔ 6G(H8), 7G(H8) ↔ 8C(H6) suggesting a similar base-base orientation of the 7G and 1A bases in mismatched duplex (II) in comparison with the stackings in the counterpart 6G ↔ 7G ↔ 8C in the matched duplex (I) as would be expected from a classical B-DNA type conformation. The interproton distances between the H8/H6 of the base and its own H1' is much longer (3.70 Å) than the interproton distance between H5 and H6 of cytosines (2.45 Å). For syn conformation, the two distances are approximately...
TABLE 9
Intraresidual proton distances (Å) calculated from the NOESY spectra of the duplexes (I), (II), (III) and (IV) using two-proton approximation
(Reference for C(H5)-C(H6) distance of 2.46 Å in the NOESY experiments.)

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<th>H8/H6-H2'</th>
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equal. We have compared the cross-peak intensities of $^6\text{C}(\text{H5,H6})$ relative to H8/H6 $\leftrightarrow$ H1’ in the NOESY spectra of duplex (I) and (II) at mixing times of 80 and 200 ms which also allowed us to make an assessment of the effect of the spin diffusion at $\tau_m = 80$ ms in the estimation of distances. It is clear from Table 9 that all the H8/H6 $\leftrightarrow$ H1’ intraresidue distances estimated from the two-proton approximation, including those for $^7\text{G}$ and $^1\text{A}$ for both duplexes (I) and (II) are much longer than 2.45 Å consistent with the *anti* orientation about the glycosidic bond which again shows that both matched duplex (I) and mismatched duplex (II) adopt a B-DNA type structure. As it was noted above, the cross-peaks $^7\text{G}(\text{H8})$ $\leftrightarrow$ $^7\text{G}(\text{H1'})$ and $^8\text{C}(\text{H6})$ $\leftrightarrow$ $^8\text{C}(\text{H1'})$ in the duplex (II) are crowded at 294 K which, however, separated at 285 K. The chemical shifts of the duplex (II) observed at 294 K are collected in Table 8.

(B) $^1\text{H}$ resonances of DNA duplexes (III) and (IV) containing phenazinium ion at the 5’-end. The assignment of the nonexchangeable protons of the matched duplex (III) and mismatched duplex (IV) tethered with Pzn ion at the 5’-end have been performed at 294 K (Table 8). Some NOESY spectra of the duplexes (III and IV) are shown in Fig. 5a1, a2, b1, b2, c1, c2. The assignment of nonexchangeable protons of duplex (III) and (IV) have been performed in a similar manner as described above for the duplexes (I) and (II). The main problem in the assignment of nonexchangeable protons involved the assignment of aromatic and sugar ring protons of residues neighbouring to the Pzn ion and of mismatched base-pair. We have observed similar differences in the chemical shifts in H8/H6 $\leftrightarrow$ H1’ cross-peaks of $^7\text{G}$, $^6\text{G}$, $^8\text{C}$ residues (Table 8, Fig. 5a1, a2,) for the pairs of duplexes (III), (IV) and (I), (II). Following upfield (indicated by ‘+’) and downfield (indicated by ‘−’) shifts for aromatic protons in both mismatched duplexes (II) and (IV) have been observed in comparison with the counterparts in duplexes (I) and (III): for $^6\text{G}$ ($\Delta\delta_{\text{H8}} = +0.3$, $\Delta\delta_{\text{H6}} = +0.3$, $\Delta\delta_{\text{H5}} = +0.4$) and for $^7\text{G}$ ($\Delta\delta_{\text{H8}} = -0.3$). The aromatic protons of $^8\text{C}$ (H5 and H6) in spectrum of matched duplex (III) bearing Pzn residue move downfield by more than 0.3 ppm in comparison with the corresponding protons for matched duplex (I). On the other hand, the aromatic

### Table 9 (continued)

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Fig. 4. Regions from NOESY (80 ms) and DQF-COSY spectra are put together for assignment purposes between H8/H6 aromatic protons and H2' protons in the duplex (II), p(d(TGTVFGGC)) : p(d(ACAAACA)), solid line shows the connectivities in the heptamer and dashed lines for the octamer. In the NOESY spectrum successive H2'(i-1) ↔ H8/H6(i) and H8/H6(i) ↔ H2'(i) cross-peaks are connected by vertical lines. In the DQF-COSY spectrum the H1' ↔ H2' and H1' ↔ H2" cross peaks of the each nucleotide are connected by vertical lines. Sequence-specific assignments for the base protons and the H1' protons are indicated. Horizontal lines show the H8/H6(i) ↔ H2'(i) connectivities in the NOESY spectrum with the H1' ↔ H2' in the DQF-COSY spectrum, and (H1' ↔ H2")i(i-1) connectivities in DQF-COSY with the H2'(i-1) ↔ H8/H6(i) cross peaks derived from nOe spectrum. The sequential assignment pathway can be followed from 1T to 8C for octamer and from 1A to 7A for heptamer of the duplex (II) following the arrows shown at 1A (first nucleotide of heptamer) and 1T (first nucleotide of octamer).

protons of 1C and 2C move upfield in the Pzn-linked duplex (III) compared to the matched duplex (I). We have seen the same influence of Pzn residue on the 2C (H6,H5) aromatic protons in the Pzn-linked duplex (IV). Note that the aromatic and H1' protons of dangling 8C residue can not be assigned unambiguously for the duplex (IV).

Assignment of exchangeable imino protons
Assignment of the exchangeable NH⋯N imino protons in duplexes (I), (II), (III) and (IV) have been accomplished via inspection of NOESY spectra at a
TABLE 10

Interresidue distances (Å) calculated from the NOESY spectra of the duplexes (I), (II), (III) and (IV) using two-proton approximation

(Reference for C(H5)-C(H6) distance of 2.46 Å in the NOESY experiments.)

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<th>Residue</th>
<th>( \text{H}<em>2^\prime \leftrightarrow \text{H}6/\text{H}8</em>{i+1} )</th>
<th>( \text{H}1^\prime \leftrightarrow \text{H}6/\text{H}8_{i+1} )</th>
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mixing time of 400 ms (Fig. 6). They were acquired in a mixture of H$_2$O-D$_2$O (9:1, v/v) between 273 and 278 K in order to prevent broadening due to exchange of imino protons with H$_2$O and D$_2$O. The scheme for the assignment of imino protons is shown in Scheme 1. The assignment procedure using the NOESY spectra is briefly illustrated on the duplex (II) (Fig. 6a$_1$,a$_2$). The NOESY spectrum exhibits seven large cross-peaks between δ10.5 and 14.5, which show slow exchange process with water signal at ~δ5.05 (Fig. 6a$_1$). These cross-peaks correspond to the seven imino protons $^2$G, $^6$G, $^7$G, $^1$T, $^3$T, $^4$T, $^5$T observed in the 1D NMR spectra at the same temperature (Fig. 7). Two sharp resonances at δ12.80 and δ12.68 assigned to the imino protons of Watson-Crick hydrogen bonded $^6$G and $^2$G, respectively, show cross-peaks (a) to the imino protons of $^5$T and $^3$T (δ14.26 and 14.11), (b) to the amino protons of $^6$C and $^2$C, and (c) to the H2 protons of adenine moiety shown by solid lines in Fig. 6a$_1$ and Scheme 1. Note that the H2 protons of adenine residues were already assigned on the basis of the NOESY connectivities to its own H1' (i) and to the H1' of the 3'-end residue (i + 1) (Fig. 3a). Hence, the $^5$A(H2) (δ7.75) is connected to the upfield imino guanine signal (δ12.68) and $^3$A(H2) (δ7.35) with downfield resonance at δ12.80. By using these data and Scheme 1, three downfield signals of imino protons in the region δ14.0–14.2 can be clearly assigned as $^5$T (δ14.26), $^4$T (δ14.08) and $^3$T (δ14.11). The assignment of the last five imino protons in the spectra of duplexes (I), (IV), and (III) have been accomplished in the same manner (Fig. 6b$_1$, b$_2$, 6c$_1$, c$_2$, 6d$_1$, d$_2$), respectively, and the data are collected in Table 11.

In accordance with the nucleotide compositions in duplexes (I)–(IV), four signals of the imino protons of thymidine and three signals of guanosine are expected. Usually the signals from terminal dangling base are not seen in the spectra due to the effect of rapid melting and fast exchange with the solvent [37–39]. Indeed in the spectra of matched duplex (I) imino proton signals of $^1$T and $^7$G are quite broadened (Fig. 7). In the spectra of mismatched duplex (II) at 275 K, there are two additional very broad signals at δ13.16 and 10.89 (Fig. 7). NOe-connectivities and the results from temperature-dependent chemical shift measurements were used for assignments of these signals. The temperature dependence of the imino proton of duplex (II) is shown in Fig. 8a. Note that these resonances from imino protons are broad even at 275 K and they broaden out
Fig. 5. Comparison of the essential parts in the NOESY spectrum (800 ms) of the duplexes (III) and (IV), p(d(TGTGTTGGC)): p(d(CCAACCA)) and p(d(TGTGTTGGC)): p(d(ACAAACA)), respectively, in 0.1 M NaCl, 10 mM NaD2PO4, 10 mM EDTA (pH 7.4) in D2O at 294 K. (a1) and (a2) show the sequential H1'/H6(0) → H1(0) connectivities for octamer (solid line) and heptamer (broken line) (H8/H6 (F1 axis) → H1'/H5 (F2 axis)), cross-peaks between 6C(H5,H6), 8C(H5,H6), 2C(H5,H6) are also marked by i, j, k, respectively. Cross-peaks between H8/H6, → H5(i+1) base protons are also shown: (1) 7G(H8) → 6C(H5); (2) 5A(H8) → 6C(H5); (3) 1A(H8) → 2C(H5). (b1) and (b2) show the sequential assignment (H8/H6 (F1 axis) → H2'/H2"/CH3 (F2 axis)) (intraresidue interactions are shown by solid line between the H8/H6 and H2'/H2" protons). H8/H6(i−1) → CH3(i+1) connectivities are shown also in (b1) and (b2) by dashed lines. (c1) and (c2) show the sequential assignment by solid lines between H3′-H2'/H2" protons (H1'/H5 (F1 axis) → H2'/H2"/CH3 (F2 axis)).
rapidly upon raising the temperature. This indicates that these two protons exchange much more rapidly with H$_2$O in contrast to five other imino protons from the core residues which can be still observed at 298 K. Moreover the downfield signal from both terminal imino protons move upfield considerably ($\Delta\delta = +0.28$) upon raising the temperature from 275 to 285 K. Irradiation of this imino proton gives weak nOes to the $^7$A(H2) at $\delta 7.73$. By using this fact, the resonance at $\delta 13.16$ have been assigned to the $^1$T(NH). We did not detect any nOe of the imino proton at $\delta 10.89$ with any other resonance. Similar chemical shift for imino proton has been noted for the oligonucleotide duplex having the G-A mismatch in the core [40–42]. The imino resonance at $\delta 10.89$ corresponds to a chemical shift of a non-hydrogen-bonded imino proton as observed for loop structures or exposed imino protons in syn structures. It is noteworthy that the chemical shift of the imino protons of $^1$T and $^2$G in the mismatched duplex (II) are approximately the same as for the matched duplex (I) (Fig. 7(I) and 7(I)), respectively. But the absorption of imino protons of $^6$G and $^7$G has moved upfield to the $\Delta\delta_{6G} = +0.25$ and $\Delta\delta_{7G} = +1.94$, respectively.
Fig. 6. Comparison of the imino-aromatic (a₁, b₁, c₁, and d₁) and imino-imino regions (a₂, b₂, c₂, and d₂) in the NOESY spectrum (400 ms) of (a) duplex (II), p(d(TGTTTGCC)):p(d(ACAAACAA)), (b) duplex (I), p(d(TGTTTGCC)):p(d(CCAAACAA)), (c) duplex (IV), p(d(TGTTTGCC)): (Pzn)-p(d(ACAAACAA)), (d) duplex (III) p(d(TGTTTGCC)): (Pzn)-p(d(CCAAACAA)) in 90% H₂O/10%D₂O at 275 K. Sequential connectivities are indicated by the broken line. The two protons of the amino group of cytosine or adenosine are connected by a solid line.
Fig. 7. $^1$H-NMR spectra comparing the regions of imino protons in duplex (I) p(d(TGTTTGGC)):p(d(CCAAACA)), duplex (II) p(d(TGTTTGGC)):p(d(ACAAACA)), duplex (III) p(d(TGTTTGGC)): (Pzn)-p(d(CCAAACA)) and duplex (IV) p(d(TGTTTGGC)): (Pzn)-p(d(ACAAACA)) in 90% H$_2$O/10%D$_2$O containing 0.1 M NaCl, 10 mM Na$_2$PO$_4$, 10 mM EDTA (pH 7.4) at 275 K.

In the spectra of the Pzn-linked duplexes (III) and (IV) (Fig. 7(III), 7(IV)), four imino protons (between δ11.8 and 13.3) move upfield in comparison with the corresponding matched and mismatched duplexes (I) and (II), respectively (Fig. 7(I) and (II)). The assignment of the $^6$G and $^2$G resonances of the imino protons of the Pzn-linked matched duplex (III) have been done in the same manner (Fig. 6d1, d2) as described above for the duplex (II) (Fig. 6a1, a2). The sharp upfield imino proton of the $^7$G residue at δ12.08 has shown a connectivity with the $^6$G (Fig. 6d1, 6)

<table>
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<th>Duplex</th>
<th>$^2$G(N1H)</th>
<th>$^3$T(N3H)</th>
<th>$^4$T(N3H)</th>
<th>$^5$T(N3H)</th>
<th>$^6$G(N1H)</th>
<th>$^7$G(N1H)</th>
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<td>12.88</td>
<td>12.08</td>
<td>12.79</td>
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Fig. 8. Temperature-dependent $^1$H-NMR spectra of the exchangeable imino protons of the (a) duplex (II) $p(d(TGTTTGGC)): p(d(ACAAAAA))$, (b) duplex (III) $p(d(TGTTTGCC)): p(d(CCAAACA))$, and (c) duplex (I) $p(d(TGTTTTGGC)): p(d(CCAAACA))$ at 275, 278, 285, 289, 294, and 298 K. The assignment of imino protons is shown in the subspectra. In (a) imino protons of $^5$I, $^4$I, $^3$I can be observed between 14.5 and 13.5 ppm and between 13.5 and 12.0 ppm for the terminal $^1$I, and for the G-C base pairs $^6$G, $^2$G, but the imino protons of $^7$G have been observed at 10.89 ppm. In (b) the imino protons $^5$I, $^4$I, $^3$I can be observed between 14.5 and 13.5 ppm for the A-T base pair, and between 13.5 and 12.0 ppm for the terminal $^1$I, $^6$G, $^2$G, $^7$G (G-C base pairs); (c) shows that the imino protons of $^5$I, $^4$I, $^3$I absorb as usual for duplex (I), a standard B-DNA, between 14.5 and 13.5 ppm for the A-T base pair, and between 13.5 and 12.0 ppm for the terminal $^1$I, and for the G-C base pairs ($^6$G, $^2$G, $^7$G).

d$_2$). The last signal in the spectrum of the duplex (III) (Fig. 7(III)) at $^8$12.79 have been assigned to the imino proton of the $^1$I because it quickly broadened and moved more upfield than the other three signals in this region upon raising the temperature from 275 to 298 K (Fig. 8b).

A comparison of the temperature dependent spectra of imino protons in the upfield region of the Pzn-linked matched duplex (III) (Fig. 8b) and the matched duplex (I) (Fig. 8c) shows that the resonances of $^1$I ($^8$13.24) and $^7$G ($^8$12.83) in duplex (I) broaden more rapidly than the imino protons of $^1$I ($^8$12.79) and $^7$G ($^8$12.08) in duplex (III) indicating that the former pair of protons in duplex (I) exchange much more rapidly with solvent in contrast to $^2$G and $^6$G which can be well observed at 298 K. Note that for the duplex (III), the imino resonance of $^7$G do not broaden even upon raising the temperature from 275 to 298 K. This suggests that the Pzn residue in duplex (III) has indeed stabilized the Watson-Crick hydrogen bonding in the $^7$G-$^1$C base pair.

There are comparable signals for the Pzn-linked mismatched duplex (IV) at 275 K (Fig. 7(IV)) and duplexes (II) and (III). The signals for $^1$I, $^6$G and $^2$G have been assigned (Fig. 6c$_1$, c$_2$) in the same manner as for the imino protons of the mismatched duplex (II) and they absorb more upfield than the corresponding protons in the mismatched duplex (II). These upfield shifts in the Pzn-linked mismatched duplex (IV) is most probably dictated by the diamagnetic influence of the Pzn residue in the same manner as for the Pzn-linked matched duplex (III). The chemical shift of the imino proton resonances of $^2$G in duplexes (III) ($^8$12.54)
TABLE 12
Chemical shifts of the proton signals of phenazinium residue in the match Pzn-NH-(CH₂)₂-NH-p(d(CCAAACA)) : p(d(TGTTTGGC)) (III), and mismatch Pzn-NH-(CH₂)₂-NH-p(d(ACAAACA)) : p(d(TGTTTGGC)) (IV) duplexes and Pzn-N(CH₃)₂-C1⁻ (V) [43], Pzn-NH-(CH₂)₂-NH-p(d(CCAAAC-A)) (VI), [43] (T = 294 K)

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* The signal of the minor conformers.

and (IV) (δ12.51) is pretty close. But the 6G resonance in duplex (IV) has moved more upfield (Δδ = +0.16) than the corresponding proton in the the duplex (III) which is clearly due to the influence of 7G-1A mismatch in this duplex (IV). But it is noteworthy that the imino resonance at δ12.72 for 6G residue in the duplex (IV) has broadened more significantly than the corresponding signal in the spectrum of the mismatched duplex (II) (Fig. 7(IV)). Moreover, the chemical shifts of the imino resonance for the 7G in the mismatched duplex (II) and Pzn-linked mismatched duplex (IV) are almost identical (δ10.90) which suggest that the Pzn ion do not particularly stabilize the 1A-7G mismatch. On the other hand, the imino resonance for 7G for Pzn-linked duplex (III) move more downfield as a sharp signal to δ12.08 which also suggest that the Pzn ion do not particularly stabilize the 1A-7G mismatch in the duplex (IV).

Assignment of the N-(2-hydroxyethyl)phenazinium protons in the duplexes (III) and (IV). The H1, H3, H4, H5, H6, H7 and H8 of the aromatic part of Pzn ion and -₁₁CH₂⁻⁻⁻⁻₁₂CH₂-OH and -₁₃CH₂⁻⁻⁻⁻₁₄CH₂⁻ (Table 12) protons in the linker in the modified duplex (III) were assigned (Fig. 9a) on the basis of their scalar and dipole interaction using DQF-COSY, NOESY and ¹³C-¹H correlation experiments. We have also independently confirmed the assignment for the corresponding Pzn
protons in the duplex (III) [43] at different concentrations. It may be noted that some undefined cross-peaks centered at $\delta 7.07$, $7.57$, $7.38$ and $7.73$ in the aromatic region of DQF-COSY and NOESY spectra of the duplex (III) (Fig. 10a$_1$,a$_2$) have been observed. They absorbed close to the cross-peaks of H3, H4, H5, H6 of the Pzn residue, but the intensities of these peaks are only $\sim 30\%$ of the corresponding cross-peaks arising from the major conformer. Two other groups of signals have been also observed: (1) the proton at $\delta 6.59$ has connectivity with $\delta 4.63$ and $\delta 4.01$ and (2) $\delta 6.54$ has connectivity with $\delta 4.77$ and $\delta 3.80$. The cross-peaks between these protons are not observed in the COSY spectra. These absorptions could not be assigned to the oligonucleotide protons and they have chemical shift values very close to the $-^{11}\text{CH}_2^{-12}\text{CH}_2^{-}\text{OH}$ protons of the Pzn linker arising from the major conformer of the duplex (III). It is noteworthy that the chemical shifts of these unassigned absorptions are distinctly different from the protons of the Pzn residue attached to the free single stranded Pzn-NH$\left(\text{CH}_2\right)_2\text{NH-PO}_{2}^{-}$ (d(CCAAACA)) (VI) (Table 12). The occurrence of these additional absorptions clearly suggests the presence of a minor conformer of the duplex (III) with different stereochemical orientation of Pzn residue. For the minor conformer, no cross-peaks between protons of Pzn residue and the oligonucleotide have been observed as in the major conformer (Fig. 9a) (vide infra).

The protons of Pzn residue in the mismatched duplex (IV) were assigned by the NOESY connectivities (Fig. 9b and 10b$_2$) and DQF-COSY (Fig. 10b$_1$, Table 12). The characteristic signals of the hydroxyethyl group $-^{11}\text{CH}_2^{-12}\text{CH}_2^{-}\text{OH}$ (Fig. 9b) were found at about $\delta 4.70$ and $4.00$. The signal for $^{12}\text{CH}_2^{-}$ absorbed at $\delta 4.00$ while the $^{11}\text{CH}_2^{-}$ protons absorbed at $\delta 4.70$ because they are close to the endocyclic imminium ion. The protons of the methylene chain $-^{13}\text{CH}_2^{-14}\text{CH}_2^{-}$ were assigned from the nOe connectivities with the aromatic protons of the Pzn ion. The more intense cross-peak showed connectivities at $\delta 6.13$ assigned to H1 of Pzn residue and $\delta 3.18$ assigned to $^{13}\text{CH}_2^{-}$ and the lesser intense cross-peak showed connectivities to $\delta 6.13$ and $3.01$ with H1 of Pzn residue and $^{14}\text{CH}_2^{-}$, respectively (Fig. 9b, the cross-peaks are marked by '3', '4'). NOe connectivities between the $^{11}\text{CH}_2^{-}$, $^{12}\text{CH}_2^{-}$ and $^{13}\text{CH}_2^{-}$ protons have also been observed which are marked by symbols '5' and '6' in Fig. 9b. The minor conformation of the duplex (IV) was also detected as for the duplex (III). It is easy to see from the chemical shifts of the proton signal of the Pzn residue of the minor conformation in Table 12 (the data are marked by *) that they are approximately the same for duplexes (III) and (IV). The aromatic protons of the Pzn residue of the duplex (IV) could not be assigned unambiguously. The aromatic-aromatic region of the DQF-COSY spectrum of the duplex (IV) was quite complex because of the presence of cross-peaks from more than one conformer due to different stereochemical orientation of Pzn residue. This is evident from the fact that the nOe cross-peaks have been found in the NOESY spectra at the different mixing times between the H8/H7 of aromatic Pzn proton and $-^{11}\text{CH}_2^{-12}\text{CH}_2^{-}\text{OH}$ for both major and minor conformers: $\delta 4.71$ and $\delta 4.00$ with $\delta 7.81$ (designated as '1' in Fig. 9b); $\delta 4.76$ and $\delta 4.04$ with $\delta 7.68$ (designated as '2' in Fig. 9b). Both signals at $\delta 7.81$ and $\delta 7.68$ have cross-peaks with H1 aromatic protons $\delta 6.13$, assigned to the major conformer, and $\delta 6.51$ assigned to the minor
conformer, respectively. A similar attempt for the assignment of the aromatic protons of Pzn residue in duplex (III) (Fig. 9a) clearly shows at least two different conformations (Table 12): δ7.81 (H8); 7.53 (H7); 7.07 (H6); 7.08 (H5) and δ7.68

Fig. 9. (a) Expanded contour plot of $^1$H homonuclear NOESY NMR spectrum (800 ms) of duplex (III), p(d(TGTTTGGC)):p(d(CCAAACA)), in 0.1 M NaCl, 10 mM NaD$_2$PO$_4$, 10 mM EDTA (pH 7.4) in D$_2$O recorded at 294 K (total oligomer concentration 10 mM). The dashed lines follow the connectivities between Pzn protons: (1) H8 ↔ 11CH$_2$, (2) H8 ↔ 12CH$_2$, (3) H7 ↔ 11CH$_2$, (4) H7 ↔ 12CH$_2$, (5) H1 ↔ 11CH$_2$, (6) H1 ↔ 12CH$_2$, (7) H1 ↔ 13CH$_2$, (8,9) H1 ↔ 14CH$_2$, (10) 11CH$_2$ ↔ 13CH$_2$. The cross-peaks between the protons of oligonucleotide and Pzn residues (i) 1(CH6) ↔ 13CH$_2$, (ii) 1(CH6) ↔ 14CH$_2$, (j) 1(CH5) ↔ 13CH$_2$, (j', j'') 1(CH5) ↔ 14CH$_2$, (j') 1(CH6) ↔ 14CH$_2$, (ii') 1(CH5) ↔ 14CH$_2$, (ii'') 1(CH5) ↔ 14CH$_2$ are marked. (b) Expanded contour plot of $^1$H homonuclear NOESY NMR spectrum (800 ms) of duplex (IV), p(d(TGTTTGGC)):p(d(ACAAACA)), in 0.1 M NaCl, 10 mM NaD$_2$PO$_4$, 10 mM EDTA (pH 7.4) in D$_2$O recorded at 294 K (10 mM). Note that at least three sets of cross-peaks for all (Pzn-aromatic)-(Pzn-aromatic), (Pzn-linker)-(Pzn-linker), (Pzn-aromatic)-(Pzn-linker) and between 11CH$_2$ and 12CH$_2$ of (Pzn-hydroxyethyl group) and (Pzn-aromatic) or (Pzn-linker): (1) H8 ↔ 11CH$_2$, (2) H8 ↔ 12CH$_2$, (3) H1 ↔ 11CH$_2$, (4) H1 ↔ 12CH$_2$, (5) H1 ↔ 13CH$_2$, (6) H1 ↔ 14CH$_2$, (7) 11CH$_2$ ↔ 13CH$_2$, (8) 11CH$_2$ ↔ 14CH$_2$. 
(H8); 7.35 (H7); 7.07 (H6); 7.08 (H5). We were also able to define a third minor conformer with the following aromatic absorptions: 87.55 (H8); 7.42 (H7); 6.97 (H6); 7.19 (H5). All these data suggest that Pzn residue adopts a few different stereochemical orientations in the duplex (IV) with almost equal possibility. It is likely that the stereochemical orientations of the Pzn residue is not at all rigid, and it exists in a dynamic equilibrium between at least two or three different conformers. One of the strongest pieces of evidence for this suggestion is that we have seen NOESY cross-peaks between the aromatic protons of Pzn and also between the aromatic protons of Pzn and its linker protons in the spectrum of the duplex (IV) both in duplicates, and even in triplicates (Fig. 9b). We have however not observed any NOESY cross-peaks between Pzn-aromatic and oligonucleotide protons as in the case with duplex (III) (Fig. 9a). These are the reasons why in the MD calculation of duplex (IV), no constrains for the Pzn residue have been used.
Determination of stereochemical orientation of the phenazinium residue in the matched duplex (III) based on the nOe data. The stereochemical orientation of the Pzn residue in the duplex (III) was determined by the nOe connectivities of its aromatic and aliphatic protons with the nucleic acid residues. Interactions between the Pzn and oligonucleotide protons from ¹C, ⁷G and ⁸C residues in the duplex (III) have been observed at 10 mM concentration: the H₅ (δ7.34) and H₆ (δ7.21) of Pzn moiety show a nOe connectivity with the ⁷G(H'I') and ⁷G(H"I"). In contrast, at a 25 mM concentration of duplex, we observed nOe connectivity of the H₈ of Pzn moiety (δ7.91) with ⁸C(H'I') at δ6.22, suggesting clearly the effect of inter-molecular association. As mentioned above, the H₆ and H₅ resonances of ⁸C in duplex (III) absorb at a more downfield region compared to the corresponding resonances in duplex (I) suggesting a diamagnetic deshielding effect due to the Pzn residue. Similarly, an analysis of the imino protons also suggests that Pzn residue stabilises the ²C-⁶G and particularly the ¹C-⁷G base pairs. It is also important to note that the expected nOe connectivities from a B-DNA type helix such as nOes of H₁'/H₂'/H₂" protons of ⁷G in the matched duplex (I) with the H₆ base proton of the ⁸C nucleotide of the 3'-end has not been detected. Other nOes were however found between the -¹³CH₂,¹⁴CH₂- of the linker (δ3.44 and 3.17) and H₆ (δ7.71) and H₅ (δ5.85) aromatic protons of the ¹C residue (marked by i, i"; j, j' and j", respectively, in Fig. 9a). We have observed nOe cross-peaks between the H₇, H₈ of Pzn (δ7.75 and 7.91) and its -¹¹CH₂-¹²CH₂-OH (numbered (1), (2), (3) and (4), respectively, in Fig. 9a). The nOe cross-peaks between H₁ (δ6.35) and the -¹¹CH₂-¹²CH₂-OH have been marked by (5) and (6) in Fig. 9a.

The ¹³CH₂ of the methylene chain (δ3.44) are located approx. 2.3 Å from H₁ of Pzn residue in a cis-conformation (φ(C1-C2-N2-CC) = 0°) while this distance is more than 3.5 Å in trans-conformation (φ(C1-C2-N2-CC) = 180°). In the NOESY spectrum of the duplex (III) there is an intense cross-peak with coordinates δ6.13 and δ3.44 which are assigned to the protons H₁ and -¹³CH₂, respectively, (in Fig. 9a), these cross-peaks are marked by '7' suggesting the cis-conformation of the Pzn residue. This has been subsequently used as a constraint in the MD calculation. The distances between the protons of the Pzn residue and the oligonucleotide have been estimated using the two proton approximation despite the inherent problems associated with such an approximation. An attempt to estimate the correlation time (τₑ) for the protons of the Pzn residue and of the nucleotides revealed that τₑ

Fig. 10. Comparison of the aromatic-aromatic proton regions of the DQF-COSY (a₁ and b₁) and NOESY (a₂ and b₂) spectra of the Pzn-linked duplexes: Pzn-matched duplex (III) (DQF-COSY and NOESY in subspectra a₁ and a₂, respectively), and Pzn-mismatched duplex (IV) (DQF-COSY and NOESY in subspectra b₁ and b₂, respectively) in 0.1 M NaCl, 10 mM NaD₂PO₄, 10 mM EDTA (pH 7.4) in D₂O recorded at 294 K. H₁, H₃, H₄, H₅, H₆, H₇, and H₈ correspond to the Pzn protons. Solid and dashed lines indicate NOE connectivities between Pzn-Pzn protons. Note that in the subspectra a₁ and a₂ for Pzn-matched duplex (III), predominantly one set of sharp cross-peaks arising from Pzn aromatic protons are observed, suggesting one major conformation. Subspectra b₁ and b₂ for Pzn-mismatched duplex (IV), however, show the presence of at least three sets of cross-peaks for each Pzn aromatic protons (marked by solid, dot and dashed-star lines) of comparable intensities which suggest that at least three different stereochemical orientations of the Pzn ion is feasible.
Fig. 11. Chemical shift vs. temperature profiles of the base proton resonances (a) and (b), H1' resonances (c) and CH₃ protons (d) of the duplex (II), p(d(TGTGGGC)):p(d(ACAAACA)), (10 mM). Chemical shift was scaled with respect is relative DSS.

for these protons varied less than 0.5 ns which is within the experimental error. We have therefore estimated some proton-proton distances within the Pzn moiety, and from the Pzn to the nucleotide using the H6-H7 aromatic Pzn protons (r = 2.5 Å) as a reference. Since it was not possible to distinguish between the methylene protons for the -1¹CH₂-, 1²CH₂-, 1³CH₂- and 1⁴CH₂-groups, we have used a wide constraint of 2.0–5.0 Å for the distances between methylene protons of the linker and any other protons of oligonucleotide or Pzn residue for the MD calculation. These data suggested that the Pzn residue is indeed intercalated between 8C residue and 7G-1C base pair. Appropriate constraints were therefore employed to implement this Pzn stereochemistry in the starting structure for MD calculations (vide infra).

Chemical shift vs. temperature profiles. It is well known that the chemical shifts (δ) of the base and H1' protons are highly sensitive to ring-current effects exerted by the adjacent bases. Therefore, the δ-values provide a suitable probe to monitor stacking interactions in oligonucleotides. The chemical shift (δ) vs. temperature profiles of the nucleobase and H1' resonances of the duplex (II) (Fig. 11) display a roughly sigmoidal shape. These profiles reflect the gradual shift of the duplex-monomer equilibrium at low temperature (283 K) to the monomer at high
temperature (353 K). The plateau observed at low temperature then corresponds to the intact duplex (II) and the one observed at high temperature to the single stranded monomer. Some interesting deviations from a pure sigmoidal shape of the chemical shift profiles for the duplex (II) are noted (Fig. 11a-d). Although the sigmoidal melting profiles of 5'T, 4'T, 1'A and 6'C (Fig. 11b) suggest Watson-Crick base-pairing respectively with 3'A, 4'A, 7'G and 2'G (Fig. 11a) but this is not clearly evident from the temperature dependent profiles of the latter group of protons. They in fact show upfield shift as the temperature increases. The shift of H5 and H6 resonances of 2'C which is next to 5'-terminal mismatch residue 1'A are strikingly different from those displayed by the corresponding resonances of 6'C located next to 3'-terminal residue 7'A. The chemical shift profiles for the 3'-terminal 8'C(H6, H5), (Fig. 11b,c) appear to display a pure sigmoidal shape as for 6'C(H6), 6'G(H8) and other core residues. For the duplexes (III) and (IV), the temperature dependencies of only some of the base protons were followed because of the problems due to broadening as a result of slow exchange and overlap of too many protons.

Conformation of the sugar ring. The conformational analysis of the sugar ring rests upon the analysis of vicinal proton-proton couplings. In cases where the five individual couplings $J_{1'2'}$, $J_{1'2''}$, $J_{2'3'}$, $J_{2'2''}$ and $J_{3'4'}$ are available, these are analysed in terms of the relative fractions as well as of the geometry of the N- and S-type conformers with the aid of the program PSEUROT [44]. In cases where all five coupling constants cannot be determined, recourse is taken to a graphical method [45], which utilises the information content embedded in sums of couplings, viz $\Sigma_{1'} = (J_{1'2'} + J_{1'2''})$, $\Sigma_{2'} = (J_{1'2'} + J_{2'3'} + J_{2'2''})$, $\Sigma_{2''} = (J_{1'2''} + J_{2''3'} + J_{2'2''})$. The values for $J_{1'2'}$, $J_{1'2''}$, $J_{2'3'}$, $J_{2'2''}$, for most residues except for some of the duplex (I) where the H2' and H2'' resonances are near isochronous have been extracted [46] from DQF-COSY and E-COSY spectra, whereas $J_{3'4'}$ have been estimated [45] from $\Sigma_{4'}$. The analysis of the matched duplex (III) as an example is briefly described below. Fig. 12d shows the H1'-(H2', H2'') regions of the experimental DQF-COSY spectrum of the duplex (III). The boxed sets of cross-peaks patterns in the figure show the characteristic phase-sensitive multiplets of the H1'-H2', H1'-H2'' cross peaks belonging to 1'T residue, the slices of which have been shown in Figs. 12a-c.

The (H2''(ω₁-axis))-(H1'(ω₂-axis)) cross-peaks (Fig. 12a) along the ω₂-axis have the information about the vicinal proton-proton coupling for the H1' proton. The values of $J_{1'2'}$ and $J_{1'2''}$ have been obtained from the slices through the (H2''(ω₁-axis))-(H1'(ω₂-axis)) cross-peak along ω₂-axis as shown in Fig. 12a. The subspectrum is composed [46] of a small active $J_{1'2''}$ coupling marked (i) and a large $J_{1'2'}$ coupling marked (j). The values of $J_{2'2''}$, $J_{2'3'}$, and $J_{2'2'}$ have been determined by the analysis of H1'-H2' and H1'-H2'' cross-peaks in the H2'/H2'' region along ω₂-axis (Fig. 12b,c). The value of $J_{2'3'}$ is too small to be resolved under the experimental conditions. To obtain more accurate coupling constants, the cross-peaks in 1'2' and 1'2'' regions and 3'2' and 3'2'' regions were simulated by SMART [17]. It should be pointed out [47] that the cross-peak patterns are very sensitive to the parameters involved, namely, vicinal and geminal coupling con-
constants, line width, line shape, filter functions, acquisition times, digital resolution and phase. The \(1', 2'; 1', 2''\), \(2', 1'; 2'', 1'\) and \(3', 2'\) and \(3', 2''\) regions of the spectrum of the duplex (II) are shown in Fig. 13 as examples. The overlapping \(2'\) and \(2''\) protons were not considered further in this investigation. The line widths used for the simulation were taken from studies reported in the literature [47–49]: the \(H1', H2'\) and \(H2''\) line widths are 7, 9–11 and 8 Hz, respectively. The \(J_{2'2''} = -14\) Hz was kept constant. The chemical shifts of the \(2', 2''\) and \(3'\) protons were included exactly for every residue. The \(1', 2''\); \(1', 2''\); \(2', 1'\) and \(2', 1''\) peaks were simulated using the starting experimental values of \(J_{1'2'}, J_{1'2''}, J_{2'3'}\) and \(J_{2'2''}\) obtained from the experimental spectra in the manner described before. Fig. 13 shows the comparison of some experimental and calculated DQF-COSY cross-peaks for purines \(2G, 7G, 4A\) and \(7A\) in the duplex (II). These residues are chosen because of the following reasons: For the \(2G\) residue, the simulations of the cross-peaks were straightforward and gave unambiguous results while the cross peaks of the \(4A\) residue have shown problems due to the poor quality of the experimental cross peaks. The \(H2'\) and \(H2''\) sugar protons of the \(7G\) and \(7A\) residues have reverse chemical shifts compared to the other residues. One comes across this quite commonly for the 3'-terminal \(7A\) residue, but for the \(7G\) residue this reversal of the chemical shifts of \(H2'\) and \(H2''\) sugar protons seem to be unusual. The same situation has been observed for the \(7G\) residue in the matched Pzn-linked duplex (III). In this case, it was not possible to extract all coupling constants from the pattern of the cross peaks \(H1'\)-\(H2', H2''\). Nevertheless we could measure the sums of couplings, \(\Sigma_1' = (J_{1'2'} + J_{1'2''})\), \(\Sigma_2' = (J_{2'3'} + J_{2'3''})\), \(\Sigma_2'' = (J_{2''3'} + J_{2''3''})\) with an experimental precision of 0.9 Hz for \(J_{1'2'}, J_{1'2''}\)-coupling constants (Table 13). The \(\Sigma_1'\) of more than 13.3 Hz shows that the sugar of \(7G\) residue adopts predominantly a south conformation. Using the equation [45], \(\rho_S = (\Sigma_1' - 9.8)/5.9\), where \(\rho_S\) is the mole fraction of south population) the fraction of S-type conformer is roughly computed as to be 69%. It means that the sugar of \(7G\) in the duplex (III) adopts a north conformer for an appreciable time (31%). It has been found that when \(\Sigma_1'\) shows a preference for a south conformer, the sums \(\Sigma_2'\) and \(\Sigma_2''\) also suggest the same (\(H2'\) and \(H2''\) are assigned as downfield and upfield, respectively). Unfortunately, the same could not be done for the \(7G\) residue of the duplexes (I) and (IV) in which \(H1'\) and \(H2', H2''\)
Fig. 13. (A) and (B) show the cross-peaks of H2'/H2"-H1 and H1'-H2'/H2" regions from the DQF-COSY experiments on duplex (II); (C) shows the comparison of the experimental DQF-COSY (subspectra are marked by 'a') and best-fit simulations cross-peaks (subspectra are marked by 'b') of the mismatched duplex (II) as example for the 2G, 7G, 7A and 4A residues which are chosen for the following reasons: (1) the H2' and H2" protons of the 7G and 7A residues have reverse chemical shifts compared to other residues, (2) cross-peaks for 2G have been chosen to show where the simulation of the cross-peak is straightforward and gives unambiguous results, while the cross-peak of 4A have been exhibited to show the problems associated in the simulation of an inferior quality of experimental cross-peaks. Only the cross-peaks which did not overlap with other cross-peaks from other residue were simulated.
cross peaks overlapped with other resonances. The chemical shifts of H2' and H2" of 7G residue of the mismatched (II) and matched Pzn-linked (III) duplexes show the strong influence of the mismatch base pair and Pzn residue on these protons. The coupling constants of the sugar protons were interpreted with the aid of the program PSEUROT [44] in terms of P (phase angle of pseudorotation), \( \Psi_m \) (puckering amplitude) (Table 13) along with the population of the major S-type (C2'-endo) conformer (data not shown). The population of the S-type were more than 90% for most residues except for the terminal nucleotides 8C (~50%) and 7G. Note that during the least-squares fit of the coupling constants the minor N-type conformation was fixed at \( P_N = 9^\circ \) and \( \Psi_m = 35.6^\circ \).

The sugar conformation could also be determined independently by nOe intensities in the NOESY (80 ms) spectra of the duplexes (I), (II), (III) and (IV) using H1'-H4' region [46]. The distances H1'-H4' show the most variation with P and provide quite accurate diagnostic information concerning sugar conformers [50,48]. The calculated distances of H1'-H4' using two proton approximation are listed in Table 9. The intraresidue distance between H4' and H1' of ~2.9 Å is typical of S-type sugar (C2'-endo) pucker [46]. The data on nOe-derived distances are shown in Table 9 which are in agreement with the coupling constant data (Table 13). Assessment of DNA duplexes on the basis of nOe and \( 3J_{\text{HH}} \) coupling constants suggest that they have sugar geometries similar to those found in B-DNA. The backbone torsion angle \( \delta(\text{C5'-C4'-C3'-O3'}) \) is directly related to the pseudorotation parameters [51], P and \( \Psi_m \) [44]. The \( \delta \) (S-type conformation) values of the duplexes (I), (II), (III) and (IV) are shown in Table 13. They range from 120° to 140° with exceptions for the terminal nucleotides for 7A, 1T, 8C and 7G. It is likely that the increase of the \( \delta \) for 7A, 1T, 8C may be related to the fact they are terminal residues. To estimate the torsion angle \( \gamma(\text{O5'-C5'-C4'-C3'}) \), the approach [52] of using the sum of J-coupling of H4' (\( \Sigma_{4'} = (J_{3'4'} + J_{4'5'} + J_{4'5''}) \)) has been employed combined with the analysis of lower bound nOe distances to any protons in the 4.1–4.5 ppm (H5'/H5") region for the duplexes (I), (II), (III) and (IV). The outer peak separations in the H4' dimension in DQF-COSY experiment and the estimation of \( \Sigma_{4'} \) values for the majority of residues in duplexes (I), (II), (III) and (IV) were found to lie in the range of 4.9–6.2 Hz except for the terminal 8C residue. Using the contour map of the sum of J-couplings of H4' as a function of the pseudorotational and \( \gamma \) torsions [52] and our experimental data \( \Sigma_{4'} \) (Table 14) and P (Table 13), the \( \gamma \) torsions were found to be either 60 ± 15° or 240 ± 15°. We have then assessed the the \( \gamma \) torsion on the basis of H8/H6-H5'/H5" and regions of the NOESY spectra. We have observed that almost all H8/H6-H5'/H5" cross peaks are weaker than H8/H6-H1' cross peak in the anti conformation. These data are consistent with the \( \gamma^+ \) rotamer. The population of the \( \gamma^+ \) rotamer could be calculated by means of sum rule [51] from the sum of the \( J_{3'4'} \) and \( J_{4'5'} \) coupling constants using \( \Sigma_{4'} \) and \( J_{3'4'} \) data (Table 14): \( P_{\gamma^+} = (13.3-(\Sigma_{4'}-J_{3'4'}))/9.7 \). The values of \( P_{\gamma^+} \) range from 94 to 100%.

The backbone angle \( \epsilon(\text{C4'-C3'-O-P}) \) are related to the coupling constant between the H3' and the nearest phosphate [53]. For this purpose, we have used H2'-H3' cross-peak in DQF-COSY experiment as a consistency check for \( \Sigma_{4'} \) and


<table>
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<th>Residue</th>
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<th>Error $^a$</th>
<th>$J_{H1'-H2''}$</th>
<th>Error $^a$</th>
<th>$J_{H2'-H3'}$</th>
<th>Error $^a$</th>
<th>$J_{H2''-H3'}$</th>
<th>Error $^a$</th>
<th>$J_{H3'-H4'}$</th>
<th>Error $^a$</th>
<th>$\Sigma_1$</th>
<th>$\Sigma_2$</th>
<th>$\Sigma_2^*$</th>
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* Table 13: $J$-coupling constants (Hz) of the duplexes (I), (II), (III) and (IV)
|  | 10.3  | ± 0.5 | 5.9  | ± 0.3 | 7.3  | ± 0.2 | 1.0  | ± 0.5 | 2.3  | ± 0.6 | 15.4 | 29.1 | 20.0 | 140  | 32  | 0.25 | 130  |
|  | 10.2  | ± 0.5 | 5.5  | ± 0.3 | 7.2  | ± 0.2 | 1.0  | ± 0.5 | 2.3  | ± 0.6 | 15.7 | 27.4 | 22.5 | 140  | 34  | 0.22 | 131  |
|  | 10.3  | ± 0.5 | 5.9  | ± 0.5 | 7.3  | ± 0.5 | 1.0  | ± 0.5 | 3.0  | ± 0.6 | 15.4 | 29.1 | 20.0 | 133  | 34  | 0.17 | 126  |
| 3T I | 9.9  | ± 0.5 | 5.6  | ± 0.3 | 6.0  | ± 0.3 | 1.5  | ± 0.5 | 2.0  | ± 0.6 | 15.4 | 22.2 | 153  | 36   | 0.03 | 139  |
|  | 10.3  | ± 0.5 | 5.9  | ± 0.3 | 6.3  | ± 0.2 | 1.2  | ± 0.5 | 2.0  | ± 0.6 | 15.6 | 31.9 | 22.0 | 150  | 34  | 0.12 | 137  |
|  | 9.9  | ± 0.5 | 5.9  | ± 0.3 | 6.3  | ± 0.3 | 1.5  | ± 0.3 | 2.0  | ± 0.6 | 15.4 | 27.4 | 22.2 | 153  | 34  | 0.05 | 138  |
|  | 10.3  | ± 0.5 | 5.9  | ± 0.3 | 6.3  | ± 0.5 | 1.2  | ± 0.5 | 2.0  | ± 0.6 | 15.6 | 31.9 | 22.0 | 150  | 34  | 0.12 | 137  |
| 4T I | 9.9  | ± 0.5 | 5.6  | ± 0.3 | 6.5  | ± 0.2 | 1.2  | ± 0.5 | 2.0  | ± 0.6 | 15.4 | 22.2 | 149  | 34   | 0.09 | 136  |
|  | 10.5  | ± 0.5 | 5.6  | ± 0.3 | 7.0  | ± 0.2 | 1.2  | ± 0.5 | 3.0  | ± 0.6 | 15.3 | 31.9 | 22.0 | 134  | 37  | 0.12 | 127  |
|  | 9.8  | ± 0.5 | 5.9  | ± 0.3 | 6.5  | ± 0.3 | 1.2  | ± 0.3 | 2.0  | ± 0.6 | 15.4 | 29.0 | 22.5 | 151  | 33  | 0.05 | 137  |
|  | 10.5  | ± 0.5 | 5.9  | ± 0.3 | 7.0  | ± 0.5 | 1.2  | ± 0.5 | 3.0  | ± 0.6 | 15.3 | 31.9 | 22.0 | 134  | 37  | 0.12 | 127  |
| 5T I | 9.9  | ± 0.5 | 5.6  | ± 0.3 | 6.2  | ± 0.2 | 1.4  | ± 0.5 | 3.4  | ± 0.6 | 15.4 | 30.2 | 21.7 | 126  | 34  | 0.26 | 121  |
|  | 9.9  | ± 0.5 | 5.6  | ± 0.3 | 7.9  | ± 0.2 | 1.2  | ± 0.3 | 3.2  | ± 0.6 | 15.4 | 32.7 | 22.5 | 130  | 31  | 0.14 | 124  |
|  | 9.9  | ± 0.5 | 5.6  | ± 0.3 | 8.2  | ± 0.2 | 1.4  | ± 0.3 | 3.4  | ± 0.6 | 16.1 | 29.6 | 23.2 | 126  | 34  | 0.26 | 121  |
|  | 9.9  | ± 0.5 | 6.2  | ± 0.3 | 7.9  | ± 0.6 | 1.2  | ± 0.5 | 3.2  | ± 0.6 | 15.4 | 32.7 | 22.5 | 130  | 31  | 0.14 | 124  |
| 6G I | 9.8  | ± 0.9 | 4.3  | ± 0.9 | **   | **   | **   | **   | **   | **   | **   | 15.4 | **   | **   | **   | **   | **   | **   |
|  | 9.5  | ± 0.5 | 6.5  | ± 0.3 | 7.7  | ± 0.6 | 1.4  | ± 0.5 | 2.8  | ± 0.6 | 15.1 | *    | 21.4 | 138  | 29  | 0.05 | 12   |
|  | 11.0 | ± 0.9 | 4.3  | ± 0.9 | **   | **   | *    | **   | **   | **   | **   | 16.0 | 29.5 | 22.1 | **   | **   | **   | **   |
| 7G I | **  | **   | **   | **   | **   | **   | **   | **   | **   | **   | **   | 15.4 | **   | **   | **   | **   | **   | **   |
|  | 10.5 | ± 0.5 | 5.5  | ± 0.3 | 5.0  | ± 0.6 | 1.5  | ± 0.5 | 1.0  | ± 0.6 | 15.4 | 28.0 | 21.5 | 167  | 39  | 0.09 | 149  |
|  | 10.0 | ± 0.9 | 4.0  | ± 0.9 | **   | **   | **   | **   | **   | **   | **   | 13.9 | 32.0 | 21.3 | **   | **   | **   | **   |
| 8C I | 7.8  | ± 0.5 | 5.9  | ± 0.3 | 5.8  | ± 0.3 | 4.0  | ± 0.5 | 2.8  | ± 0.6 | 13.7 | 27.2 | 22.9 | 165  | 40  | 0.2   |
|  | 5.9  | ± 0.5 | 6.8  | ± 0.3 | 7.0  | ± 0.3 | 4.0  | ± 0.5 | 2.8  | ± 0.6 | 14.8 | 27.0 | 25.7 | 188  | 30  | 0.48 | 150  |
|  | 6.8  | ± 0.5 | 4.9  | **   | **   | **   | **   | **   | **   | **   | **   | 13.3 | **   | **   | **   | **   | **   | **   |
| IV  | 7.0  | ± 0.5 | 4.9  | **   | **   | **   | **   | **   | *    | **   | 13.9 | **   | **   | **   | **   | **   | **   |

*a* Error estimates for the coupling constants were obtained as follows. To find the upper and lower limit the simulations were repeated for each coupling constant by the values of experimental J coupling constants (DQF COSY) in increments of 0.3 Hz. *b* Root-mean-square deviation between observed and calculated coupling constants. P, phase angle of pseudorotation; \( \varphi_m \), puckering amplitude; \( \Sigma_1 \) = \((J_{1/1'2} + J_{1/2'})\); \( \Sigma_2 = (J_{1/1'2} + J_{2/2'} + J_{2/3'}) \); \( \Sigma_2 = (J_{1/1'2} + J_{2/2'} + J_{3/2'}) \). *P*, \( \varphi_m \) and \( \delta \) are in degrees. **J** constants have not been determined because of crowding. \( \delta \), torsion angle for the south conformation C5'-C4'-C3'-O3'.

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TABLE 14

Sums of the coupling constants (Hz) of the duplexes (I), (II), (III) and (IV) from DQF-COSY experiments with and without \^3P decoupling

\(\Sigma_3 = (J_{2'3'} + J_{2'5'} + J_{2'4'})\), \(\Sigma_3' = (J_{2'3'} + J_{2'5'} + J_{3'4'})\), \(\Sigma P_3 = (J_{2'3'} + J_{2'5'} + J_{3'4'} + J_{3'5'})\)

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<th>Duplex (I) without J_{HP}</th>
<th>with J_{HH} and J_{HP}</th>
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<td>11.1</td>
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H3'-P coupling constants. The sum of coupling constants to the H3', \(\Sigma P_3 = (J_{2'3'} + J_{2'5'} + J_{3'4'} + J_{3'5'})\) with the active coupling \(J_{2'3'}\), giving the antiphase characteristic to the cross peak pattern is reflected by the outer separation of the multiplet lines of a non-terminal H2'-H3' cross peak. The values of these sums have been obtained from the DQF-COSY spectra recorded with and without \^3P decoupling (\(\Sigma P_3\) and \(\Sigma_3\), respectively). The difference between \(\Sigma P_3\) and \(\Sigma_3\) allow the estimation of the highest limit of the \(J_{3'5'}\) coupling constant. Another way to estimate \(J_{3'5'}\) is to use data \(\Sigma P_3\) and \(J_{2'3'}, J_{2'5'}, J_{3'4'}\) (Table 13). These procedures gave the \(J_{3'5'}\) for all non-terminal residues smaller than 5 Hz, and between 5 and 6 Hz for the terminal residues 1T, 8C, 7A and 1C (or \(\lambda A\)) which are consistent with the observed \(J_{3'5'}\) coupling constants of less than 5 Hz for all residues in B-DNA sequence published in the literature [53,52]. From a plot of of \(\Sigma P_3\) as a function of phase angle of pseudorotation (\(P\)), the range of torsion angle \(\epsilon\) has been found to be limited [52] to 130–200° for a B_I type phosphate, or 280–350° for a B_{II}-type phosphate and the corresponding H3'-P distance bounds are found to be 2.80–3.25 Å. This distance limit is common to the both B_I and B_{II}-type conformation and have been compared with distances calculated in the MORASS algorithm. The torsion angle \(\beta(P-O5'-C5'-C4')\) is monitored by the \(J_{3'5'}\) and \(J_{5'5'}\) coupling constants, which in practice is a difficult task to measure. The sum rule, \(\Sigma_{5'} = (J_{5'5''} + J_{5'4'} + J_{5'p})\) and \(\Sigma_{5''} = (J_{5'5''} + J_{5'4'} + J_{5'p})\), is an interesting approach [52] which can
be estimated from the line width of the H5' and H5" multiplet resonances determined from NOESY data at longer mixing times (200–500 ms). In the same manner the highest limit of the line widths [52] of the H5'/H5" peaks were roughly estimated experimentally to be less than 28 Hz in NOESY spectra [52]. Hence, the β torsion could be limited to the range 105°–255° which corresponds to the H5'-P and H5"-P distances of 2.65–3.40 Å. No constraint for β, ε and γ have been used in any of our MD/MORASS calculations. We have, however, compared all experimental torsion angles (vide supra) with those of the calculated data from MD/MORASS calculations (vide infra).

Comparison of structures. To obtain more accurately integrated NOESY cross-peak volumes by AURELIA [17] for refinement of the structures, we have used the 200 ms NOESY spectrum. The hybrid matrix procedure was employed to correct for spin diffusion effects at this mixing time as described by Gorenstein et al. [54,55]. Only those cross-peaks that were adequately resolved were used for the NOESY constraints. For the mismatched duplex (II) adequately resolved diagonal peaks have also been included. In the matched duplexes (I) and (III), the additional constraints on seven imino hydrogen bonds were included but for the mismatched duplexes (II) and (IV) only constraints on six hydrogen-bonds were kept with 1.93 ± 0.3 Å and a force constant of 25 kcal.mol⁻¹ Å⁻² was used except for the terminal residue where a force constant of 10 kcal.mol⁻¹ Å⁻² was used. This is because we observed only six imino protons in the downfield region of the spectra (Fig. 7) suggesting their involvement in the Watson-Crick base pairing. The seventh imino proton was found in the upfield region (vide supra), hence this could not be attributed unambiguously as hydrogen-bonded imino proton. The basic iterative refinement scheme of Gorenstein’s hybrid matrix approach using his Multiple Overhauser Relaxation Matrix (MORASS) computer program was employed [54,55]. This has been used by Gorenstein et al. for the assessment of conformation of the DNA duplex with G-A mismatch in the core [54,55]. The iterative refinement scheme was followed until the %RMS converged to a limiting value as outlined in Tables 15–18. It has been demonstrated [54,55] that the convergence between theoretical and experimental volumes could be achieved with as low as 15% residual error using ‘perfect volumes’ simulated from a single correlation time for a model structure. But the actual experimental data allow only an upper limit of %RMS error (25–50%) between theoretical and experimental volumes. Indeed for the matched duplex (I), the %RMS errors between theoretical and experimental volumes after refinement have reached approx. 69/58%, respectively (Table 15), starting from the B-type DNA duplex conformation. The reason for the choice of a starting B-type DNA duplex conformation for the matched duplex (I) for the hybrid matrix/MORASS refinement approach is supported by our experimental NMR data (vide supra). A single isotropic correlation time ($\tau_c$) of 2 ns has been assumed. We have based this on the experimental data for the mismatched duplex (II) using the resolved diagonal peaks and corresponding cross-peak in the NOESY spectra. The correlation time we have obtained varied between 1.5 and 2 ns depending upon which protons were used for the calculation. However, it was shown [54,55] that with the scaling of the experimental to
TABLE 15
MORASS/Restrained MD refinements of matched duplex (I)

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<th>Structure</th>
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<th>$E_{\text{tot}}$ $b$ (no constraint)</th>
<th>$E_{\text{tot}}$ $c$</th>
<th>constraint energy $d$</th>
<th>%RMS$<em>{\text{vol}}$ $e$ ($% R</em>{\text{the}} / % R_{\text{exp}}$)</th>
<th>Flatwell $f$ Parameter $g$ (%)</th>
<th>$R$ factor $h$</th>
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<td>-110.4</td>
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<td>109.4</td>
<td>92/72</td>
<td>18/20</td>
<td>0.365</td>
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</table>

$^a$ Structure of the matched duplex (I) at various refinement cycles from 1 to 10; $^b$ total energy (kcal/mol) including the NOESY distance constraint term; $^c$ potential energy (kcal/mol) not including NOESY distance constraints; $^d$ NOESY distance constraint energies (kcal/mol); $^e$ RMS error in NOESY volumes calculated from MORASS, %RMS$_{\text{vol}}$ = $((1/N)\sum((v^m_i - v^n_i)/v^m_i)^2)^{1/2} \times 100$ where %RMS$_{\text{vol}}$ = $%R_{\text{the}}$ when $m$ = the calculated volume and $n$ = the experimental volume and %RMS$_{\text{vol}}$ = $%R_{\text{exp}}$ when $m$ = the experimental volume and $n$ = the calculated volume; $^f$ flatwell potential function parameters ($k$, harmonic force constant in kcal.mol$^{-1}$ Å$^{-2}$ and % error, permitted error); $^g$ $R$ factor = $\sum|v^m_i - v^n_i|/\Sigma v^m_i$ where $m$ = the experimental volume, $n$ = the calculated volume [65].

Theoretically calculated volumes an incorrect estimate of $\tau_c$ has negligible effects on the derived distances. We have done the refinement of the matched duplex (I) with three different $\tau_c$ (1, 2 and 3.6 ns) and all of them have shown successful convergence (1.0–1.5%RMS difference between final converged structures).

Mismatched duplex (II). Table 16 shows the %RMS errors in calculation of theoretical and experimental volumes during the refinement [54,55] of the two different starting structures (GA(anti-anti), GA(anti-syn)) at each stage of the refinement process. The unusually large %RMS for the first and third syn structures are the result of the $^1$A and $^7$G residues being in syn orientation and clearly indicates that a syn geometry of the mismatched $^1$A and $^7$G residues is not in agreement with NMR data. All final structures result in approx. 55%RMS$_{\text{the}}$ and 46%RMS$_{\text{exp}}$ deviations. To compare data, calculations for both syn and anti structures have been performed with hydrogen-bond constraints on six imino protons only and without constraints on endocyclic sugar torsions for the $^1$A in order to allow this residue to be restrained only by the nOe constraints. This means that the mismatched $^1$A-$^7$G base pair was unconstrained. The total potential energies with and without constraints as well as the constraint energies of the different structures at each iteration step of the refinement procedure are also listed in Table 16. The potential and constraint energies of the constrained minimized structures increase only moderately as the force constants increase from 10 to 40 kcal.mol$^{-1}$ Å$^{-2}$ and the allowed variations of the distance constraints is decreased from 15 to 6% (Table 16). By incorporation of NOESY distance
TABLE 16
MORASS/Restrained MD refinements of mismatched duplex (II)

<table>
<thead>
<tr>
<th>Structure a</th>
<th>( E_{\text{tot}} ) b (kcal/mol)</th>
<th>( E_{\text{tot}} ) c (no constraint)</th>
<th>( \text{Constraint energy} ) d</th>
<th>( % \text{RMS}<em>{\text{vol}} ) c (% ( R</em>{\text{the}} ) / ( R_{\text{exp}} ))</th>
<th>Flatwell Parameter f</th>
<th>R factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA1(anti-syn)(^{1}) starting in Syn conformation</td>
<td>( -121.6 )</td>
<td>151/471901</td>
<td>( 10.7 )</td>
<td>( 98/69 )</td>
<td>( 10/14 )</td>
<td>( 15/12 )</td>
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<td>GA2(anti-syn)</td>
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<td>73/47</td>
<td>12/16</td>
<td>13/10</td>
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<tr>
<td>GA4(anti-syn*)</td>
<td>-30.5</td>
<td>-110.6</td>
<td>47.5</td>
<td>96/44</td>
<td>14/18</td>
<td>12/8</td>
</tr>
<tr>
<td>GA5(anti-syn*)</td>
<td>-16.9</td>
<td>-105.5</td>
<td>53.6</td>
<td>85/44</td>
<td>16/20</td>
<td>10/7</td>
</tr>
<tr>
<td>GA6(anti-anti)</td>
<td>119.7</td>
<td>101/786084</td>
<td>38.8</td>
<td>50/45</td>
<td>20/40</td>
<td>6</td>
</tr>
<tr>
<td>GA7(anti-anti)</td>
<td>-5.1</td>
<td>-114.1</td>
<td>50.0</td>
<td>56/39</td>
<td>20/40</td>
<td>6</td>
</tr>
<tr>
<td>GA8(anti-anti)</td>
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<td>-107.9</td>
<td>87.0</td>
<td>59/40</td>
<td>20/40</td>
<td>6</td>
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<table>
<thead>
<tr>
<th>Structure a</th>
<th>( E_{\text{tot}} ) b (kcal/mol)</th>
<th>( E_{\text{tot}} ) c (no constraint)</th>
<th>( \text{Constraint energy} ) d</th>
<th>( % \text{RMS}<em>{\text{vol}} ) c (% ( R</em>{\text{the}} ) / ( R_{\text{exp}} ))</th>
<th>Flatwell Parameter f</th>
<th>R factor</th>
</tr>
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<tbody>
<tr>
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<td>119.7</td>
<td>101/786084</td>
<td>76.7</td>
<td>85/48</td>
<td>10/14</td>
<td>15/12</td>
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<td>14/10</td>
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<td>34.9</td>
<td>68/44</td>
<td>12/16</td>
<td>13/10</td>
</tr>
<tr>
<td>GA4(anti-anti)</td>
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<td>25.1</td>
<td>64/43</td>
<td>14/18</td>
<td>12/8</td>
</tr>
<tr>
<td>GA5(anti-anti)</td>
<td>-28.5</td>
<td>-113.9</td>
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<td>62/41</td>
<td>16/20</td>
<td>10/7</td>
</tr>
<tr>
<td>GA6(anti-anti)</td>
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<td>55.0</td>
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<td>18/20</td>
<td>8/6</td>
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<tr>
<td>GA7(anti-anti)</td>
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<td>64.6</td>
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<td>20/40</td>
<td>6</td>
</tr>
<tr>
<td>GA8(anti-anti)</td>
<td>-4.1</td>
<td>-119.1</td>
<td>54.6</td>
<td>58/41</td>
<td>20/40</td>
<td>6</td>
</tr>
<tr>
<td>GA9(anti-anti)</td>
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<td>-126.8</td>
<td>63.8</td>
<td>55/46</td>
<td>20/40</td>
<td>6</td>
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</table>

<table>
<thead>
<tr>
<th>Structure a</th>
<th>( E_{\text{tot}} ) b (kcal/mol)</th>
<th>( E_{\text{tot}} ) c (no constraint)</th>
<th>( \text{Constraint energy} ) d</th>
<th>( % \text{RMS}<em>{\text{vol}} ) c (% ( R</em>{\text{the}} ) / ( R_{\text{exp}} ))</th>
<th>Flatwell Parameter f</th>
<th>R factor</th>
</tr>
</thead>
<tbody>
<tr>
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<td>-4.0</td>
<td>-126.8</td>
<td>63.8</td>
<td>55/46</td>
<td>20/40</td>
<td>6</td>
</tr>
</tbody>
</table>

a Structure of mismatched duplex (II) at various refinement cycles from 1 to 10; GA(anti-anti) and GA(anti-syn) mismatched base pair 7G-1A in anti, anti and anti, syn conformation, respectively; b total energy (kcal/mol) including the NOESY distance constraint term; c potential energy (kcal/mol) not including NOESY distance constraints; d NOESY distance constraint energies (kcal/mol); e RMS error in NOESY volumes calculated from MORASS, \( \% \text{RMS}_{\text{vol}} = (\langle 1/N \rangle \Sigma_m (v_m^\text{calc} - v_m^\text{exp})^2)^{1/2} \times 100 \) where \( \% \text{RMS}_{\text{vol}} = \% R_{\text{the}} \) when \( m = \) the calculated volume and \( n = \) the experimental volume and \( \% \text{RMS}_{\text{vol}} = \% R_{\text{exp}} \) when \( m = \) the experimental volume and \( n = \) the calculated volume [55]; f flatwell potential function parameters (k, harmonic force constant in kcal.mol\(^{-1}\) Å\(^{-2}\)) and \% error, permitted error); g R factor = \( \Sigma_m |v_m^\text{calc} - v_m^\text{exp}|/\Sigma_m (v_m^\text{calc}) \) where \( m = \) the experimental volume, \( n = \) the calculated volume [65].

The MD calculations clearly were able to locate the anti conformation (Fig. 14). The transition from the 1A (syn) to the 1A (anti) conformation during the MD run was accomplished within the first 20 ps of restrained MD refinement. Energy terms (Table 16) do not allow more than crude discrimination between starting structures. Note that the structures at the tenth iteration step (i.e., GA10(anti-anti)) exhibit the smallest r.m.s.d deviation (less than 1 Å) although they arose from the starting models GA1(anti-anti), GA1(anti-syn), with the largest r.m.s. deviation (2.7 Å).

Phenazinium-linked matched (III) and mismatched (IV) duplexes. Four different starting structures of the matched (III) and mismatched (IV) duplexes bearing Pzn residue were generated for the molecular dynamics computations as a test for
TABLE 17
MORASS/Restrained MD refinements of phenazinium-linked matched duplex (III)

<table>
<thead>
<tr>
<th>Structure a</th>
<th>$E_{\text{rot}}$ b (kcal/mol)</th>
<th>$E_{\text{rot}}$ c (no constraint)</th>
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<th>$%\text{RMS}_{\text{olv}}$ e</th>
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<td>10/14</td>
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<td>63/64</td>
<td>12/16</td>
<td>14/10</td>
<td>0.369</td>
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<td>12/16</td>
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<td>18/20</td>
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<td>45/67</td>
<td>20/40</td>
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<td>0.303</td>
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<td>111.8</td>
<td>51/55</td>
<td>20/40</td>
<td>6</td>
<td>0.300</td>
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</tbody>
</table>
the convergence characteristics of the final structures. All starting structures for the matched duplex were generated as follows: the p(d(TGTGGGC)):p(d(CCA-
AACA)) duplex was built from B-form DNA by using Amber 4.0 [24]. The Pzn residue was specially created as described above and attached to the 5'-terminal 1C residue and positioned for intercalation such that the Pzn residue is sandwiched between 7G-1C base pair and 8C in accordance with the experimental data discussed above. We have established (Fig. 9a) that H1 of Pzn residue and -13CH2 are located in spatial proximity (< 3.5 Å). Corresponding strong cross-peak have been observed in the NOESY spectra (τm = 200 ms) for the duplex (III) (Fig. 9a). In the trans orientation (ϕ(C1-C2-N2-CC) = 180°), the protons of 13CH2 are located more than 6 Å away from the H1 aromatic proton. The distance found using two protons approximation were not more than the 3 Å which is consistent with the conformation of the Pzn residue as shown in Fig. 15 (termed as cis orientation of H1 and -13CH2 (ϕ(C1-C2-N2-CC) = 0°)). We have tried to calculate the starting structures ‘model 1’ of the duplex (III) with both cis and trans orientation of H1 and 13CH2. For the ‘model 1’ with trans linker, a high %RMS values for first two steps of MD/MORASS refinement was observed due to the incorrect orientation of the 13CH2 moiety in the linker with respect to the H1 aromatic proton of the Pzn group. After the first 10 ps of restrained MD refinement, a transition from trans to cis orientation was, however, observed. The cis conformation of the Pzn residue has therefore been used for all starting structures of the matched and mismatched duplexes (III) and (IV), respectively, and kept constrained during MD/MORASS calculation for the matched duplex (III) (Figs. 15,16).

All starting structure were minimized until an r.m.s gradient of 10^-4 kcal.mol^-1 Å^-1 was achieved for the successive steps. This process removed bad contacts between Pzn ion and DNA and pushed the base-pairs apart to form an intercalated structure. The starting structures have been found after minimization to have approximately the same energy (Table 17–18). Then these structures served as starting models for structural refinement using MD/MORASS approach with NOE distance constraints (200 distances) and torsion angle constraints ν2 (C2-C3, rotation) and ν3 (C3-C4, rotation). The torsion angle constraints were fixed during MD calculation without changing the force constant (40 kcal.mol^-1.rad^-2) and flatwell of ±10° for all residues except for 8C was used. For the 6G and 7G

Notes to Table 17:

a Structure of phenazinium-linked matched duplex (III) at various refinement cycles from 1 to 10:
b Total energy (kcal/mol) including the NOESY distance constraint term; c potential energy (kcal/mol) not including NOESY distance constraints; d NOESY distance constraint energies (kcal/mol); e RMS error in NOESY volumes calculated from MORASS, %RMSvol = (1/N)Σu(vu - vui)2/N 1/2 × 100 where %RMSvol = %Rexp when m = the calculated volume and n = the experimental volume and %RMSvol = %Rexp when m = the experimental volume and n = the calculated volume [55]; f flatwell potential function parameters (k, harmonic force constant in kcal.mol^-1 Å^-2 and % error, permitted error); g R factor = Σu|vu - vui|/Σu(vu) where m = the experimental volume, n = the calculated volume [65].
### Table 18

MORASS/Restrained MD refinements of phenazinium linked mismatched duplex (IV)

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Fig. 14. Snapshots of the trajectory of the NOESY-distance-restrained (MORASS hybrid matrix) dynamics run showing the transition from the \textit{syn} to the \textit{anti} conformation for $^1\text{A}$ in the mismatch duplex (II). Expanded stereoview of the mismatched base-pair and its neighbours in the duplex (II): (a) starting structure after minimization, (b) after the second cycle of MORASS/MD refinements and (c) forth cycle of MORASS/MD refinements.

residues in matched duplex (III), according to the experimental data described above, the south conformation of the sugar residue has been fixed with a phase angle of pseudorotation, $P = 160^\circ$, and puckering amplitude, $\Psi_m = 35^\circ$. Flat well of $\pm 10^\circ$ has been chosen according to the estimation of the fluctuation of the torsion angles when the experimental errors of the coupling constants were included in the PSEUROT [44] calculations. As has been pointed out, the $^7\text{G}$ residue of the duplex (III) has only 69% of south population. For all four starting models the first refinement has been done with south conformation of the sugar of $^7\text{G}$ residue, but we have also done a second set of calculations without torsional constraints of the $^6\text{G}$ and $^7\text{G}$ residues. In the last set of calculations, all four starting models have

Notes to Table 18:
\begin{itemize}
  \item[a] Structure of phenazinium-linked mismatched duplex (IV) at various refinement cycles from 1 to 10;
  \item[b] total energy (kcal/mol) including the NOESY distance constraint term; \item[c] potential energy (kcal/mol) not including NOESY distance constraints; \item[d] NOESY distance constraint energies (kcal/mol); \item[e] RMS error in NOESY volumes calculated from MORASS, $\%\text{RMS}_{voI} = ((1/N)\sum((v_m^m - v_n^n)/v_n^m)^2)^{1/2} \times 100$ where $\%\text{RMS}_{voI} = \%\text{R}_{\text{the}}$ when $m =$ the calculated volume and $n =$ the experimental volume and $\%\text{RMS}_{voI} = \%\text{R}_{\text{exp}}$ when $m =$ the experimental volume and $n =$ the calculated volume [55]; \item[f] flatwell potential function parameters (k, harmonic force constant in kcal.mol$^{-1}$ Å$^{-2}$ and $\%$ error, permitted error); \item[g] $R$ factor $= \Sigma |v_m^m - v_n^m|/\Sigma (v_n^m)$ where $m =$ the experimental volume, $n =$ the calculated volume [65].
\end{itemize}
Fig. 15. Expanded stereoview of the four models of the duplex (III) bearing Pzn residue at the 5'-end of the heptamer showing two base-pairs and intercalated geometry of Pzn residue. Top part: initial models (a) Pzn-gc-1, (b) Pzn-gc-2, (c) Pzn-gc-3, (d) Pzn-gc-4 in classical B-DNA conformation and with four different positions of intercalating Pzn residue after minimization until an RMS gradient of $10^{-4}$ kcal.mol$^{-1}$Å$^{-1}$ was achieved. Bottom part: final MD/MORASS refined structures (e) Pzn-gc-1, (f) Pzn-gc-2, (g) Pzn-gc-3, (h) Pzn-gc-4, respectively. The initial structure (b) Pzn-gc-2, was converted to the structure (d) Pzn-gc-4, already after second step of refinement.
Starting structures for the duplex (IV)

Pzn-ga-1
(a)

Pzn-ga-2
(b)

Pzn-ga-3
(c)

Pzn-ga-4
(d)

Final structures after MORASS/MD refinement

Pzn-ga-1
(e)

Pzn-ga-2
(f)

Pzn-ga-3
(g)

Pzn-ga-4
(h)

Fig. 16. Expanded stereoviews of the four starting structures, (a) Pzn-ga-1, (b) Pzn-ga-2, (c) Pzn-ga-3, (d) Pzn-ga-4) and the four corresponding final models ((e) Pzn-ga-1, (f) Pzn-ga-2, (g) Pzn-ga-3, (h) Pzn-ga-4, showing the orientation of intercalated Pzn residue in the duplex (IV). Starting structures (a–d) have been generated upon minimization of classical B-DNA conformation incorporating four different stereochemical orientations of the intercalating Pzn residue. Final structures (e–h) are the result of 9 cycles of MD/MORASS refinements.
been converted to the north sugar conformation for $^6$G and $^7$G which is not completely consistent with the experimental data (vide infra). All experimental nOes found amongst Pzn protons and between Pzn-DNA protons were initially translated into distance restraints based on their intensities measured from the volume integrals of the Pzn cross-peaks normalized to the reference distances H8-H7 aromatic Pzn protons. Then during the MD calculations, nOes of the -NH-(CH$_2$)$_2$-NH- linker were assigned values of 3.5 $\pm$ 1.5 Å to allow for the uncertainties in the stereochemical orientations arising from indefinite assignment. This includes any indirect effects that may be more significant in the data examined at the longer mixing time. We excluded all constraints between -CH$_2$CH$_2$-OH group and other Pzn-protons in two starting structures in which the stereochemical orientation of this group was above the plane of Pzn residue in which it was situated between aromatic rings of Pzn residue and $^7$G-$^1$C base pair of matched duplex (III) (or $^7$G-$^1$A mismatched duplex (IV)), two other starting structures were also used as starting points for calculations in which the -CH$_2$CH$_2$-OH group was below the plane of Pzn residue as shown in Figs. 15 and 16.

Discussion

The oligonucleotide duplexes (I), (II), (III) and (IV) were studied in detail by $^1$H-NMR spectroscopy and MD-calculations. The experimental data show several important points regarding the influence of the oligonucleotide modification with 5'-tethered Pzn residue.

(1) The duplexes appear to be in a normal B-DNA form (NOESY data); the relative intensities of most intranucleotide nOes (H8/H6-H2' > H6/H8-H1', H8/H6-H3' and H1'-H2'' > H1'-H2' (Tables 9–10)) observed in the 80 ms NOESY spectrum are consistent with the south sugar geometries for the majority of sugar moieties. The sigmoidal melting curves observed for most protons of mismatched (II) duplexes are classical evidence for a cooperative duplex to single strand transition. Most resonances moved upfield at lower temperature which reflects that their stereochemical orientations are either above or below the aromatic rings of neighbouring bases in the duplex [56]. However, some protons move upfield upon duplex melting, $^3$A(H8), $^4$A(H8), $^2$G(H8) and $^7$G(H8), in the mismatched duplex (II) which can not be explained at the present time [57]. Note that the clear sigmoidal melting curves have been observed for the resonances of other terminal residues, $^1$A(H8) and $^7$A(H8), of the duplex (II). It is reasonable to suggest that quite good stacking may exist for the terminal residues in the duplex (II) due to Watson-Crick base-pairing between $^6$G-$^2$C and the terminal $^7$G-$^1$A mismatch base pair. These data are consistent with the data from MD calculations presented in Fig. 17d.

(2) There are large chemical shift changes of the nucleobase protons as well as the sugar residues flanking the Pzn or mismatch base-pair (Fig. 18) in both strands of the duplexes (II), (III) and (IV) in comparison with the duplex (I).
Fig. 17. Stereoviews of the final structures obtained after 9 steps of restrained MD with distance constraints derived from MORASS hybrid matrix approach and torsion angle restraints for (a) the matched duplex (I) and (c) the mismatched duplex (II). (b) and (d) show the overlays of the 6 structures of the matched duplex (I) and the mismatched duplex (II) during last 50 ps (collected after each 10 ps) upon restrained MD calculation starting from the final structure (a) and (c), respectively. MD calculation were performed during the trajectory of 100 ps with soft constraints on NOESY-derived distances and torsion constraints (force constant for the NOESY volumes constraints were 10 kcal.mol$^{-1}$ Å$^{-2}$, and error bars for the flat-well potential were ±15%, respectively. Force constant for torsion constraints is 40 kcal.mol$^{-1}$ rad$^{-2}$ accomodating a dihedral angle variation of ±10°).
Fig. 18. Histogram of differences in the H6/H8 and H1' chemical shifts $\delta$ (ppm) showing the comparison of the matched duplex (I) with the mismatched duplex (II), Pzn-linked matched duplex (III) and Pzn-linked mismatched duplex (IV). Positive shifts correspond to the resonances that move up-field compared to the same resonances of the matched duplex (I); negative shifts correspond to the resonances that move in the down-field region.
(3) $^7\text{G}$ and $^8\text{C}$ base-sugar interaction in the Pzn-linked duplexes (III) and (IV) are disrupted by the Pzn residue.

(4) The pairing of a guanine to cytosine (Fig. 7) appears to be considerably strengthened by the Pzn residue in the matched duplex (III) compared to matched duplex (I), whereas in the mismatched duplexes the influence of the Pzn attached to the 5'-end of the hexamer oligonucleotide is not so significant.

(5) All our NOESY and DQF-COSY data show that in $^7\text{G}$-$^1\text{A}$ mismatch base pair, both purines adopt anti conformation. Both $^1\text{A}$ and $^7\text{G}$ show H8-sugar proton intraresidue nOe connectivities with the increase of relative intensities in the following order $\text{H}2' > \text{H}2'' > \text{H}1'$, which is characteristic of the anti conformation. The nOe intensities of all nucleotide residues for all matched and mismatched duplexes indicate anti conformations which is typical of B-DNA like structures (Tables 9-10).

(6) A detailed comparison of the individual values for the torsion angles and sugar pucker from the lowest energy structure after nine steps of MORASS/MD refinements is given in Fig. 19. The left column in Fig. 19 shows a comparison of all torsions between starting (marked in Fig. 19 by 'gc-min' and 'ga-min') and final structures (marked in the figure by 'gc' and 'ga') of matched and mismatched duplexes (I) and (II) showing the deviation from a standard canonical Arnott's B-DNA. For the starting matched (gc-min) and mismatched DNA (ga-min) structure most torsion angles ($\alpha, \beta, \gamma, \epsilon, \zeta, \chi$) fall close to the typical values for B-DNA. But the final structure of these duplexes obtained after the MORASS/MD refinements show some interesting features. The values for torsion angle ($\alpha, \beta, \gamma, \zeta, \chi$) for the mismatched and matched duplexes are very similar to typical B-DNA structures except for the terminal residues. For the duplex (I) there are, however, several exceptions as seen in the unusual behavior of the torsion angles of the residues $^6\text{G}$ ($\epsilon, \zeta$), $^2\text{C}$ ($\alpha, \gamma$), and $^3\text{A}$ ($\epsilon, \zeta$): (i) The $\alpha$ torsion of $^2\text{C}$ residue has $g^+$ conformation compared to the rest which have $g^-$ conformation. (ii) The $\gamma$ torsion of $^2\text{C}$ is in the trans range. (iii) For $^4\text{G}$ and $^3\text{A}$ residues, the rotation about the $\epsilon$ and $\zeta$ torsions are found to be in the unusual $g^-$ and trans conformation, respectively, which are characteristic of rare $B_{11}$ backbone conformation. The sugar pucker obtained from the MORASS/MD refinements are consistent with the experimental data (Table 13). All values except for the $^8\text{C}$ are located in the S-range.

(7) Finally, $\chi$ is predominantly in anti (270–210°) conformations for all residues except for $^1\text{C}$, which is high anti, in the matched duplex (I). Thus, both the experimental data and results of MORASS/MD refinements unambiguously show that mismatched $^1\text{A}$ and $^7\text{G}$ base-paired residues adopt anti conformation. In neutral aqueous buffer with the standard tautomers of adenine and guanine, there are four types of G-A mismatch base pairs possible [58]. The syn-anti conformation for G-A base-pairing is unambiguously ruled out by our experimental data (vide supra). The G(anti)-A(anti) conformations can be distinguished on the basis of hydrogen-bonding either through 'imino' [58] or 'amino' (Fig. 2) functions of guanine residue. An ‘imino’ G(anti)-A(anti) species in $5'$-d(CGAGAATTTCGCG) [59] and in $5'$-d(CCAAGATTGG) [60–62] and ‘amino’ G(anti)-A(anti) in $5'$-
d(ATGAGCGCCTA) [41] have already been observed. In order to characterize the type of 'imino' or 'amino' G(anti)-A(anti) conformation, the unequivocal assignment of the imino proton of \(^7\)G has been performed. The observation of an imino proton resonance at 10–11 ppm is usually assigned either to the imino proton of the mismatched guanine residue in the syn conformation (which has been excluded, vide supra), or to the non-hydrogen bonded imino proton (which if hydrogen-bonded should move downfield to 12 ppm region) [41]. Note that the imino protons of \(^7\)G in the mismatched duplex (II) absorbs at 10.89 ppm (Table 11) which suggest that this must be a non-hydrogen bonded imino proton. These conclusions are supported by MD-calculation of the mismatched duplex (II) (Fig. 17). Despite the bulging of the mismatched bases, the conformation of the duplex

![Fig. 20. Potential energy (E_{pot} (kcal/mol)) (top line) and cartesian coordinate deviation (bottom line) (r.m.s. deviation (\AA)) during 100 ps of restrained molecular dynamics on the final structures generated after 9-step of MD/MORASS refinement procedures: (a) matched duplex (I), (b) mismatched duplex (II), (c)-(e) matched duplex (III) bearing Pzn residue for three final structures (Table 17: (c) Pzn-gc-1, (d) Pzn-gc-2, (e) Pzn-gc-3), (f)-(i) mismatch duplex (IV) bearing Pzn residue for the four final structures (Table 18: (f) Pzn-ga-1, (g) Pzn-ga-2, (h) Pzn-ga-3, and (i) Pzn-ga-4).](image-url)
(II) calculated during 100 ps appear to be quite stable and is very similar to the native duplex (I). Indeed, neither duplex (I) nor duplex (II) show any greater variation in the r.m.s. deviation or in potential energy fluctuation (Fig. 20). The MD/MORASS refinement data of the final structure of mismatched duplex (II) show (Fig. 17) that the 1A and 7G indeed form stable base pairs through inter-strand hydrogen bonds with N7(A) ⋅⋅⋅HN2(G) (1.92 Å) with a subtended angle of 176° and the hydrogen bond between N3(G) ⋅⋅⋅HN6(A) (2.01 Å) has a subtended angle of 153° (propeller-twist angle: 36°). It may be noted that the starting structure for the MD/MORASS refinement for the mismatched duplex (II) was an imino-type G(anti)-A(anti) conformation (without any constraint) but the structure that was finally produced at the end of MORASS/MD refinement exhibited amino-type conformation of the G(anti)-A(anti) base pair.

(8) Fig. 19 illustrates that the unconstrained γ torsion angle is mainly in the γ⁺ mode except for 7G, 1A, 1C, and 2C residues in duplexes (III) and (IV) in which γ is around 200°. It may be noted that the behaviour of γ is coupled with the mobility of torsion angle α. This cooperative mobility of γ with respect to α has been observed [63] earlier for the B-type DNA due to the ‘crankshaft’ motion about the intermediate β bond in trans orientation. Estimation of J coupling of H4' (Σ₄' = (J₄'₃' + J₄'₅' + J₄'₅'') ≈ 4.9—6.2 Hz, except for the terminal 8C residue) and the NOESY spectra in the H8/H6-H5'/H5'' region suggest that all residues in all duplexes are in γ⁺ orientation. The only exception is the terminal 2C residue (γ') in the matched duplex (I) which is evident from its NOESY spectrum (the cross peak H6-H5'/H5'' have been clearly observed with the intensity comparable to H8/H6-H1’ supporting γ⁺ orientation. This suggest that although Pzn-gc-1/Pzn-gc-3 and Pzn-gc-2/Pzn-gc-4 (Table 17 and Fig. 19) have shown comparable experimental and theoretical %RMS, but the former two structures (i.e., Pzn-gc-1/Pzn-gc-3) are less preferable because of the fact that for the 2C and 3T residues in the duplex (III), γ⁺ orientation can not be experimentally supported because of absent of the cross peak H6-H5'/H5'' although all other cross peaks (H8-H1', H8-H3', H8-H4') for this residue has been assigned without any problem. Note that although the starting structures for Pzn-gc-2/Pzn-gc-4 (with the hydroxyethyl moiety of phenazine residue ‘up’ and ‘down’, respectively) for MORASS/MD refinements were different (see Fig. 15b,d and Table 17) but they converged to the identical final structure in which the hydroxyethyl moiety of Pzn residue is in the ‘down’ orientation.

(9) The variation of ε and ζ for the Pzn-linked matched and mismatched duplexes are shown in the middle and right column of Fig. 19. The identification of torsion angles ε and ζ have been found to be a useful way [64,52,63] for discriminating between B₁ and B₁Ι phosphate conformation. The difference between ε and ζ torsion angles should be −90° for B₁ and +90° for B₁Ι DNA conformations. However, the chaotic difference between ε and ζ torsions have also been observed [61] to take place for the asymmetrical duplexes. The MORASS/MD refinement (Fig. 19) shows that the phosphates 7G, 6G and 2C flanking the Pzn residue exhibit the less common B₁Ι-type DNA conformation. It has been predicted [65,64] that predominantly B₁Ι-type DNA conformation should
have a phosphorus chemical shift downfield compared to B₁ conformation. The $^{31}$P chemical shift can therefore potentially provide evidence of the conformation of the phosphate ester backbone in oligonucleotide duplexes and complexes. In the $^{31}$P-NMR spectra of the matched duplex (I), the $^1P$ and $^9P$ phosphates and matched duplex with Pzn residue (III), the $^1P$ phosphate resonances are shifted downfield from $-2$ ppm to $0.5$ ppm. These downfield phosphodiester reso-

Fig. 21. Stereoview of the superimposition of the structures of the duplex (III) (collected after each 10 ps) derived from 100 ps of restrained molecular dynamics with soft distance constraints (force constants and error bars for the flat-well were $10$ kcal.mol$^{-1}$ Å$^{-2}$ and $±15\%$, respectively) and torsion constraints ($40$ kcal.mol$^{-1}$ rad$^{-2}$ and $±10°$) have been utilized: (a) Pzn-gc-1, (b) Pzn-gc-2, (c) Pzn-gc-3, during last 50 ps.
Fig. 22. Stereoview of the superimposition of the structures of the duplex (IV) (collected after each 10 ps) derived from 100 ps of restrained molecular dynamics with soft distance constraints (force constants and error bars for the flat-well were 10 kcal.mol\(^{-1}\) Å\(^{-2}\) and ±15%, respectively) and torsion constraints (40 kcal.mol\(^{-1}\) rad\(^{-2}\) and ±10°) have been utilized: (a) Pzn-ga-1, (b) Pzn-ga-2, (c) Pzn-ga-3, and (d) Pzn-ga-4 during last 50 ps.
nances have been shown ($^{31}$P-H correlation experiment) to belong to the terminal $^1$T, $^1$C and $^1$T residues, respectively. This clearly means that none of our duplexes belong to the B$_{II}$-type DNA family.

(10) Typically, intercalating drugs cause 1–2 ppm downfield shifts of the neighbouring phosphate resonances of DNA, which exhibit a local unwinding of the helix and the phosphorus conformation is changed from a gauche, gauche to a gauche, trans conformation [64]. But in the case of duplex (III) the large upfield shift of $^9$P, which belongs to the 5'-end of $^1$C residue, (to $\sim -2$ ppm) was obviously induced by the diamagnetic anisotropy of Pzn residue. This clearly rules out any intercalated structure for phenazium residue with DNA bases, and is oriented perpendicular to the main axis of the DNA duplex (Fig. 21). This may explain why Pzn residue strongly stabilizes imino-type hydrogen bond in the flanking $^1$C-$^7$G base pair (Fig. 7) which in turn enhances the stability of the duplex.

(11) The $^{31}$P spectra of the mismatched duplex (II) and the mismatched duplex with Pzn residue (IV) suggest, as in the case of matched duplexes (I) and (III), that they do not belong to the B$_{II}$-type DNA family.

(12) Four final structures of the mismatched Pzn-linked duplex (IV) appear completely different with respect to the stereochemical orientation of the Pzn residue (Fig. 16, Table 18). In all structures Pzn-ga-1, Pzn-ga-2, Pzn-ga-3 and Pzn-ga-4, good stacking interaction between $^6$G, $^7$G and $^8$C in the octamer chain have been found. But it is only in the structure Pzn-ga-3, that the mismatched base pair $^7$G-$^1$A exist, the Pzn residue in this case shield only part of the mismatched base pair by stacking with the flanking $^1$A residue (propeller-twist angle: 36°). MD-calculation during 100 ps (Fig. 22) without constraints for the Pzn residue has shown that it fluctuates near the $^1$A residue position, but never completely stabilizes the mismatched $^7$G-$^1$A base pair. It is likely that Pzn residue may be partly influencing the hydration behaviour of the duplex (IV) compared to its parent duplex (II).

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