The solution structure of a 3'-phenazinium (Pzn) tethered DNA – RNA duplex with a dangling adenosine: r(5'-G-AUUGAA3'):d(5'-TCAATC3' – Pzn)

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Received September 24, 1994; Revised and Accepted October 20, 1994

ABSTRACT

The 3'-Pzn group tethered to an oligo-DNA stabilizes a DNA – RNA hybrid duplex structure by 13°C compared to the natural counterpart. This report constitutes the first full study of the conformational features of a hybrid DNA – RNA duplex, which has been possible because of the unique stabilization of this rather small duplex by the tethered 3'-Pzn moiety ($T_m \approx 40^\circ\text{C}$ from NMR). In this study, a total of 252 inter- and intra-strand torsional and distance constraints along with the full NOE relaxation matrix, taking into account the exchange process of imino and amino protons with water, have been used. The 3'-Pzn-promoted stabilization of the DNA – RNA hybrid duplex results in detailed local conformational characteristics such as the torsion angles of the backbone and sugar moieties that are close to the features of the other natural DNA – RNA hybrids (i.e. sugars of the RNA strand are 3'-endo, but the sugars of the DNA strand are intermediate between A- and B-forms of DNA, $72^\circ < P < 180^\circ$; note however, that the sugars of our DNA strand have a C_{1'}-exo conformation: $131^\circ < P < 154^\circ$). This study suggests that 3'-Pzn-tethered smaller oligo-DNA should serve the same purpose as a larger oligo-DNA as an antisense inhibitor of the viral mRNA. Additionally, these types of tethered oligos have been found to be relatively more resistant to the cellular nuclease. Moreover, they are taken up quite readily through the cellular membrane (14) compared to the natural counterparts.

INTRODUCTION

Ribonucleic acids (RNAs) play a wide range of important roles in living cells, including the traditional roles in transcription and translation (tRNA, mRNA and rRNA) of genetic information. Excitement in RNA chemistry has also grown with the discovery

of RNA sequences with catalytic activity, the formation of 'lariats' during the processing of pre-rRNA, and the development of recombinant RNA technology. In addition, it has been found that many families of retroviruses, including the HIV retrovirus, use RNA to carry the genetic material to the infected host cells. The reverse transcription of the viral RNA by the viral reverse transcriptase gives the DNA – RNA hybrids which are specifically degraded by the inherent RNase H activity of the viral reverse transcriptase giving proviral DNA that then finally becomes integrated into the host DNA. Thus the inhibition of any of the above key processes will be able to suppress the activity of retroviral polymerases and thus viral replication. The binding of DNA ligands such as intercalators or minor groove binding ligands have been studied and reviewed extensively (1). Recently, an attempt to design an RNA – DNA hybrid binding agent has been partly successful (2). It has been known for some time now that a covalently linked agent (5h) such as cholesterol (3), ethidium bromide (4), acridine (5a–c) or phenazinium (Pzn) (6a) derivative stabilizes the DNA duplex considerably in comparison with their natural counterparts. Most of the conclusions of these studies are based upon UV spectrometric data, except for a few NMR studies with acridine (5d–g) and Pzn (6b–g). Only recently have we begun to understand the mechanism of stabilization with our 5'-Pzn-tethered matched and mismatched duplex (6b,c). We showed (6b) that the Pzn tether increases the thermodynamic stability of the duplex by 9–20°C, compared with the natural counterparts, this stability however decreases as the number of mismatches increases with the target DNA sequence. In addition, we found that the Pzn-mediated stabilization of the DNA duplex depends both on the stacking ability (6b) with the neighbouring nucleobases in both strands as well as on the hydration pattern in the minor and the major grooves of DNA (6c). Interestingly, we found (6c) by comparing the extent of hydration (by NOESY and ROESY experiments) in matched and single-mismatched DNA duplexes with or without the Pzn tether that the amount of hydration decreases as the T_m

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stability of the Pzn-tethered DNA duplex increases compared to the non-tethered counterpart. These studies have led us to address the question if it is possible to stabilize an otherwise unstable small DNA–RNA hybrid duplex by using 3'-Pzn-tethered DNA. We here report our high-resolution NMR studies in combination with the MD/MARDIGRAS refinement procedure to determine the conformational properties of the 3'-Pzn-tethered DNA–RNA hybrid, $r(5'GAUUGAA3') : d(5'TCAATC3' - \text{Pzn})$, which is 13°C more stable (Fig. 1A) than the natural counterpart without the Pzn tether.

MATERIALS AND METHODS

Synthesis and sample preparation

Oligonucleotide $5'r(GAUUGAA)3'$ was synthesized according to the standard H-phosphonate methodology (7) using a manual preparative solid phase synthesis (8a) protocol (400 mg of nucleoside-bound CPG with loading of 50 $\mu\text{mol/g}$). Preparative purification of the $5'r(GAUUGAA)3'$ was carried out by successive anion-exchange HPLC [Polysil CA (8b) (4.6×250 mm), 3 M KH_2PO_4 in 30% acetonitrile] and reverse phase HPLC [Lichrosorb RP-18 (4.6×250 mm), 0–20% acetonitrile containing 0.05 M LiClO_4]. Oligonucleotide was isolated as lithium salt (300 OD₂₆₀ units).

$5'p[d(TCAATC)]3' - \text{Pzn}$ was synthesized by using a standard protocol (8c) and isolated by anion-exchange and reverse phase HPLC as described above. For NMR measurements, the oligonucleotides were purified additionally by successive passage through a small column of Dowex 50 (Na^+) and Chelex 100 (Na^+) resins. The NMR sample was dissolved in 0.4 ml of the buffer (0.1 M NaCl, 10 mM, NaH_2PO_4 , 10 mM EDTA, pH 7.4) and twice lyophilized to dryness from D_2O and dissolved in D_2O (99.96% D). The duplex, $\{5'r(G^1A^2U^3U^4G^5A^6A^7)3': 5'p[d(T^8C^9A^{10}A^{11}T^{12}C^{13})]3' - \text{Pzn}\}$, concentration for NMR measurements was 10 mM and 25 μM for the UV measurement. Molar extinction coefficients of $5'r(GAUUGAA)3'$ and $5'p[d(TCAATC)]3' - \text{Pzn}$ were estimated (8d) at 260 nm ($\epsilon = 77900 \text{ M}^{-1}\text{cm}^{-1}$ and $56900 \text{ M}^{-1}\text{cm}^{-1}$, respectively).

Nuclear magnetic resonance spectroscopy

^1H -NMR spectra were recorded on a Bruker AMX NMR spectrometer operating at 500.13 MHz. NOESY experiments in D_2O at 12°C (with mixing times of 5 μs and 150 ms) and in 90% H_2O –10% D_2O mixture (6c) at 0°C (with A mixing time of 200 ms), respectively, have been performed with a final spectra of 2000×2000 real data points after Fourier transformation. After Fourier transformation the spectra in 90% H_2O –10% D_2O were corrected for the excitation profile (6c). The volumes of all crosspeaks of all NOESY experiments were calculated and normalized using the volumes of the diagonal peaks of the 5 μs NOESY (in D_2O) using the AURELIA (9a) program supplied by Bruker. DQF-COSY spectra were recorded with and without phosphorus decoupling at 12°C. The crosspeaks were then

simulated using SMART (9a). In all NOESY and DQF-COSY experiments a relaxation delay of 5 s was used. The T_1 was measured both by the inversion recovery and saturation recovery methods, the T_2 was measured by using the cpmg pulse sequence (9b). In all cases, a 20 s relaxation delay was used. Correlation times (τ_c) were calculated by two different ways: (i) using the intensity of the reference H5–H6 protons of U^3 , U^4 , C^9 and C^{13} and of the H2'–H2' crosspeaks of the DNA strand, assuming isotropic motion, which have been normalized to the corresponding intensity of the diagonal peaks (9a), and (ii) also by using the dipolar relaxation times T_1 and T_2 , which are related for an isolated homonuclear two-spin system (10) at 12 and 0°C. Thus, τ_c for the H5–H6 and H2'–H2' crosspeaks in DNA or RNA were 2.0 ns at 12°C, whereas τ_c at 0°C for H2A and the amino protons of A and C were found to be 5 ns and 0.8 ns for the H2'–H2' crosspeaks. Note that the shorter τ_c for the H2'–H2' crosspeaks at 0°C was due to the spin diffusion (10).

Model building and molecular mechanics/dynamics calculation

The starting Cartesian coordinates of the duplexes were generated using Amber 4.0 from Arnott A-RNA and B-DNA coordinates because of the qualitative similarity of our NMR data in the hybrid DNA–RNA in which the RNA strand showed similarity to A-type RNA and the DNA strand to the B-type DNA; one of the strands was thereafter changed to DNA or RNA, respectively, and the parameters of the Pzn residue (6b) were added to the Amber 4.0 force-field. To correct for spin diffusion of the NOESY volumes the hybrid matrix procedure of MARDIGRAS (ver. 5.0) (10) was used in which crosspeaks involving exchangeable imino and amino protons were included. The rates of exchange of imino or amino protons were calculated from the combination (6d) of NOESY and ROESY spectra in H_2O – D_2O (9:1 v/v) at 0°C. For MARDIGRAS runs we used isotropic single experimentally derived τ_c of 2 ns for all the base protons and sugar protons at 12°C, whereas we used τ_c of 2 ns for base protons, 0.8 ns for the sugar protons and 5 ns for H2A and the amino protons of A and C at 0°C to obtain the effective correlation time (τ_c^*) (10,11n) for any two-proton dipole–dipole interaction: $\tau_c^* = \{ \frac{1}{2}[(1/\tau_{ci}) + (1/\tau_{cj})] \}^{-1}$ where τ_{ci} or τ_{cj} are the correlation times of the *i* and *j* protons. Subsequently, the results were combined and averaged by AVGMARD (10) for all interproton distances using the NOESY spectra in H_2O – D_2O (9:1 v/v) at 0°C and in D_2O at 12°C at the mixing times 200 and 150 ms, respectively, in order to cover the conformational space at a temperature range from 0 to 12°C, which is still much below the T_m of the duplex (40°C). The results were used as an input in our MD calculations. In our 7-step protocol of MD calculations (6b), we used 105 intra-residual NOEs, 55 sequential NOEs, 24 cross-strand NOEs and 7 NOEs between Pzn to duplex as distance constraints (a total of 191 NOE constraints). In addition, we have employed 6 constraints defining the Watson–Crick base pairing ($1.93 \pm 0.3 \text{ \AA}$) of the duplex and 55 torsional angle constraints from DQF-COSY experiments (an average of 17.2 constraints per nucleotide residue). Eight hybrid DNA–RNA structures represent two families of starting models: the first is based on A-type RNA in both strands, and the second is based on B-type DNA models also in both strands. Each of this family has 4 structures (q1–q4) encompassing four different stereochemical orientations of the Pzn residue (see the footnote to Table 4).

Figure 1. (A) Comparison of the first derivative ($\Delta A_{260}/\Delta T$ where A indicates UV absorption at 260 nm) of the UV melting curve (25 μ M) of (1) the unmodified hybrid DNA–RNA duplex, r(GAUUGAA):d(TCAATC) ($T_m = 5^\circ\text{C}$) with (2) 3'-Pzn-tethered DNA–RNA hybrid, r(5'GAUUGAA3'):d(5'TCAATC3'-Pzn) ($T_m = 18^\circ\text{C}$). Under the conditions of the melting point, the 3'-Pzn-tethered DNA–RNA hybrid [i.e. duplex (2)] is found to be stable by 13°C compared to the natural duplex (1). (B–D) Chemical shifts versus the temperature profiles of some protons of hybrid DNA–RNA duplex, r(5'GAUUGAA3'):d(5'TCAATC3'-Pzn). The base proton resonances: (B) H6T¹² (■), H6C¹³ (●), H2A⁷ (○) and H3Pzn (□), (C) the methyl groups of T¹² (●) and T⁸ (○), and (D) the H1' of T⁸ (■) and A¹¹ (□) and H5 of U⁴ (●) and C¹³ (○) and H1Pzn (◇). The transition temperature is at $\sim 40^\circ\text{C}$.

RESULTS AND DISCUSSION

Assignment of the non-exchangeable protons

The assignment of the non-exchangeable protons (Table 1) in the duplex was made in the usual sequential manner from a NOESY spectrum. The four strong H5–H6 crosspeaks from the uridines and cytidines and the two methyl resonances from the thymidines were easily identified from both NOESY and DQF-COSY spectra. These peaks then served as the reference points for the sequential assignments. Each H6/H8 base proton showed a crosspeak with both its own (H6/H8_(i) $\leftrightarrow$ H1'_(i)) and its 5'-linked residue H1' (H6/H8_(i) $\leftrightarrow$ H1'_(i-1)). This allowed us in a straightforward way to connect all residues in both the RNA (G¹ $\rightarrow$ A⁷) and the DNA strand (T⁸ $\rightarrow$ C¹³) (Fig. 2A). In the RNA strand the overlapping peaks of H8G¹ (8.13 ppm) $\leftrightarrow$ H1'G¹

(5.71 ppm) and H8A² (8.16 ppm) $\leftrightarrow$ H1'G¹ (5.71 ppm) (Fig. 2A) were resolved on the other side of the diagonal and could thus be assigned unequivocally. The crosspeaks of H8G⁵ (7.65 ppm) $\leftrightarrow$ H1'U⁴ (5.69 ppm) and H8G⁵ (7.65 ppm) $\leftrightarrow$ H1'G⁵ (5.69 ppm) are overlapped (Fig. 2A) in 12°C NOESY spectra, nevertheless they could be unambiguously assigned at 0°C . The assignment of the DNA strand could however be done in a straightforward manner starting either at the 5' end [H6T⁸ (8.07 ppm) $\leftrightarrow$ H1'T⁸ (6.31 ppm)] or at the 3' end [H6C¹³ (7.49 ppm) $\leftrightarrow$ H1'C¹³ (5.99 ppm)] (Fig. 2A). This assignment was further corroborated by the H6/H8_(i) $\leftrightarrow$ H2'_(i, i-1) and H6/H8_(i) $\leftrightarrow$ H3'_(i, i-1) pathways of the RNA strand (Fig. 2B, C) and by the H6/H8_(i) $\leftrightarrow$ H2'/2'_(i, i-1) pathway of the DNA strand (Fig. 2D). Fig. 2 also shows severe overlaps in the aromatic to H2'/H3' area of the RNA strand, which becomes

Table 1. ^1H chemical shifts of hybrid DNA–RNA duplex $r(5'\text{GAUUGAA}3')\cdot d(5'\text{TCAATC}3'-\text{Pzn})$ at 12°C

Residue	H2/H6	H8/H5/CH ₃	H1'	H2'	H2'	H3'	H4'	H5' /H5'	NH	NH ₂ ^a
G ¹		8.13	5.71	4.88		4.72	^b	^b	11.33	7.28/6.16
A ²	7.68	8.16	5.95	4.64		4.72	^b	^b		7.50/6.56
U ³	7.67	5.08	5.42	4.34		4.64	4.45	^b	13.99	
U ⁴	8.07	5.58	5.69	4.45		4.61	^b	^b	13.28	
G ⁵		7.65	5.69	4.64		4.45	^b	^b	11.92	7.20/5.35
A ⁶	7.71	7.78	5.74	4.52		4.67	^b	^b		^b
A ⁷	7.94	7.86	5.88	4.47		4.63	4.51	^b		^b
T ⁸	8.07	2.09	6.31	2.60	2.78	5.02	4.46	4.15, 4.29	14.05	
C ⁹	7.75	5.85	6.04	2.41	2.70	^b	4.35	4.30		8.44/7.15
A ¹⁰	6.88	8.19	6.10	2.77	2.92	5.10	4.48	4.33		7.89/6.24
A ¹¹	7.65	8.06	6.22	2.59	2.92	5.09	4.51	4.20, 4.33		7.78/6.37
T ¹²	7.22	1.34	5.92	2.12	2.49	4.86	4.25	^b	13.73	
C ¹³	7.49	5.55	5.99	2.12	2.24	4.92	4.24	4.17		8.19/7.10
Pzn	H1	H3	H4	H5	H6	H7	H8			
	6.72	7.27	7.50	7.69	7.18	7.32	7.81			

^afor NH₂ the first chemical shift is for the hydrogen bonded proton and the second is for the non-hydrogen bonded proton.

^bCould not be obtained due to overlap of signals.

Table 2. Coupling constants obtained in Hz after simulation of the observable experimental crosspeaks of the DQF-COSY spectra and calculated best fit sugar pucker of the hybrid DNA–RNA duplex $r(5'\text{GAUUGAA}3')\cdot d(5'\text{TCAATC}3'-\text{Pzn})$ at 12°C

Residue	$J_{1'2'}$	$J_{1'2'}$	$J_{2'3'}$	$J_{2'3'}$	$J_{3'4'}$	$J_{4'5'} + J_{4'5'} P_S$	Φ_S	%S	ΔJ_{max}	RMS
T ⁸	9.1 ± 0.5	5.7 ± 0.5	5.2 ± 0.5	2.2 ± 0.5	3.3 ± 1.0	3.8 ± 1.0 150.7 ± 10.9°	37.3 ± 7.8°	81	0.41	0.293
C ⁹	9.2 ± 0.5	5.2 ± 0.5	4.8 ± 0.5	2.8 ± 0.5	3.4 ± 1.0	^a 150.9 ± 12.6°	42.6 ± 6.5°	77	0.39	0.296
A ¹⁰	9.2 ± 0.5	5.2 ± 0.5	5.3 ± 0.5	2.4 ± 0.5	3.9 ± 1.0	2.8 ± 1.0 139.8 ± 6.4°	41.3 ± 9.6°	79	0.33	0.243
A ¹¹	9.2 ± 0.5	4.9 ± 0.5	4.8 ± 0.5	2.6 ± 0.5	3.5 ± 1.0	^a 147.0 ± 7.2°	44.1 ± 8.3°	77	0.29	0.226
T ¹²	9.2 ± 0.5	5.0 ± 0.5	4.9 ± 0.5	2.1 ± 0.5	3.3 ± 1.0	4.1 ± 1.0 148.2 ± 6.4°	41.9 ± 8.1°	80	0.33	0.242
C ¹³	9.9 ± 0.5	4.7 ± 0.5	5.2 ± 0.5	2.7 ± 0.5	2.2 ± 1.0	^a 154.5 ± 6.4°	42.8 ± 5.8°	85	0.50	0.309

^aCould not be observed.

The program PSEUROT was run with P_N and Φ_N fixed at different values ($9^\circ < P_N < -9^\circ$, $32^\circ \Phi_N < 42^\circ$) and they had no effect on the final result while P_S , Φ_S and %S were iterated by the program. All permutations using the maximum and minimum possible coupling constants were also assessed and the errors presented in P_S and Φ_S are the standard deviations of those runs.

ΔJ_{max} is the largest difference between the coupling constants calculated by PSEUROT and the experimental coupling constants.

still more intricate with a larger RNA strand making it a challenging task (11) to extract any useful information (see the legend to Fig. 2 for full assignments).

Assignment of the exchangeable protons and H2A protons

The chemical shifts of the exchangeable protons are given in Table 1. The H2A protons were assigned from the imino protons following the $d/rA(H2_{(i)}) \leftrightarrow dT/rU(\text{imino}_{(i+1 \text{ or } j)})$ pathway (data not shown). The N1HG¹ (11.33 ppm) imino proton has a crosspeak with H2A² (7.68 ppm) which in turn has a crosspeak with N3HT¹² (13.73 ppm). Similarly the N3HU³ (13.99 ppm) $\leftrightarrow$ H2A¹¹ (7.65 ppm) $\leftrightarrow$ N3HU⁴ (13.28 ppm), N3HU⁴ (13.28 ppm) $\leftrightarrow$ H2A¹⁰ (6.88 ppm) $\leftrightarrow$ N1HG⁵ (11.92 ppm) and N1HG⁵ (11.92 ppm) $\leftrightarrow$ H2A⁶ (7.71 ppm) $\leftrightarrow$ N3HT⁸ (14.05 ppm) connections could be established and the H2 protons assigned. The remaining H2 resonance (detected in the inversion recovery experiment) was assigned as H2A⁷.

Assignment of the N-(2-hydroxyethyl)phenazinium protons

The assignment of all coupled protons H1, H3, H4, H5, H6, H7, H8, $^{-13}\text{CH}_2$ - and $^{-14}\text{CH}_2$ - protons of the Pzn residue were made from both NOESY and DQF-COSY spectra (6b,e). In NOESY additional crosspeaks could be found between H1 and H8, between H1 and $^{-13}\text{CH}_2$ - and $^{-14}\text{CH}_2$ - and between H3 and

$^{-14}\text{CH}_2$ - of the linker. The protons of the hydroxyethyl group could not be assigned.

Stereochemical orientation of the phenazinium ring

There are several crosspeaks in the NOESY spectra that have enabled us to determine the stereochemical orientation of the Pzn ring. Both the H7 and H8 protons of Pzn have crosspeaks to the H1' proton of G¹. The H3Pzn proton has crosspeaks to the H2', H2' and H5 protons of ^{13}C and with the slowly exchangeable imino proton of the linker (visible in the NOESY spectra recorded in 90% H₂O). This allowed us to stereochemically orientate the Pzn ring with respect to the linker and also unambiguously set the linker to the *cis* orientation with respect to H1 and $^{-13}\text{CH}_2$ - or ^2CNH and H3 [$\phi(^{13}\text{C}-^2\text{C}-^2\text{CNH}-^{13}\text{C}) = 0^\circ$] (6b) which is consistent with our earlier observations for the DNA-tethered Pzn duplex (6b).

Conformation of the sugar rings

The conformation of the sugar rings of the DNA strand could be determined from the $J_{H1'-H2'}$, $J_{H1'-H2'}$, $J_{H2'-H3'}$, $J_{H2'-H3'}$ and $J_{H3'-H4'}$ coupling constants obtained from DQF-COSY. These coupling constants were then further refined by simulation with the SMART program (9a) and are presented in Table 2. The phase angles (P) and the puckering amplitudes (Φ) of each sugar

Figure 2. Expanded plots of the NOESY spectra (150 ms) of the hybrid r(5'GAUUGAA3'): d(5'TCAATC3'-Pzn) in D₂O at 12°C. Crosspeaks in (A) show the sequential aromatic to H1' connectivities for both the RNA and the DNA strand. The assignment of the RNA strand starts with the 5'-end, i.e. from H8G¹ (8.13 ppm)–H1'G¹ (5.71 ppm) crosspeak. Similarly, we also start with the 5'-end for the DNA strand, i.e. H6T⁸ (8.07 ppm)–H1'T⁸ (6.31 ppm) crosspeak. Both are marked with arrows showing the direction of the assignment. The assignment of the inter-residual crosspeaks are as follows (marked by arabic numbers): (1) H1'T⁸–H6C⁹, (2) H1'C⁹–H8A¹⁰, (3) H1'A¹⁰–H8A¹¹, (4) H1'A¹¹–H6T¹², (5) H1'T¹²–H6C¹³, (6) H1'G¹–H8A², (7) H1'A²–H6U³, (8) H1'U³–H6U⁴, (9) H1'U⁴–H8G⁵, (10) H1'G⁵–H8A⁶ and (11) H1'A⁶–H8A⁷. The subspectra (B) and (C) show the same part of the spectrum. In (B), the sequential H6/H8 to the H2' crosspeaks of the RNA strand are traced and starts with the 5'-end: H8G¹ (8.13 ppm)–H2'G¹ (4.88 ppm) crosspeak. The assignment of the inter-residual crosspeaks are as follows: (1) H2'G¹–H8A², (2) H2'A²–H6U³, (3) H2'U³–H6U⁴, (4) H2'U⁴–H8G⁵, (5) H2'G⁵–H8A⁶ and (6) H2'A⁶–H8A⁷. In (C), the sequential H6/H8 to the H3' crosspeaks of the RNA strand are marked and starts with the 5'-end: H8G¹ (8.13 ppm)–H3'G¹ (4.72 ppm) crosspeak. The assignment of the inter-residual H6/H8 to the H3' crosspeaks are as follows: (1) H3'G¹–H8A², (2) H3'A²–H6U³, (3) H3'U³–H6U⁴, (4) H3'U⁴–H8G⁵, (5) H3'G⁵–H8A⁶ and (6) H3'A⁶–H8A⁷. In (D), the sequential H6/H8 to the H2'/H2' crosspeaks of the DNA strand are traced and starts with the 5'-end: H6T⁸ (8.07 ppm)–H2'/H2'T⁸ (2.60, 2.78 ppm) crosspeak. The assignment of the inter-residual crosspeaks are as follows: (1, 2) H2'/2'T⁸–H6C⁹, (3, 4) H2'/2'C⁹–H8A¹⁰, (5, 6) H2'/2'A¹⁰–H8A¹¹, (7, 8) H2'/2'A¹¹–H6T¹², (9, 10) H2'/2'T¹²–H6C¹³. Note that only the intra-residue H6/H8 to H1'/H2'/H2'/H3' crosspeaks are labeled in the figure.

Table 3. Torsional constraints calculated from pseudorotational parameters (P , Φ) and $^3J_{\text{HH}}$ (Table 2) and distance constraints for ϵ used in MD calculations

Residue	C2'–C3'	C3'–C4'	ϵ^a (Å)	β	γ
G ¹	35.5 ± 5.5°	–30.5 ± 6.5°	2.45–3.07		60 ± 40°
A ²	35.5 ± 5.5°	–30.5 ± 6.5°	2.45–3.07	180 ± 75°	60 ± 40°
U ³	35.5 ± 5.5°	–30.5 ± 6.5°	2.45–3.07	b	b
U ⁴	35.5 ± 5.5°	–30.5 ± 6.5°	2.45–3.07	b	b
G ⁵	35.5 ± 5.5°	–30.5 ± 6.5°	2.45–3.07	b	b
A ⁶	35.5 ± 5.5°	–30.5 ± 6.5°	2.45–3.07	b	b
A ⁷	35.5 ± 5.5°	–30.5 ± 6.5°		180 ± 75°	60 ± 40°
T ⁸	–6.2 ± 32.1°	17.8 ± 7.3°	2.80–3.25	180 ± 75°	60 ± 40°
A ¹⁰	–31.4 ± 6.5°	9.8 ± 4.2°	2.80–3.25	180 ± 75°	60 ± 10°
A ¹¹	–36.8 ± 6.2°	15.7 ± 4.9°	2.80–3.25	180 ± 75°	60 ± 40°
T ¹²	–35.5 ± 5.9°	15.8 ± 4.4°	2.80–3.25	180 ± 75°	60 ± 10°
C ¹³	–38.6 ± 4.4°	20.4 ± 3.3°	2.80–3.25	180 ± 75°	60 ± 40°

^afor ϵ the distance between P and H3' is constrained (13). All aromatic to H3' crosspeaks had linewidths that were clearly smaller than the 20 Hz which allowed us to exclude conformational space with $\epsilon = 60^\circ$ (the H3' linewidth is dependent on $J_{2'3'}$, $J_{2'3'}$, $J_{3'4'}$, $J_{3'P}$ and contributions from $1/\pi T^*_2$ and $\epsilon = 60^\circ$ requires the $J_{3'P}$ to be larger than 20 Hz). The distances for ϵ include both B_I and B_{II} phosphate conformations with the exclusion of ϵ^+ rotamer.

^bCould not be calculated due to the overlap of the crosspeaks of interest.

C2'–C3': $\nu_2 = \Phi \cdot \cos(P)$ and C3'–C4': $\nu_3 = \Phi \cdot \cos(P + 4\pi/5)$. The errors in C2'–C3' and C3'–C4' are calculated using the maximum errors from Table 2.

Table 4. Atomic RMSD (Å) comparison of starting and final structures^a

	A-RNA_q1	A-RNA_q2	A-RNA_q3	A-RNA_q4	B-DNA_q1	B-DNA_q2	B-DNA_q3	B-DNA_q4
A-RNA_q1		0.601 (0.118)	1.531 (0.239)	1.548 (0.311)	3.800 (3.474)	3.810 (3.466)	4.098 (3.490)	4.191 (3.506)
A-RNA_q2	0.612 (0.301)		1.305 (0.195)	1.349 (0.268)	3.726 (3.467)	3.711 (3.459)	3.981 (3.483)	4.048 (3.499)
A-RNA_q3	1.407 (0.450)	1.362 (0.485)		0.396 (0.125)	3.862 (3.435)	3.891 (3.426)	3.749 (3.450)	3.776 (3.467)
A-RNA_q4	1.392 (0.593)	1.433 (0.613)	0.667 (0.389)		3.823 (3.393)	3.819 (3.386)	3.701 (3.411)	3.742 (3.428)
B-DNA_q1	0.806 (0.576)	0.938 (0.606)	1.226 (0.486)	1.086 (0.548)		0.378 (0.104)	1.230 (0.258)	1.412 (0.239)
B-DNA_q2	0.771 (0.503)	0.713 (0.496)	1.273 (0.553)	1.265 (0.687)	0.678 (0.527)		1.265 (0.288)	1.393 (0.294)
B-DNA_q3	1.419 (0.648)	1.439 (0.638)	0.695 (0.437)	0.497 (0.505)	1.140 (0.632)	1.185 (0.534)		0.592 (0.181)
B-DNA_q4	1.484 (0.688)	1.436 (0.672)	0.478 (0.437)	0.686 (0.325)	1.188 (0.524)	1.225 (0.523)	0.580 (0.391)	

^aRMSD values for all atoms or all atoms except Pzn (data presented within brackets) for the structures with different orientation of Pzn. Eight starting structures have been compared to each other above the diagonal and those after MD/MARDIGRAS refinement below the diagonal. The eight starting structures represent two families of A- and B-type models. Each of this family has 4 structures (q1–q4) encompassing four different stereochemical orientations of the Pzn residue: The Pzn has been stacked according to the NOESY data with the neighbouring base pair G¹–C¹³ while its *N*-hydroxyethyl group is orientated either to the minor groove in q1 and q2 or to the major groove in q3 and q4. In q1 and q3, the terminal hydroxymethylene moiety of *N*-hydroxyethyl is facing out from the duplex, whereas in q2 and q4 it is faces into the duplex. The comparison within the q1 and q2 and the q3 and q4 groups of structures are shown by boxes and shading, respectively.

moiety were obtained from the program PSEUROT (9b) using the three-bond coupling constant information, and the endocyclic torsional angles were derived (Table 3). All sugar residues of the DNA strand were found to be in a South-type conformation ($140^\circ < P_s < 155^\circ$ and $37^\circ \Phi_s < 44^\circ$). No $J_{\text{H1}'-\text{H2}'}$ crosspeaks could be observed for the RNA strand in the DQF-COSY spectra, which is probably due to both smaller coupling constants (< 1 Hz) and the line-broadening. The small $J_{\text{H1}'-\text{H2}'}$ coupling constant implies that all the sugar residues of the RNA strand adopt an N-type conformation ($P = 8 \pm 28^\circ$ and $\Phi = 36.5 \pm 6.5^\circ$) (12b), and thus are accordingly constrained in our calculation.

Conformation of the ϵ , β and γ torsional angles

These torsional angles were defined using the method of Kim *et al.* (13). The γ torsional angles were constrained using both the sum of the $J_{\text{H4}'-\text{H5}'}$ and $J_{\text{H4}'-\text{H5}'}$ coupling constants (when available from DQF-COSY, see Table 2) and from the NOE

volumes (NOESY spectra at 150 ms) of the H6/H8 ↔ H5'/5' crosspeaks and from the NOE crosspeak linewidths of the aromatic to H4' crosspeaks (the H4' linewidth is dependent on $J_{3'4'}$, $J_{4'5'}$, $J_{4'5'}$, $J_{4'P}$ and $1/\pi T^*_2$). The volumes of all H6/H8 ↔ H5'/5' crosspeaks are smaller than the volumes of the H6/H8 ↔ H1' (i.e. 3.2–3.9 Å for B-DNA). The linewidths lie in the range 11–15 Hz and the sum of the $J_{\text{H4}'-\text{H5}'}$ and $J_{\text{H4}'-\text{H5}'}$ coupling constants are less than 4.2 Hz. These data are consistent with a γ in gauche⁺ range (13) of either $60 \pm 10^\circ$ (when both coupling constant, distance and linewidth information were available) or $60 \pm 40^\circ$ (when only distance and/or linewidth information were available). The ϵ and β torsional angles were constrained using the linewidths of crosspeaks of H6/H8 to H3' or H5'/H5' and H1' to H3' or H5'/H5'. The linewidths of H5'/H5' protons is dependent on $J_{5'5'}$, $J_{5'5'4'}$, $J_{5'5'P}$ and $1/\pi T^*_2$ which were found to be less than 28 Hz. Hence, we constrained β to $180 \pm 75^\circ$ in accordance with the procedure of Kim *et al.* (13). All linewidths of H6/H8 to H3' crosspeaks were clearly

Figure 3. (A) The plot of the atomic RMSD deviations (Å) for each residue upon comparison of the starting structures of the A-type with the B-type of duplex (●), the RMSD deviations of the final structures (q1–q4) obtained after refinement from the A- and B-type of duplex (■), the RMSD as a result of the comparisons of the starting and final structures of the A-type (Δ), and the starting and final structures of the B-type (◇). (B) and (C) are the plots of 6th root squared R^2 values (10) for the inter-residual (B) and intra-residual (C) distances for each residue of the average of the final four structures in each group: (i) A-RNA_q1/q2 and B-DNA_q1/q2 (■ and □) and (ii) A-RNA_q3/q4, B-DNA_q3/q4 (◆ and ◇). The filled symbols (■ and ◆) are the R^2 values of the average of four structures compared with the experimental NOESY spectra recorded in H_2O (including exchangeable protons), whereas the empty symbols (□ and ◇) are the R^2 values of the average of four structures compared with the NOESY spectra recorded in D_2O . (D) Stereoviews of the superimposition of the eight final structures shown in Table 4 after 7 steps of MD/MARDIGRAS refinement of the hybrid DNA–RNA duplex with the atomic RMSD deviations 1.5 Å with all atoms and 0.65 Å excluding the Pzn residue. (E) Two different stereoviews of superimposition of the final four q1/q2 type structures after 7 steps of MD/MARDIGRAS refinement of the hybrid DNA–DNA duplex with the atomic RMSD deviations <0.9 Å (for all atoms including the Pzn residue).

smaller than 20 Hz, which suggests that the rotamer distribution with $\epsilon = 60^\circ$ should be excluded and accordingly the H3'–P distance was constrained to 2.80–3.25 Å and for the RNA residues 2.45–3.07 Å. These distances include in both cases both B_I and B_{II} phosphate conformations.

Melting properties

Chemical shifts of nucleobase and H1' protons are sensitive to the ring current effects exerted by the adjacent nucleobases. Therefore, these chemical shifts are suitable probes to investigate the stacking interactions in oligonucleotides. The chemical shift versus temperature profiles of the DNA–RNA duplex are shown in Fig. 1B–D, and all are sigmoidal in shape. These profiles show that the duplex–monomer equilibrium shifts from the duplex structure at <20°C to the monomer structures at >50°C. Interestingly, the profile of the H3Pzn proton also shows a sigmoidal shape, indicating that it is stacked. A perusal of these sigmoidal melting curves suggest that the T_m of the Pzn-tethered

DNA–RNA duplex and the T_m of the stacking–destacking equilibrium of the Pzn residue is $\approx 40^\circ C$ under the NMR measurement conditions. This means that both the stacking–destacking equilibrium of the Pzn residue and the duplex–monomer equilibrium of the duplex are parts of the same process.

Analysis of the structures resulting from the MD/MARDIGRAS refinement

To find structures that are consistent with the NMR constraints, we have performed a restrained molecular dynamics (MD) calculation. In the present study, complete relaxation matrix analysis has been employed using the MARDIGRAS algorithm (10) to generate distance constraints (197 NOE constraints). The distance constraints between the aromatic to exchangeable protons were corrected (10) by using the rates of exchange of imino or amino protons with water, which also allowed us to constrain the interstrand distances. The coupling constant information from

Table 5. Energy terms (in kcal mol⁻¹, AMBER force field) of minimized structures before and after MD and squared sixth root total indices *R*² analysis using CORMA (10) for the eight starting and final structures after MD/MARDIGRAS refinement (10)

Structure	Potential energy (no constraint) ^a	Potential energy (with constraint) ^b	Constraint energy	Potential energy after minimization ^c	<i>R</i> ² of the starting structures ^d	<i>R</i> ² of the final structures ^d
A-RNA_q1	-122	269	149	-115	0.231 (0.192)	0.134 (0.088)
A-RNA_q2	-122	274	149	-115	0.227 (0.188)	0.133 (0.088)
A-RNA_q3	-116	311	157	-110	0.231 (0.194)	0.132 (0.090)
A-RNA_q4	-121	308	157	-109	0.230 (0.195)	0.135 (0.092)
B-DNA_q1	-124	287	156	-93	0.178 (0.194)	0.134 (0.089)
B-DNA_q2	-124	291	158	-93	0.177 (0.195)	0.135 (0.087)
B-DNA_q3	-119	317	156	-84	0.188 (0.204)	0.133 (0.091)
B-DNA_q4	-114	304	153	-83	0.186 (0.205)	0.133 (0.091)

^aNot including constraints in the process of minimization after MD.^bIncluding constraint energy in the process of minimization after MD.^cPotential energy of the starting structures after minimization without constraints.^d $R^2 = [(\sum_i |I_0/6 - I_c/6|)/(\sum_i |I_0/6|)]^2$ (10) where I_0 and I_c are the intensities of observed non-zero and calculated NOE crosspeaks. The result of the *R*² analysis of the starting and final structures are based on the NOE data in H₂O while the data in parentheses show a similar analysis in D₂O (see 10 for the procedure of analysis). Note that the *R*² improves by c. 2-fold in D₂O.**Table 6.** Average backbone torsion angles, glycosidic angle (χ) and pseudorotation phase angle (*P*) with standard deviations for 8 final structures after MD/MARDIGRAS refinement

Nucleotide	α (°)	β (°)	γ (°)	δ (°)	ϵ (°)	ζ (°)	χ (°)	<i>P</i> (°)	Φ_m
rG ¹	^a	^a	59 ± 1	79 ± 1	174 ± 2	-70 ± 8	-134 ± 7	45 ± 7	41.3 ± 0.8
rA ²	-80 ± 4	170 ± 2	61 ± 2	80 ± 4	178 ± 2	-70 ± 1	-161 ± 3	46 ± 3	36.0 ± 4.0
rU ³	-75 ± 2	170 ± 0.5	62 ± 2	83 ± 0.9	-173 ± 1	-59 ± 1	-167 ± 1	5 ± 0.5	36.0 ± 1.2
rU ⁴	-106 ± 1	-161 ± 2	61 ± 2	89 ± 0.9	-178 ± 1	-79 ± 1	-156 ± 1	31 ± 1	30.1 ± 1.5
rG ⁵	-75 ± 1	174 ± 0.4	60 ± 0.8	90 ± 0.6	-158 ± 0.8	-56 ± 0.8	-155 ± 1	9 ± 3	30.2 ± 0.8
rA ⁶	-93 ± 1	174 ± 0.6	59 ± 0.4	82 ± 0.7	-175 ± 0.5	-77 ± 0.4	-154 ± 0.1	46 ± 0.6	36.2 ± 0.4
rA ⁷	-80 ± 0.5	179 ± 0.3	51 ± 0.2	74 ± 0.5	134 ± 100	^a	-167 ± 0.7	25 ± 0.9	40.3 ± 0.4
dT ⁸	^a	174 ± 2	55 ± 2	130 ± 2	175 ± 0.8	-90 ± 0.6	-127 ± 0.4	154 ± 2	31.0 ± 1.0
dC ⁹	-66 ± 1	-179 ± 0.5	52 ± 0.5	129 ± 0.7	-169 ± 1	-91 ± 1	-118 ± 0.6	140 ± 0.7	44.4 ± 0.5
dA ¹⁰	-100 ± 3	-176 ± 0.9	53 ± 0.4	121 ± 2	168 ± 2	-95 ± 2	-118 ± 0.7	139 ± 2	37.6 ± 0.53
dA ¹¹	-61 ± 2	-175 ± 1	58 ± 0.5	127 ± 1	-170 ± 3	-87 ± 0.8	-119 ± 2	143 ± 1	35.2 ± 0.4
dT ¹²	-78 ± 3	170 ± 2	51 ± 1	116 ± 0.7	-178 ± 3	-102 ± 6	-121 ± 3	131 ± 0.4	44.0 ± 1.5
dC ¹³	-88 ± 5	-184 ± 8	61 ± 6	141 ± 6	-177 ± 5	^a	-139 ± 5	145 ± 2	40.0 ± 5.0

^aAngle doesn't exist.

DQF-COSY experiments were used to employ the constraints for the conformation of the sugar moiety (55 dihedral angle constraints).

Analysis of both DNA and RNA strands in the hybrid DNA-RNA duplex. Eight starting structures have been used representing A- and B-type DNA or RNA (see Materials and Methods). Each of this family has 4 structures (q1-q4) encompassing four different stereochemical orientations of the Pzn residue (see the legend to Table 4). The atomic RMSD values before and after the MD/MARDIGRAS refinement procedure for all eight models are also presented in Table 4. As expected, the large differences (RMSD ~ 3.5-4.2 Å) for the starting geometries were observed between A- and B-type families. The DNA-RNA part alone of these eight structures converged to an RMSD of ~ 0.39-0.69 Å, whereas the RMSD including the Pzn residue varied between 0.497 and 1.433 Å. The analysis of RMSD values of each residue of the duplex in Fig. 3A shows the largest RMSD for all residues of the starting A- and B-type structures, which is reduced considerably in the final structures independent of whether one started from the A-RNA or B-DNA type structures. One of the important criteria to estimate the correctness of refinement procedure is the total sixth-root residual index *R*², which is

obtained from a direct comparison of the theoretical 2D NOE spectrum with the experimental data (10). Table 5 lists total residual indices *R*² for the 150 ms 2D NOE data set at 0°C in H₂O and 12°C in D₂O calculated with the program CORMA (10) for all eight starting models and the resulting NMR-constrained MD structures after 7 steps of refinement. It may be noted that the NOE data from H₂O mostly exhibit the interaction between the exchangeable protons describing both the inter- and intra-strand base-base interaction in the duplex, whereas the spectra in D₂O give informations regarding the intra-strand sugar and sugar-base conformation. The *R*² analysis of the final structure obtained from the NOE data sets from D₂O and H₂O experiments when fitted with the NOE data set in D₂O or H₂O shows that the *R*² factor from the D₂O data set gives at least two-fold improvement compared to that from H₂O (Table 5).

Analysis of the stereochemical orientation of the 3'-Pzn residue. A perusal of the relative energetics in Table 5 shows that the structures q1 and q2 with the hydroxyethyl group of the Pzn residue orientated in the minor groove are energetically more preferred to q3 and q4 in which the hydroxyethyl group is orientated in the major groove. This is also consistent with the

Figure 4. Plot of the torsional angles α , β , γ , ϵ , ζ and χ and the pseudorotation phase angle (P) for each residue of the average structure of the final four q1/q2 type structures shown in Fig. 3E obtained after 7 steps of MD/MARDIGRAS refinement ($\blacklozenge$) and their comparison with the starting structures A- ($\circ$), and B-type ($\square$) conformations.

total R^2 values for q1 and q2 (Table 5). The analysis of R^2 values per residue in Fig. 3B and C, where the coordinates of all q1 and q2 structures and q3 and q4 structures have been combined and averaged, shows the following. (i) The R^2 values for all constituent residues of DNA and RNA (except for the Pzn residue) in all final structures in D_2O or H_2O are similar but the R^2 values are in most cases better in D_2O than in H_2O . (ii) The inter- and intra-residual distances between Pzn and the neighbouring C^{13} residues of q1 and q2 types of structures are always less than q3 and q4 types of structures, which are consistent with the NOE data measured under the same conditions. Note that the data on the change of chemical shifts as a function of temperature show (Fig. 1B and D) that H3Pzn and H1Pzn have the same sigmoidal melting profile ($T_m \approx 40^\circ C$) as the H2/H6/H8 and the methyl protons of nucleobases, suggesting that the Pzn residue should be involved in a stacked conformation with the neighbouring G^1-C^{13} base pair in the same way as other nucleobases. The inspection of eight q1/q2 and q3/q4 types of structures clearly shows that while the Pzn residue is indeed planar with the neighbouring base pair G^1-C^{13} in the former group, it is clearly non-planar in the latter. Thus the stereoviews of the superimposition of the eight final structures described in Table 4 after MD/MARDIGRAS refinement of the hybrid DNA-RNA duplex with the atomic RMSD deviations 1.5 Å with all atoms and 0.65 Å excluding the Pzn residue are shown in Fig. 3D. The distinction between q1/q2 and q3/q4 types of structures was finally based on our observation of a sharp resonance in the 1D spectrum and a crosspeak with water in our NOESY spectrum for the imino proton (NH-CH₂CH₂O-) of Pzn in H_2O at $0^\circ C$, suggesting a slow exchange process of this imino proton with water. Clearly, this is only possible if this imino proton is hydrogen bonded to some other function. In agreement with this experimental observation, we have observed that this imino proton is indeed hydrogen bonded to the non-bridging oxygen of the linker phosphate group in the final q1/q2

types of structures, lending additional support to their structural integrity. Thus all four superimposed final q1/q2 types of structures in Fig. 3E (RMSD $\approx 0.7-0.9$ Å including all atoms of the Pzn-tethered DNA-RNA duplex) represent the NMR time-averaged conformers that can be attributed to the Pzn-tethered DNA-RNA hybrid duplex.

Analysis of the nucleotide conformations of the final q1/q2 type structures. A detailed comparison of the conformational preferences for the torsion angles and sugar pucker of the average from final q1/q2 type structures is given in Fig. 4 and Table 6. This shows a very small range of preference for the individual backbone angles (α , β , γ and ϵ) for the q1/q2 structure as found in the canonical A-RNA or B-DNA conformation. Note that α has not been constrained at all in our model building, the conformation space for ϵ^+ has been excluded and β/γ are constrained to *trans* and *gauche*⁺ conformations, respectively, for all DNA and some RNA residues. It means that the torsional preferences found for α , β , γ and ϵ merely represent the lowest energy conformation of the molecules. Nevertheless, both backbone torsion angles which define the P-O diester bond, α and ζ are in the *gauche*⁻ ($-sc$) range. The values for ζ for the ribo strand are similar to the regular A-RNA and for the deoxyribo strand they are similar to the B-DNA type conformation. The torsion angles which define the C-O-P ester bonds (β and ϵ) are close to the *trans* ($+ap$) value. For γ , *gauche* ($+sc$) values are preferred. For torsion angle δ (Table 6) the values for the deoxyribo strand are in the *gauche-trans* range ($+as$), and for the ribo strand in the *gauche* ($+sc$) range. The structural parameters of sugar pucker and the glycosidic torsion (χ) angles show a more significant discrepancy between the ribo and deoxyribo strands than for the backbone torsion angles. The χ angles are all predominantly in the *anti* conformation ($-ac$) range: -118 to -127° for the deoxyribonucleotides (except for the terminal dC¹³ residue which has $\chi \approx 139^\circ$), which are quite close to a B-type structure ($\chi \approx -110^\circ$). The χ angles for the ribonucleotide residues vary from -154 to -167° in the RNA strand (Table 6) (except for the terminal rG¹ residue which has $\chi \approx 134^\circ$), which are closer to A-RNA type structure ($\chi \approx -150^\circ$). The pseudorotational characteristics for RNA show in part North-type sugars (close to C_3' -endo, $P = 5-55^\circ$, $\Phi_m = 30-45^\circ$), which are typical for A-type structures, whereas for the deoxyribonucleotides residues the phase angle varies in the South range ($P = 131-154^\circ$, $\Phi_m = 31-45^\circ$), which suggests a C_1' -exo type conformation.

CONCLUSIONS

This report constitutes a study of conformational features of a stable DNA-RNA hybrid using torsional and distance constraints (total of 252 inter- and intra-strand constraints) with a complete matrix NOE relaxation approach taking into account the exchange process with water. Several structures of DNA-RNA hybrids have been examined by NMR and X-ray crystallography. These studies suffered from two severe problems in that they had to employ a larger duplex for stability reasons, which also created inherent complexities from overlapping resonances, that, in turn, reduced the number of total constraints in the model building. These studies mainly fall into three categories: (i) the RNA and DNA strands of the chimeric duplex are similar to the corresponding A-RNA or the B-DNA (11e,f), (ii) the RNA strand of the duplex is similar to A-RNA and the DNA strand is

somewhat intermediate between A- and B-forms of DNA (11a–d,k), and (iii) in one crystalline duplex, the conformation of both ribose and deoxyribose sugars were in the C_3' -endo conformation, but the sugar residues in the DNA strand underwent a conformational transition to C_2' -endo in solution (11m). The occurrence of all the above variations in the DNA–RNA hybrid clearly shows that the RNA sugars are invariably 3'-endo but the DNA sugars are flexible and can indeed take up various conformations (O_4' -endo to C_1' -exo to C_2' -endo, $72^\circ < P < 180^\circ$).

The present investigation shows for the first time that the 3'-Pzn group tethered to an oligo-DNA has the unique ability to stabilize an otherwise unstable DNA–RNA hybrid duplex structure. Clearly, this finding opens enormous possibilities for the design of various heterocyclic molecule-tethered antisense DNA oligomers for use as viral RNA-directed drugs. The 3'-Pzn-promoted stabilization of the DNA–RNA hybrid duplex results in detailed local conformational characteristics such as the torsion angles of the backbone and sugar moieties that are close to the features of the other normal DNA–RNA hybrids, which do not have any tethered ligand (11a–m) (i.e. sugars of the RNA strand are 3'-endo, but the sugars of the DNA strand are intermediate between A- and B-forms of DNA, $72^\circ < P < 180^\circ$; note that the sugars of our DNA strand are C_1' -exo conformation: $131^\circ < P < 154^\circ$). This means that 3'-Pzn-tethered smaller oligo-DNA could serve the same purpose as a larger oligo-DNA as an antisense inhibitor of viral mRNA. The advantage of such a DNA-tethered ligand is that they can be engineered in such a manner that it both stabilizes the DNA–RNA hybrid as well as promoting its cellular uptake. Interestingly, some initial data show that ligand-tethered oligo-DNA is more nuclease resistant (14) than the natural counterpart.

ACKNOWLEDGEMENTS

The authors thank the Swedish Board for Technical Development (NUTEK), Swedish Natural Science Research Council (NFR) and Wallenbergstiftelsen for generous financial support. We thank Professor T.James for kindly allowing us to use his MARDIGRAS (ver. 5.0).

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