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NMR Study of the p-DNA Duplex [(4'→2')-3'-Desoxyribopyranosyl-(^{m5}CGDDTT^{m5}CG)$_2$] and Comparison with its p-RNA Analogue

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Abstract: The determination of the solution structure of small non-natural oligopeptides and oligonucleotides by NMR, which is one of the main research topics of our group, is illustrated on the example of an 8-mer p-DNA duplex. p-DNA, the 3'-desoxy analogue of p-RNA, forms an highly selective pairing system but its pairing strength is less than that of analogous p-RNA sequences. The NMR study reveals that the backbone of p-DNA corresponds more closely to the conformation predicted for pentapyranose nucleic acids by qualitative conformational analysis than p-RNA.

Keywords: p-DNA · Duplex formation · NMR solution structure · Pentopyranosyl nucleic acids · p-RNA

Introduction

As demonstrated by Eschenmoser and coworkers, p-RNA, the 4'→2' phosphodiester linked ribopyranosyl isomer of RNA (Fig. 1), forms very strong antiparallel duplexes according to the Watson-Crick pairing mode [2]. In fact, the pairing strength of such p-RNA duplexes is much higher than that of the corresponding RNA and DNA duplexes. In contrast to the naturally occurring nucleic acids, no reverse-Hoogsteen or Hoogsteen base pairs have been observed for p-RNA, which makes it a more selective pairing system than either RNA or DNA.

In the context of finding new strategies for an easy and eventually combinatorial preparation of functional RNA-structures (such as aptamers and ribozymes), two of us (D.A., S.P.) wanted to develop a general method for substituting RNA-hairpin motives by complementary duplex structures (Fig. 2). In order to avoid any interaction between the functional part of these molecules (consisting of RNA or DNA) and the hairpin-substituting duplex structures, the latter should preferentially be formed from an unnatural, autonomous pairing system that pairs only with itself, but not with RNA or DNA. As a strong, selective, and autonomous pairing system, p-RNA was in principle ideal for this purpose. Unfortunately, due to the harsh conditions required to remove the 3'-O-benzoyl protecting group, the synthesis of p-RNA oligonu-

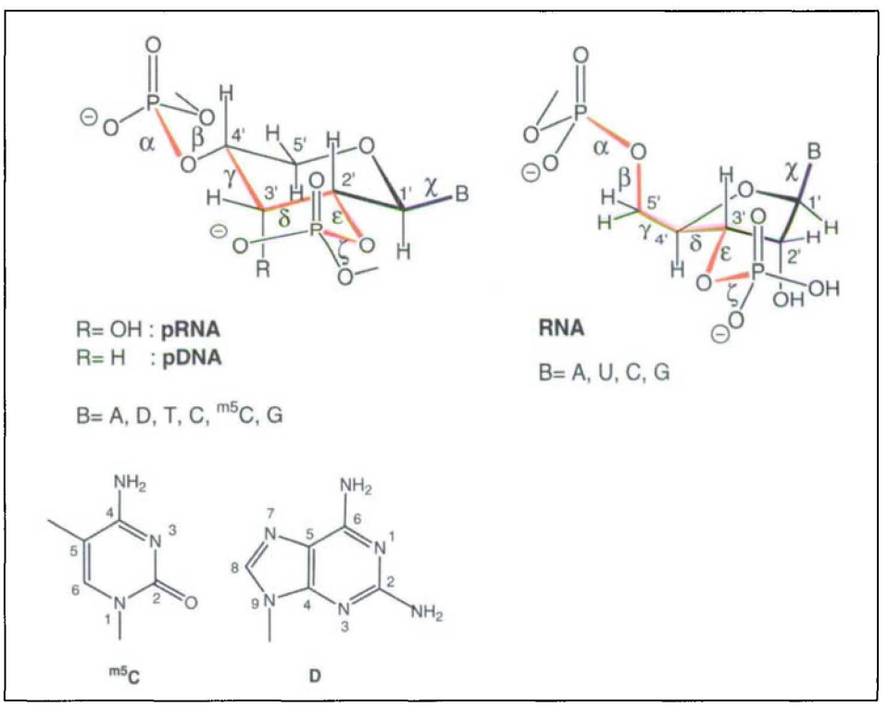

Fig. 1. Structural formulae and definition of backbone angles for p-RNA, p-DNA and RNA. The designation of backbone angles was chosen in analogy to the natural systems [1].

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cleotides was not compatible with RNA-synthesis and therefore, the corresponding 3'-desoxy analogue ('p-DNA') was prepared instead [5]. The new pairing system p-DNA ('pyranosyl-DNA') consists of 3'-desoxy-β-D-ribopyranose nucleotides which are connected *via* 4'→2'-phosphodiester moieties (Fig. 1). The conditions for their assembly from the corresponding phosphoramidite building blocks and for the final deprotection procedure are fully compatible with the conditions employed for RNA synthesis with 2'-O-TOM-protected RNA-phosporamidites [6].

With the four canonical bases, p-DNA oligonucleotides form weaker duplexes than corresponding p-RNA sequences. However, by replacing the natural nucleobases adenine and cytosine with the analogues 2,6-diaminopurine (D) and 5-methyl-cytosine (^{m5}C), respectively (Fig. 1), a substantial increase in pairing strength was achieved; p-DNA duplexes forming D-T and G-^{m5}C base pairs have almost identical pairing properties as the corresponding A-T and G-C containing p-RNA duplexes.

We have reported earlier on a detailed NMR study of the solution structure of the p-RNA octamer duplex p-(CGAATTCG)$_2$ [7]. In view of the close analogy between the constitutions of p-RNA and p-DNA, it was reasonable to presume that their solution structures would also be similar. However, the reduced pairing strength of p-DNA must be due to structural differences. These may be important for the prediction of the exact shape of p-DNA duplexes to be employed as hairpin-substituting elements in larger RNA or DNA constructs of complex tertiary structure. Here, we present the preliminary results of our NMR study of the p-DNA duplex (4'→2') 3'-desoxyribopyranosyl-(^{m5}CGDDTT^{m5}CG)$_2$, which has the same sequence than the p-RNA duplex studied earlier, except for the substitution of C by ^{m5}C and of A by D.

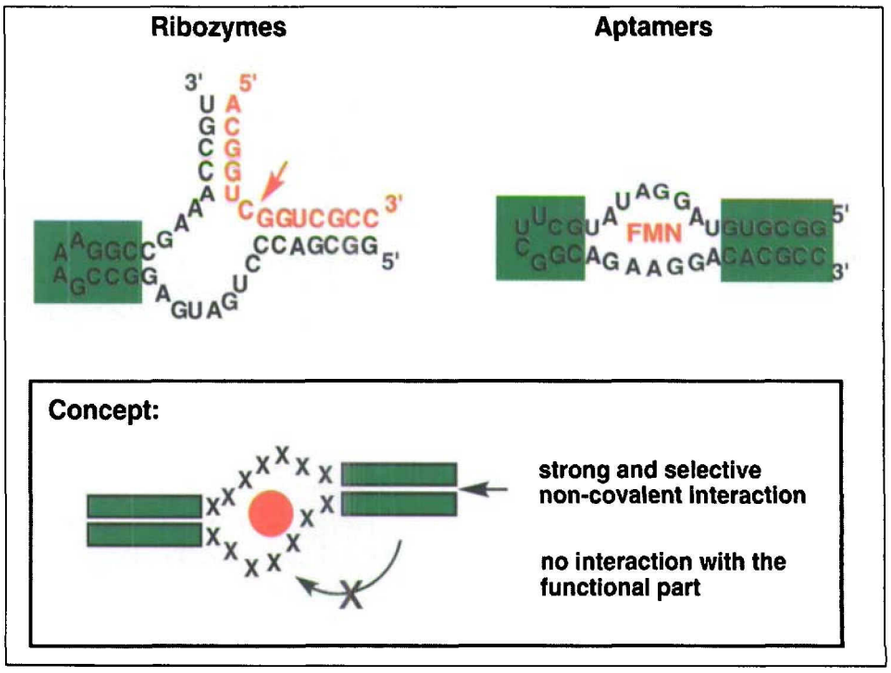

Fig. 2. Functional nucleic acids, such as ribozymes (top left: Hammerhead ribozyme [3]) and aptamers (top right: FMN-binding aptamer [4]) consist, in general, of only one oligonucleotide strand. The functional part of these compounds is usually formed by non-Watson-Crick-paired nucleosides, which form bulges or internal loops (on the level of secondary structure). These elements are able to form a diversity of functionalized, chiral and concave sites (on the level of tertiary structure) which, in analogy to proteins, are able to selectively bind other molecules by H-bonding and π-stacking. The ribozyme (top left) binds a complementary RNA-sequence (in red), which is subsequently cleaved at the position indicated by the arrow; the aptamer (top right) binds a molecule FMN (flavine mononucleotide). The spatial organization of the functional residues is provided by Watson-Crick-paired regions, forming duplex and/or stem-hairpin structures (highlighted in green). We are planning to substitute these structural elements by complementary p-DNA-duplexes (bottom, indicated in green), thus forming functional oligonucleotide-motives from several strands and avoiding interactions between the structural and functional parts.

Results

The NMR-experiments (500 MHz ^{1}H) were carried out at 26 °C with a 10 mM sample of the title compound dissolved either in D_2O or in H_2O/D_2O 9:1, each buffered with 50 mM sodium arsenate at pH = 7.0. The ^{1}H (Fig. 3), ^{13}C, and ^{31}P NMR spectra all show the number of signals expected for twofold symmetry on the NMR time scale. The sequential assignment of the non-exchangeable protons was done in a standard way by combination of COSY and NOESY. Sequential connectivities could be additionally confirmed by P,H-COSY. The stereospecific assignment of the geminal protons at C(3') and C(5') was based on the expected strong NOEs between the axial positions in the pyranose ring as well as on a qualitative analysis of the vicinal proton–proton coupling constants resulting from inspection of the peak sizes in the COSY spectrum. All findings are consistent with a chair conformation of the six-membered rings. The H-bearing carbons were assigned by HSQC. The ^{1}H NMR spectra measured in H_2O/D_2O 9:1 with solvent suppression by excitation sculpting showed three sharp resonances in the low field region which were assigned to the imino protons of the six inner base pairs. Due to fast rotation, the two NH_2 protons of guanosines and of both amino groups of 2,6-diaminopu-

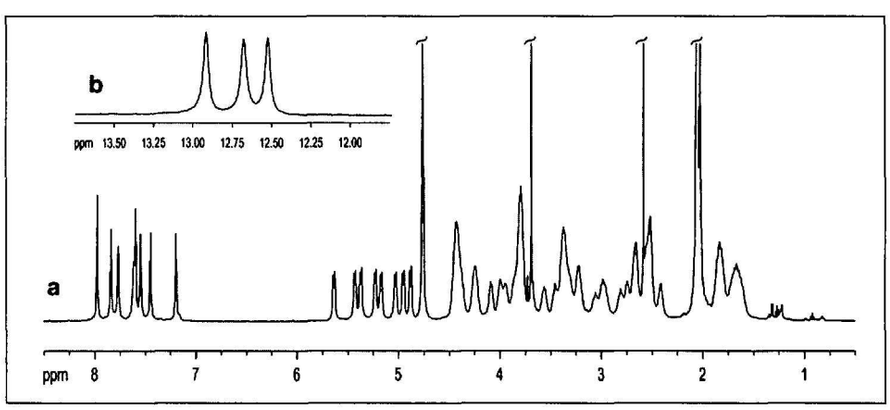

Fig. 3. ^{1}H NMR spectra of the title compound in a) D_2O and b) D_2O/H_2O 9:1. Conditions: c = 10 mM, 50 mM Na arsenate buffer, pH = 7.0, T = 300 K.

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rines appeared as single broadened signals, whereas for ^{m5}C7 , the two protons of the amino group showed distinct resonances. Vicinal ^{1}H-^{31}P coupling constants were determined from the difference in the linewidth of traces (power mode) through COSY cross peaks measured with and without ^{31}P decoupling. The resulting values were $^{3}J_{H2'P2'}$ = *ca.* 2 Hz and $^{3}J_{H4'P4'}$ = *ca.* 7 Hz. Severe overlap in the ^{13}C spectrum prohibited the determination of $^{3}J_{CP}$ coupling constants except for $J_{C1'P2'}$ which was *ca.* 12 Hz. These findings are consistent with $\varepsilon = -60°$ and $\beta = +160°$.

A series of NOESY spectra with different mixing times, both in D_2O and H_2O/D_2O 9:1, allowed a total of 140 NOE derived distance restraints to be assembled. Together with the torsional angle constraints derived from the coupling constants through Karplus relations, they were used to generate an ensemble of structures that are consistent with the experimental data by torsional angle molecular dynamics calculations [8].

Discussion

Qualitative conformational analysis predicted a quasi linear backbone for pentapyranose-derived oligonucleotides with the 2'- and 4'-OH groups in equatorial positions [9]. Our earlier NMR study of a p-RNA duplex confirmed the general aspects of this prediction, but also revealed significant deviations of the backbone angles ε and β from their idealized values of –60° and 180°, respectively (Fig. 4). With *ca.* 40°, the inclination of the mean base pair axis towards the backbone axis is stronger than in the idealized structure and the π–π stacking distance is shorter. Three possible causes for these adjustments of the backbone of p-RNA were discussed: a) avoidance of the unfavorable interaction between the 3'-hydroxy group and one of the phosphate oxygens; b) a decrease of the π–π stacking distance between neighboring base pairs towards the optimal value of *ca.* 3.5 Å; and c) an increased shift of neighboring base pairs to achieve optimal interstrand stacking.

The NMR study presented here shows that in p-DNA, the value of ε is within experimental error of the idealized value of –60°. The angle β is *ca.* 160°, about midway between p-RNA (145°) and the idealized value (180°). As in p-RNA, the pyranose sugar rings assume nearly perfect chair conformations in p-DNA. Overall, the backbone of p-DNA corresponds more closely to the idealized structure than that of p-RNA (Fig. 4). The fact that the angle ε is reduced to the value of –60° if the 3'-OH group is replaced by a hydrogen confirms hypothesis a) above. The determination of the basepair inclination proved to be more difficult in p-DNA than in p-RNA. Due to the replacement of A by D in the p-DNA duplex studied here, the H-C(2) protons of adenine, conveniently placed in the middle of the minor groove and therefore crucial for measuring interstrand distances in p-RNA, are missing. The determination of the glycosidic angle χ, which is directly correlated with inclination, had therefore to be based on NOE-derived relative interresidual distances H-C(8/6)-H-C(2') and H-C(8/6)-H-C(4'). This method of determining χ is inherently less precise than the use of interstrand correlations, because the intraresidual NOEs are much more affected by either spin diffusion or small scale dynamics. Nevertheless, simulated annealing calculations with all distance and dihedral angle restraints derived from the NMR data converged to the bundle of structures shown in Fig. 5a. With the exception of the terminal base pairs, which show the usual fraying ends, the resulting overall structure is quite well defined and no experimental constraints are violated. However, we doubt whether this result can be correct. The calculated conformation type is characterized by a glycosidic angle χ between –100° and –120°, a much smaller inclination than in p-RNA and a very large π–π distance of 4.5–5.5 Å (Fig. 5b). Such a large distance between neighboring basepairs is very difficult to accept because, not only in natural RNA and DNA but also in the other pentapyranose-NA systems studied so far [10], the π–π stacking distance was always near the ideal value of *ca.* 3.5 Å. In fact, the ideal interstrand π–π stacking between purine/purine and purine/pyrimidine is considered to be a major factor contributing to the high pairing strength in p-RNA. This raises the question whether the practically unstacked structure resulting from our calculation might be due to a systematic error in the analysis of the NOE data, in particular, the neglect of dynamic averaging. The structure shown in Fig. 6 (obtained by simple energy minimization of a starting structure that contained the experimentally determined backbone angles and hydrogen bonds as sole constraints) demonstrates that a conformation of p-DNA with strong inclination and good interstrand stacking similar to that in p-RNA would in principle be

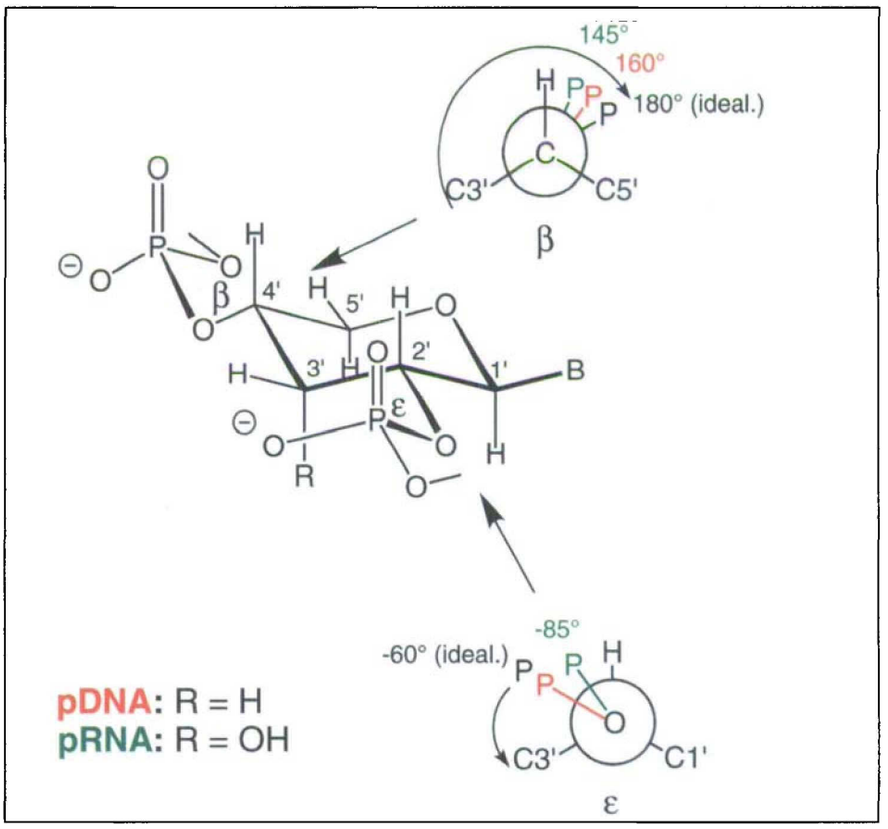

Fig. 4. Comparison between the backbone angles β and ε of p-RNA and p-DNA as determined experimentally by NMR. The values predicted for the idealized pentopyranose structure are indicated in black.

consistent with the experimentally determined backbone angles. Further work, in particular, MD calculations with dynamic averaging and NMR analysis of the same p-DNA sequence containing adenine instead 2,6-diaminopurine is needed in order to resolve these questions.

The difficulties encountered with some of the structure determinants of p-DNA illustrate one of the major problems in the determination of the solution structure of small non-natural oligonucleotides. In contrast to larger systems such as t-RNAs (or proteins) no NOE correlations between residues that are far apart in the sequence but close in space help to define the overall structure. Therefore, small errors in the determination of the local conformation propagate and make the accurate determination of parameters such as inclination or helicity very difficult.

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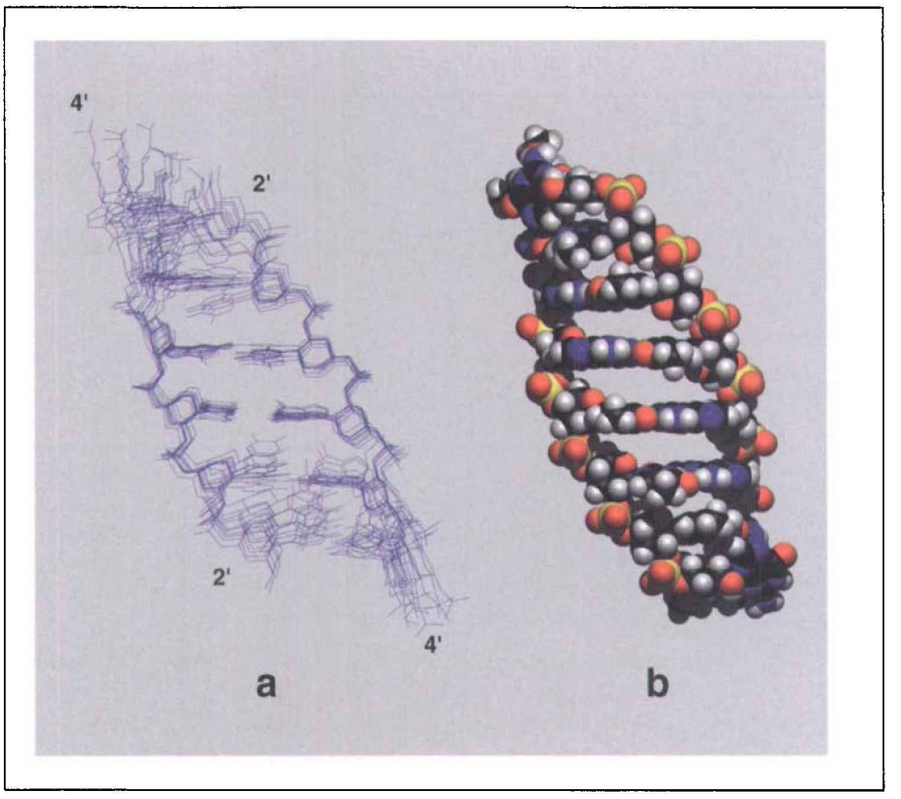

Fig. 5. a) Bundle of the ten structures lowest in energy calculated by torsion angle dynamics using all NMR-derived distance- and angle constraints. None of the structures violates the experimental constraints (the outermost base pairs are omitted for clarity). b) Space filling model of one of the structures in Fig. 5a showing the low inclination and (unrealistically?) large π–π stacking distance in the calculated structures.

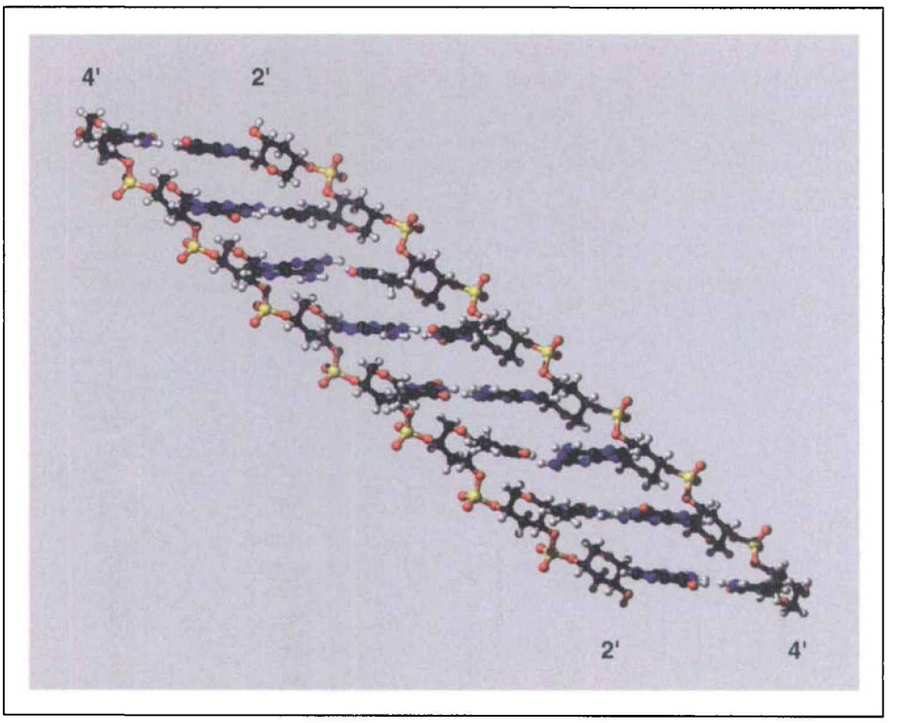

Fig. 6. Molecular model (generated by simple energy minimization) of p-DNA demonstrating that, in principle, a duplex structure exhibiting the strong inclination and short π–π stacking found in p-RNA would be consistent with the experimentally determined backbone angles β and ε.