

The effect of two antipodal fluorine-induced sugar puckers on the conformation and stability of the Dickerson–Drew dodecamer duplex [d(CGCGAATTCGCG)]₂

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Received September 25, 1997; Revised and Accepted March 9, 1998

ABSTRACT

UV thermal melting studies, CD and NMR spectroscopies were employed to assess the contribution of antipodal sugar conformations on the stability of the canonical B-DNA conformation of the Dickerson–Drew dodecamer duplex {[d(CGCGAATTCGCG)]₂, (ODN 1)}. Different oligodeoxynucleotide versions of ODN 1 were synthesized with modified thymidine units favoring distinct sugar conformations by using a 3'-endo (north) 2'-fluoro-2'-deoxyribofuranosyl thymine (1) or a 2'-endo (south) 2'-fluoro-2'-deoxyarabinofuranosyl thymine (2). The results showed that two south thymidines greatly stabilized the double helix, whereas two north thymidines destabilized it by inducing a more A-like conformation in the middle of the duplex. Use of combinations of north and south thymidine conformers in the same oligo destabilized the double helix even further, but without inducing a conformational change. The critical length for establishing a detectable A-like conformation in the middle of a B-DNA ODN appears to be 4 bp. Our results suggest that manipulation of the conformation of DNA in a sequence-independent manner is possible.

INTRODUCTION

The conformational elasticity of the DNA helix appears to be responsible for the ability of the molecule to adjust to the structural demands of a myriad of protein ligands with which it binds. Analyses of several protein–DNA complexes by X-ray crystallography and NMR spectroscopy suggest that specific structural features of the DNA double helix are perhaps the primary determinants of sequence-specific recognition by proteins, rather than the recognition of the bases themselves (1,2). Since the early description of the structure of DNA, it was known that under low relative humidity right-handed B-DNA duplexes transformed to the more paracrystalline A-form (3). In the canonical B-form, the bases are perpendicular to the helix axis and the major and minor grooves are of equal depth (4). In A-form DNA, on the other hand, the groove dimensions are in essence

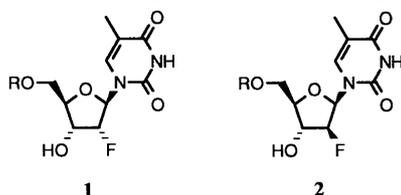
inverted, since due to the large displacement of the bases the minor groove becomes wide and shallow, and the major groove appears narrow and deep (5). The principal structural motif associated with these two distinct forms of DNA is the sugar ring pucker. In B-form DNA, the 2'-deoxyribose sugars exhibit a preferred 2'-endo (south) pucker, while A-form helices prefer the 3'-endo (north) puckering (4,5). The conformations of the 2'-deoxyribose sugars control many important structural aspects of DNA, such as longitudinal phosphate–phosphate separations, water of hydration, metal ion coordination and polyamine binding (4,5). In solution, the B-form appears to be the predominant form, and even fragments that crystallize in the A-form normally convert to the B-form in an aqueous environment (6–8). Recent evidence, however, indicates that these different helical forms of DNA can coexist in biological systems giving rise to A-B junctions. Indeed, A-B junctions appear to occur at the binding site of transcription factor IIa (9,10), in a DNA complex with HIV reverse transcriptase (11), and at the target site of the TATA-box binding protein (12–14). B to A transitions have also been detected in short DNA fragments in solution (15,16), and have been postulated on the basis of molecular mechanics simulations of specific sequences (17).

In the present work, we investigated the possibility of creating A-B junctions in a sequence-independent manner through the use of nucleosides fluorinated on the sugar ring. 2'-Fluoro-2'-deoxy *ribo* analogues of all common bases show a strong preference for a 3'-endo (N-type) conformation in the solid state (18–20), and in solution the N-type conformer often predominates by >90% (21–25). In the same manner, 2'-fluoro-2'-deoxy *ara* analogues show the opposite preference for a 2'-endo (S-type) conformation (24,25). It is not surprising, therefore, that polymers containing repeated modified units of 2'-fluoro-2'-deoxy *ribo* analogues adopt an A-form structure and show more resemblance to RNA than to DNA (26–29). Interest in inducing an A-form conformation in DNA oligomers has been spurred by research in the antisense field. Indeed, use of 2'-fluoro-2'-deoxynucleosides with a *ribo* configuration can produce segments of DNA with an increased affinity for RNA that results in the formation of more stable RNA–DNA heteroduplexes (27–29). On the other hand, 2'-fluoro-2'-deoxynucleosides with the opposite stereochemistry at C2'

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(corresponding to an *ara* configuration) have not been pursued with the same level of interest because by favoring a 2'-*endo* (south) conformation the sugars are expected to reinforce the already preferred B-form (30).

Using the well-known Dickerson–Drew dodecamer B-DNA duplex [d(CGCGAATTCGCG)]₂ (31), we have determined the thermodynamic stability of modified ODNs where the middle thymidines have been replaced by 2'-fluoro-2'-deoxyribofuranosyl thymine (**1**) and 2'-fluoro-2'-deoxyarabinofuranosyl thymine (**2**) in various combinations (see structures below). The results from thermal melting curves showed that the south thymidine conformer **2** greatly stabilized the double helix, whereas the north thymidine **1** destabilized it by inducing what would appear to be an A-B junction according to CD spectroscopy. The presence of an 'A-like' stretch of base pairs surrounding the site where the middle thymidines were replaced by **1** was also confirmed by solution NMR studies where coupling constants and nuclear Overhauser enhancement (NOE) patterns characteristic of A-type sugars were observed for the modified residues and for the base pairs flanking **1**. Use of combinations of north and south thymidine conformers in the same heteroduplex destabilized the duplex even further, but without inducing conformational changes.



MATERIALS AND METHODS

Synthetic procedures

General. All chemical reagents were commercially available. Column chromatography was performed on silica gel 60, 230–400 mesh (E. Merk), and analytical TLC was performed on Analtech Uniplates silica gel GF. NMR spectra of the synthetic nucleoside intermediates were recorded using a Bruker AC 250 spectrometer equipped with a MACSPECT computer and a ¹H,¹³C switchable probe. ¹H signals were internally referenced to TMS. One-dimensional ³¹P NMR spectra were recorded on a Bruker AMX500 using an inverse broadband probe and were referenced to external 85% phosphoric acid. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA.

1-(β-D-Ribofuranosyl)thymine (3). This compound was prepared as described by Vorbrüggen and Bennua (32).

2, 2'-Anhydro-1-(β-D-arabinofuranosyl)thymine (4). A solution of **3** (4.12 g, 16.0 mmol) and diphenyl carbonate (3.76 g, 1.1 eq.) in DMF (12 ml) was stirred at 80°C. Sodium bicarbonate (40 mg) was added to the mixture, and the temperature was raised to 130°C. After gas evolution ceased, the mixture was cooled to room temperature and poured in Et₂O (1 l). The precipitate was washed with Et₂O (2 × 300 ml) and recrystallized from MeOH (10 ml) to give **4** (2.84 g, 74%) as a brownish powder that was used directly for the next step; ¹H NMR (DMSO-*d*₆+D₂O) δ 7.73 (s, 1 H), 6.29 (d, *J* = 5.7 Hz, 1 H), 5.17 (d, *J* = 5.7 Hz, 1 H), 4.36 (s, 1 H), 4.06 (br t, *J* = 4.6 Hz, 1 H), 3.20 (m, 2 H), 1.79 (s, 3 H).

2, 2'-Anhydro-1-(3,5-di-*O*-benzyl-β-D-arabinofuranosyl)thymine (5). A solution of **4** (0.60 g, 2.50 mmol) in DMF (25 ml) at –20°C

was kept under argon and treated portionwise with NaH (0.126 g, 2.0 eq.). Immediately after, benzylbromide (1.8 ml, 6.0 eq.) was added dropwise at the same temperature, and the resulting mixture was stirred for 3 h at –20°C, and for 1 h at room temperature (rt). The reaction mixture was poured into saturated aqueous NaHCO₃ and extracted with EtOAc (3 × 200 ml). The organic layers were collected and concentrated to dryness. The residue was purified by flash chromatography (silica gel, 3% → 5% gradient of EtOH in CH₂Cl₂) to give **5** (0.97, 92%) as an amorphous powder; ¹H NMR (CDCl₃) δ 7.10–7.40 (m, 11 H), 6.17 (d, *J* = 5.9 Hz, 1 H), 5.25 (d, *J* = 5.9 Hz, 1 H), 4.56 (AB q, *J* = 11.7 Hz, 2 H), 4.20–4.40 (m, 4 H), 3.25 (AB m, 2 H), 1.94 (s, 3 H).

Anal. Calcd for C₂₄H₂₄N₂O₅ (420.469): C, 68.56; H, 5.75; N, 6.66. Found: C, 68.65; H, 5.79; N, 6.60.

1-(3,5-Di-*O*-benzyl-β-D-arabinofuranosyl)thymine (6). Anhydride **5** (1.68 g, 4.0 mmol) was dissolved in 50% aqueous ethanol (100 ml), treated with 1 N NaOH (12 ml), and stirred for 15 h at rt. The solution was neutralized with 1 N HCl and extracted with EtOAc (3 × 200 ml). The organic layers were combined and concentrated. The residue was purified by flash chromatography (silica gel, 2% → 4% gradient of EtOH in CH₂Cl₂) to give **6** as an amorphous powder in quantitative yield; ¹H NMR (CDCl₃) δ 9.69 (s, 1 H), 7.20–7.50 (m, 11 H), 6.13 (d, *J* = 3.4 Hz, 1 H), 4.40–4.80 (m, 5 H), 4.18 (dd, *J* = 6.7, 3.3 Hz, 1 H), 4.00 (br s, 1 H), 3.79 (dd, *J* = 10.5, 3.1 Hz, 1 H), 3.65 (dd, *J* = 10.5, 3.8 Hz, 1 H), 1.68 (s, 3 H).

Anal. Calcd for C₂₄H₂₆N₂O₆ (438.485): C, 65.74; H, 5.98; N, 6.39. Found: C, 65.57; H, 6.01; N, 6.44.

1-(3,5-Di-*O*-benzyl-2-*O*-trifluoromethanesulfonyl-β-D-arabinofuranosyl)thymine (7). A stirred solution of **6** (0.438 g, 1.0 mmol), dimethylaminopyridine (0.366 g, 3 eq.) and pyridine (607 μl, 7.5 eq.) in CH₂Cl₂ (20 ml) was maintained under argon and cooled to –10°C before the dropwise addition of trifluoromethanesulfonyl anhydride (253 μl, 1.5 eq.) during the course of 2 h. The entire reaction mixture was applied to a flash chromatography column (silica gel, 2% EtOH in CH₂Cl₂) to give **7** (0.560 g, 98% yield) of crude material that was used directly for the next step; ¹H NMR (CDCl₃) δ 9.09 (s, 1 H), 7.20–7.40 (m, 11 H), 6.27 (d, *J* = 3.8 Hz, 1 H), 5.38 (d, *J* = 3.2 Hz, 1 H), 4.63 (AB q, *J* = 11.8 Hz, 2 H), 4.27 (dd, *J* = 3.9, 1.3 Hz, 1 H), 4.16 (dd, *J* = 8.3, 4.3 Hz, 1 H), 3.63 (AB m, 2 H), 1.80 (s, 3 H).

1-(2-Deoxy-2-fluoro-3,5-di-*O*-benzyl-β-D-ribofuranosyl)thymine (8). A solution of **7** (1.20 g, 2.1 mmol) in THF (10 ml) at 0°C was treated with a solution of tetra-*n*-butylammonium fluoride in THF (1 M, 20 ml, 10 eq.) and stirred for 18 h at 0°C. The entire mixture was applied to a chromatography column and purified by flash chromatography (silica gel, hexanes:EtOAc, 1:1) to give **8** (0.934 g, 92%) as a foam; ¹H NMR (CDCl₃) δ 9.80 (s, 1 H), 7.55 (s, 1 H), 7.20–7.00 (m, 10 H), 6.05 (d, *J*_{HF} = 15.5 Hz, 1 H), 5.10 (dm, *J*_{HF} = 53.0 Hz, 1 H), 4.63 (AB q, *J* = 11.8 Hz, 2 H), 4.53 (AB m, 2 H), 4.27 (m, 1 H), 4.20 (dd, *J* = 7.7, 3.9 Hz, 1 H), 3.93 (d, *J* = 10.9, 1 H), 3.65 (dd, *J* = 10.9, 1.9, 1 H), 1.50 (s, 3 H).

Anal. Calcd for C₂₄H₂₅FN₂O₅ (440.476): C, 65.44; H, 5.72; N, 6.36. Found: C, 65.34; H, 5.74; N, 6.36.

1-(2-Deoxy-2-fluoro-β-D-ribofuranosyl)thymine (1). A solution of **8** (1.71 g, 3.9 mmol) in EtOH/AcOH/H₂O (30 ml, 30:1:1) was shaken in an atmosphere of hydrogen (40 psi) in the presence of 10% Pd/C (0.5 g) for 1 day at rt. The mixture was filtered through a Celite pad and the filtrate was concentrated under reduced

pressure to give **1** identical to the material of Codington *et al.* (33); $^1\text{H NMR}$ (D_2O) δ 7.67 (s, 1 H), 5.96 (d, $J_{\text{HF}} = 19.4$ Hz, 1 H), 5.16 (dd, $J_{\text{HF}} = 52.9$ Hz, $J_{\text{HH}} = 3.8$ Hz, 1 H), 4.38 (ddd, $J_{\text{HF}} = 20.9$ Hz, $J_{\text{HH}} = 8.3, 4.9$ Hz, 1 H), 4.09 (m, 1 H), 4.01 (d, $J = 13.0$ Hz, 1 H), 3.83 (dd, $J = 13.0, 3.9$ Hz, 2 H), 1.85 (s, 3 H).

1-[2-Deoxy-2-fluoro-5-O-(4,4'-dimethoxytrityl)- β -D-ribofuranosyl]thymine (9). This compound was prepared from **1**, and was obtained as a yellow foam identical to the material reported by Williams *et al.* (29).

1-[2-Deoxy-2-fluoro-5-O-(4,4'-dimethoxytrityl)-3-O-(β -cyanoethyl-N,N-diisopropylphosphoramidite)- β -D-ribofuranosyl]thymine (10). This compound was prepared from **9**, and was obtained as a white amorphous powder identical to the material reported by Williams *et al.* (29). $^{31}\text{P NMR}$ (CDCl_3) δ 149.92 (d, $J_{\text{PF}} = 9.3$ Hz), 149.63 (d, $J_{\text{PF}} = 12.3$ Hz).

1-(2-Deoxy-2-fluoro-3,5-di-O-benzoyl- β -D-arabinofuranosyl)thymine (13). This compound was prepared by a slight modification of the procedure of Tann *et al.* (34), which consisted in the recrystallization of the crude product from ethanol to give the desired pure β -anomer in 60% yield.

1-(2-Deoxy-2-fluoro- β -D-arabinofuranosyl)thymine (2). This compound was prepared by the method of Tann *et al.* (34), except that a solution of saturated methanolic ammonia was used for the removal of the ester groups instead of NaOH.

1-(2-Deoxy-2-fluoro-5-O-(4,4'-dimethoxytrityl)- β -D-arabinofuranosyl]thymine (14). This compound was prepared from **2**, and was obtained as a yellow foam identical to the material reported by Kois *et al.* (30).

1-[2-Deoxy-2-fluoro-5-O-(4,4'-dimethoxytrityl)-3-O-(β -cyanoethyl-N,N-diisopropylphosphoramidite)- β -D-arabinofuranosyl]thymine (15). This compound was prepared from **14**, and was obtained as a white amorphous powder identical to the material reported by Kois *et al.* (30). $^{31}\text{P NMR}$ (CDCl_3) δ 150.22 and 149.55.

Synthesis and characterization of oligodeoxyribonucleotides

Oligodeoxynucleotides were synthesized in a 1 μM (~ 25 O.D. units) scale by Oligos Etc., Inc. Wilsonville, OR using standard phosphoramidite chemistry. Purity of the material was assessed by analytical ion exchange HPLC ($>90\%$) and analytical gel electrophoresis. After desalting, samples were lyophilized and obtained as white powders.

Thermal denaturation studies

Extinction coefficients. Oligonucleotide solutions were prepared by dissolving each oligonucleotide in a buffer consisting of 10 mM sodium phosphate, 0.1 mM EDTA and 0.1 M NaCl. Concentrations were determined using the absorbance ($\lambda = 260$ nm) at 90°C interpolated to 25°C . Extinction coefficients were calculated using the nearest neighbor approximation (35). It was assumed that the fluorine substitutions did not change the coefficients. In all calculations $\epsilon_{260} = 110.7$ ($\text{mmol}\cdot\text{cm}$) $^{-1}$ was used for $[\text{d}(\text{CGCGAATTCGCG})]_2$. Stock solutions were stored in the freezer during the course of the experiments.

Melting curves. Absorbance versus temperature profiles were measured in 10 mm quartz cuvettes at 260 nm on a Hewlett-Packard diode array spectrophotometer (model 8452A) equipped with a

Peltier temperature controller (model 99090A). Each sample was heated to 90°C and cooled from 90° to 20°C in 1.0°C increments with 1 min equilibration. The oligomer concentration used for the UV melting studies was ~ 6.7 μM in single strands.

Thermodynamic parameters. Thermodynamic parameters for duplex formation were obtained using two procedures based on methodologies described in the literature (36–38). The first method consisted of a non-linear least-square fit of the data to a two-state model with linear sloping baselines (equation 1).

$$A(T) = lc_T[\alpha\epsilon_{\text{ds}} + (1 - \alpha)\epsilon_{\text{ss}}] \quad 1$$

where

$$\epsilon_{\text{ds}} = a_1 + b_1T$$

$$\epsilon_{\text{ss}} = a_2 + b_2T$$

$A(T)$ is the absorbance of the solution as a function of the temperature, l is the path length (1 cm), c_T is the total concentration, and ϵ_{ds} and ϵ_{ss} are, respectively, the extinction coefficients of double-stranded and single-stranded species. Variable α is the molar fraction of double-stranded form, and it is a function of temperature, according to the definition of equilibrium in a two-state system (equation 2).

$$2A \rightleftharpoons A_2$$

$$K = \frac{[A_2]}{[A]^2} = \frac{\alpha}{2(1 - \alpha)^2 C_T} = e^{-\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R}} \quad 2$$

Base slope parameters were included into the fitting function, and ΔH° , ΔS° , a_1 , a_2 , b_1 , and b_2 were used as variable parameters. A curve fitting script was written for the MLAB program (Civilized Software, Inc., Bethesda, MD) running on a Convex C3830 computer.

The second method to determine thermodynamic parameters involved rearranging equation 2 after exchanging T for T_m , for $\alpha = 0.5$. Solving for $1/T_m$ the equation can be written as equation 3.

$$\frac{1}{T_m} = \frac{R}{\Delta H^\circ} \ln C_T + \frac{\Delta S^\circ}{\Delta H^\circ} \quad 3$$

The above equation was used for the construction of linear plots of $1/T_m$ versus $\ln C_T$ (Fig. 3). From the linear fitting ΔH° and ΔS° were calculated. The T_m values were obtained from the best fit function for each melting curve by calculating the temperature value corresponding to $\alpha = 0.5$. The error of the ΔH° and ΔS° determination was $<10\%$. The thermodynamic parameters obtained from the two methods described above appear in Table 1.

Circular dichroism (CD). CD spectra were recorded on a JASCO J-715 spectrometer at 25°C between 200 and 300 nm using a quartz cell with 1 mm optical path length. Oligonucleotide concentrations were ~ 87 μM (~ 10 OD $_{260}$ /ml) in 10 mM phosphate buffer (pH 7.0) including 100 mM NaCl and 0.1 mM EDTA. CD spectra were measured in terms of molar ellipticity ($\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$).

Solution NMR studies

Samples of ODN 2 (3 mg) or ODN 3 (5 mg) were dissolved in 1 ml of 10 mM phosphate buffer (pH 7.0) which contained 0.0025 M NaCl and 0.05 mM EDTA. The solutions were incubated in a water bath at 80°C for 10 min and annealed by slow cooling to room temperature. Samples in H_2O were prepared by lyophilizing the resultant solution and redissolving it in 90% $\text{H}_2\text{O}/10\%$ D_2O . Samples in D_2O were prepared by repeated lyophilization of 100% D_2O solutions. DSS was used as internal reference. Non-exchangeable proton experiments were recorded

at 15°C and 25°C, while exchangeable proton experiments were recorded at 5°C. All experiments were performed at 500 MHz on a Bruker AMX500 spectrometer. In all cases, phase-sensitive spectra were collected using the States-TPPI method. DQFCOSY, TOCSY and NOESY experiments were collected with 2K data points for 512 t_1 values. The ^1H NOESY experiments were acquired at mixing times of 50, 100, 150 and 300 ms both in D_2O and H_2O . A spectral width of 4900 Hz and repetition delays of 2 and 2.5 s were used for the TOCSY and NOESY spectra, respectively. The water resonance was suppressed by presaturation. The data were processed using UXNMR and peak assignments and volumes were calculated with NMRCOMPASS (Molecular Simulations, Inc., San Diego, CA).

RESULTS AND DISCUSSION

Synthesis

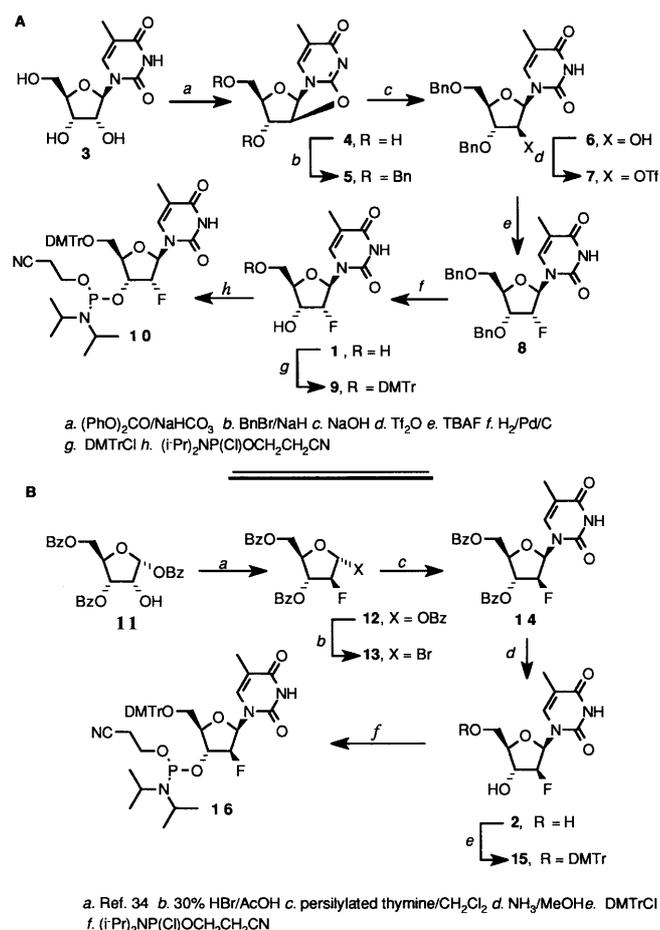
Syntheses for 1-(2-deoxy-2-fluoro- β -D-ribofuranosyl)thymine (**1**) and 1-(2-deoxy-2-fluoro- β -D-arabinofuranosyl)thymine (**2**) have been reported in the literature (29,33,34,39–42). However, we developed a new approach for the *ribo* analogue (**1**) that in our view represents an improvement over previous methodologies (Scheme 1A). Ribofuranosylthymine (**3**) was prepared according to the procedure of Vorbrüggen and Bennua (32) and converted to the corresponding 2,2'-anhydro-1- β -D-arabinofuranosylthymine (**4**) by heating in DMF in the presence of diphenylcarbonate/ NaHCO_3 (43,44). Opening of the anhydride directly with anhydrous HF in dry dioxane (33), or with 70% HF-pyridine, as reported more recently for the synthesis of 2'-deoxy-2'-fluorouridine (**1**), afforded only a low yield of the desired product (27). Instead, we proceeded to fully protect both 3'- and 5'-hydroxyl groups as benzyl ethers (92% yield) to give **5**, which under mild basic hydrolysis opened quantitatively to the corresponding 3',5'-di-*O*-benzyl-1- β -D-arabinofuranosylthymine (**6**). Treatment of **6** with diethylaminosulfur trifluoride (DAST) proceeded only in modest yield (48%) to give **8**. However, a two step sequence involving first the preparation of the triflate **7**, followed by nucleophilic displacement with tetra-*n*-butylammonium fluoride (TBAF), proceeded with yields of 98% and 92%, respectively. Finally, quantitative removal of the benzyl groups from **8** afforded the desired target **1** in 57% overall yield from ribofuranosylthymine.

The synthesis of the *ara* analogue (**2**) was more straightforward (Scheme 1B). It started with the known 1,3,5-tri-*O*-benzoyl-2-deoxy-2-fluoro- α -D-arabinofuranose (**12**) which was obtained from the commercially available ribose derivative **11** (45), according to the procedure of Tann *et al.* (34). Upon treatment with HBr (30% HBr/AcOH) the glycosyl bromide **13** was formed and subsequent glycosylation provided principally the desired β -anomer (**14**) in 60% isolated yield (34,39). After treatment with concentrated methanolic ammonia, the desired 1-(2-deoxy-2-fluoro- β -D-arabinofuranosyl)thymine (**2**) was obtained in 78% yield.

For the incorporation of **1** and **2** into oligonucleotides, the nucleosides were converted to 5'-dimethoxytrityl-3'-phosphoramidites using standard methods as outlined in steps g/h and e/f, respectively, in Schemes 1A and B (29,30).

Thermal denaturation studies

UV thermal melting studies were utilized to assess the contribution of antipodal sugar conformations on the stability of the double helix in short DNA fragments. For these studies, modified versions of the



Scheme 1.

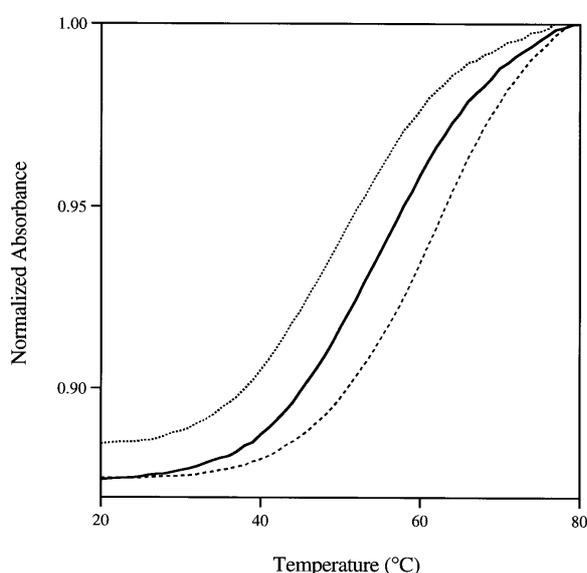
Dickerson–Drew dodecamer (ODN 1, Table 1) were synthesized having the two middle Ts adjusted to a particular conformation by using either a 2'-fluoro-2'-deoxyribose, or a 2'-fluoro-2'-deoxyarabinose moiety (ODNs 2–5). For the sake of simplicity, the letters 'S' and 'N' are used instead of T to represent the corresponding conformational preference of the substituted thymidines.

The Dickerson–Drew dodecamer duplex is known to exist in a characteristic B-form (31), and its helix to coil transitions are generally bimolecular in nature. It was felt, therefore, that this conformationally sturdy oligomer represented an ideal candidate on which to test the power of the sugar moiety to induce measurable conformational changes in an oligomeric structure. In agreement with previously reported melting data (30), the replacement of the middle Ts in ODN 1 with 2'-fluoro-2'-deoxyarabinofuranosyl thymines (TT→SS transmutation, ODN 3) significantly stabilized the already strong B-conformation characteristic of the parent ODN 1 due to the preferred 2'-*endo* (south) conformation of these nucleosides (Table 1, Fig. 1). Conversely, the replacement of the middle Ts with 2'-fluoro-2'-deoxyribofuranosyl thymines (TT→NN transmutation, ODN 2) lowered the stability of the duplex relative to ODN 1 (Table 1, Fig. 1), possibly due to the presence of 3'-*endo* (north) sugar pucker in the middle Ts which interrupt the core of a typical B-helix. The thermodynamic parameters (Table 1) indicate that the increased stability seen with ODN 3 is due to both enthalpic and entropic contributions. This oligomer appears to stack more efficiently (more negative ΔH°)

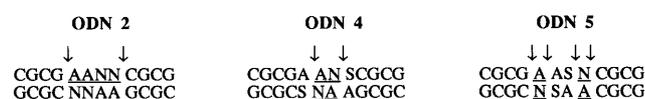
Table 1. Sequences, melting temperatures (T_m) and thermodynamic parameters for ODNs 1–5

Oligomer*	T_m (°C)	ΔT_m	Average from curve fits			$1/T_m$ vs $\log C_T$		
			ΔH° (Kcal/mol)	ΔS° (cal/mol)	$\Delta G^\circ(37^\circ\text{C})$ (Kcal/mol)	ΔH° (Kcal/mol)	ΔS° cal/mol	$\Delta G^\circ(37^\circ\text{C})$ (Kcal/mol)
d(CGCGAATTCGCG) ₂ ODN 1	51.7	—	-47.8	-123.2	-9.5	-46.8	-120.2	-9.6
d(CGCGAANNCGCG) ₂ ODN 2	47.7	-4.0	-44.4	-114.5	-9.0	-41.6	-105.5	-8.9
d(CGCGAASSCGCG) ₂ ODN 3	59.1	7.4	-48.1	-121.2	-10.5	-47.4	-119.3	-10.4
d(CGCGAANSCGCG) ₂ ODN 4	40.4	-11.3	-38.9	-100.0	-7.9	-48.8	-131.2	-8.1
d(CGCGAASNCGCG) ₂ ODN 5	42.0	-9.7	-36.5	-91.4	-8.2	-41.4	-106.8	-8.2

*The letters N and S represent corresponding substitutions of T for (north) 2'-fluoro-2'-deoxyribofuranosyl thymine and (south) 2'-fluoro-2'-deoxyarabinofuranosyl thymine, respectively.
The error of the ΔH° and ΔS° determination was <10%.

**Figure 1.** Normalized absorbance at 260 nm versus temperature for ODNs 1, 2 and 3. ODN 1 (TT) —; ODN 2 (NN); ODN 3 (SS) - - -.

and the south thymidines which are already conformationally pre-organized as 2'-endo sugars facilitate the formation of a B-type DNA (less negative ΔS°). In ODN 2, the north thymidines are also pre-organized, a fact that is probably reflected in a favorable (less negative) ΔS° . However, their north conformation leads to an 'untidy' double-helical structure in the middle of the helix as reflected by the less favorable enthalpy. This result is possibly due to the presence of a B to A transition in the center of the helix (*vide infra*) caused by the differences in sugar pucker. The two other possible mixed combinations involving TT→NS (ODN 4) and TT→SN (ODN 5) transmutations were also studied. However, the data for ODNs 4 and 5 is more difficult to interpret in terms of the individual contributions of ΔH° and ΔS° . Not surprisingly, the two resulting oligos are significantly less stable than the parent ODN 1, as reflected by their significantly lower T_m values and less favorable ΔG° values (Table 1). In each of these oligos, despite the similar ΔG° values derived from both methods, the contributions from ΔH° and ΔS° appear to move in opposite directions depending on the method of calculation.

**Figure 2.** Comparison of possible B-type and A-type conformations in ODNs 2, 4, and 5. The letters S (south) and N (north) are used instead of T to represent the conformational preference of the substituted thymidines. Base pairs with a tendency to adopt a N conformation are underlined.

The oligos where changes in the sugar pucker were expected to induce conformational alterations that deviated from B-DNA are ODNs 2, 4, and 5. To compare these oligos, it was assumed that any A/T base pair with a participating thymidine bias to a 3'-endo (north), or a 2'-endo (south) conformation would adopt the conformation induced by the more rigid thymidine partner. Therefore, in Figure 2 all base pairs with a tendency to pucker N (A-type DNA) appear underlined. It can be appreciated that ODN 2 is nicely divided into three segments where the middle four base pairs are expected to be in an A-like conformation. This produces three evenly spaced segments that mimic a B-A-B conformation with only two transition points (arrows). In ODN 4, the first five base pairs from either end of the double helix should prefer a B conformation, which is expected to be additionally reinforced by (south) thymidines 8 and 20. In this case, there should be two B-A transitions in the middle of the dodecamer, but unlike ODN 2, the A-like segment is perhaps too short to achieve a stable form, and the four bases could even occupy extrahelical positions. In ODN 5, there are four sites of A-B discontinuity (arrows) and this will provide for a poorer stacking of the duplex consistent with a less negative ΔH° . In discussing the conformational preferences of these oligos, it is important to remember that the fluorinated sugars are not totally rigid, but biased to one form of ring pucker (25). Therefore, they can probably re-adapt in response to the torque exerted by the longer DNA segments and flip to a less favorable conformation. It is possible that in ODNs 4 and 5, where the segments expected to be in an A-type conformation are too short, the 3'-endo nucleosides flip to the less favorable 2'-endo conformation, and the ODNs adopt an overall conformation more towards a B-type, but with a reduced stability (*vide infra*).

The van't Hoff plots for ODNs 1–5 (Fig. 3), which were analyzed according to equation 3, show the stability of all the new duplexes (ODNs 2–5) relative to the unmodified dodecamer,

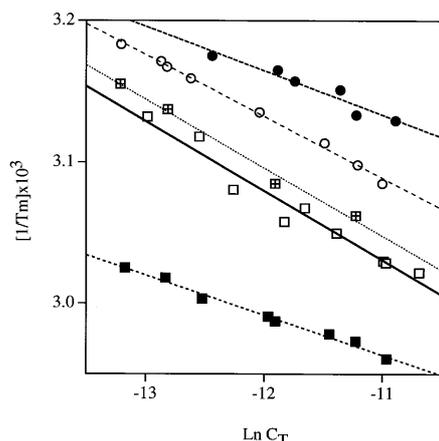


Figure 3. Plots of $1/T_m$ versus $\ln C_T$ for ODNs 1–5. Open square, ODN 1 (TT); square containing cross, ODN 2 (NN); filled square, ODN 3 (SS); filled circle, ODN 4 (NS); open circle, ODN 5 (SN).

ODN 1. As discussed above, the most stable duplex corresponded to that of ODN 3 and the least stable was that formed by ODN 4.

Circular dichroism

The changes in global conformation of a DNA sample in solution can be detected by CD spectroscopy (47). CD measurements are very sensitive to changes in the geometrical relationship between adjacent bases, which result from the different stacking interactions imparted by both B- and A-type DNA (15,16). For a pure B-type DNA, such as the Dickerson–Drew dodecamer, the CD spectrum revealed a typical B-form with a positive maximum at 280 nm, a crossover point at 269 nm, and a ratio of positive to negative lobes (the positive intensity divided by the negative intensity) of 0.55 (ODN 1, Fig. 4). The CD spectrum of ODN 3, which was also expected to be in the B-form, showed a virtually similar profile (Fig. 4). For ODN 2, however, the CD spectrum showed a conformation that is intermediate between that of B-type and A-type DNA (Fig. 4). The position of the positive maximum appeared shifted to lower wavelengths (274 nm), and the ratio of positive to negative lobes increased to almost 1 (0.94), which is much larger than that of the B-form in ODN 1. Also, the magnitude of the negative CD band at 210 nm appeared enhanced. All these features are typical of A-type DNA conformations and support the argument that north 2'-fluoro-2'-deoxyribofuranosyl thymines located in the middle of a canonical B-DNA duplex (ODN 1) force what appears to be a B-to-A conformational transition (15,16). Indeed, the shape of the CD spectrum of ODN 2 is remarkably similar to that reported for a B-A junction induced by dehydration of a duplex built with A-philic and B-philic contacts (16). On the contrary, the CD spectra of ODNs 4 and 5 do not show the same tendency as they appear to remain mostly in the B-form (Fig. 5). As discussed before, it is possible that in these two situations the A-type segments are too short to generate a stable A-form in the middle of a B-DNA duplex, and the 3'-endo 2'-fluoro-2'-deoxyribofuranosyl thymines flip to the less favorable 2'-endo conformation.

NMR spectroscopy

NMR analysis provided independent confirmation of the structural preferences of ODN 2 and ODN 3. The one-dimensional spectra

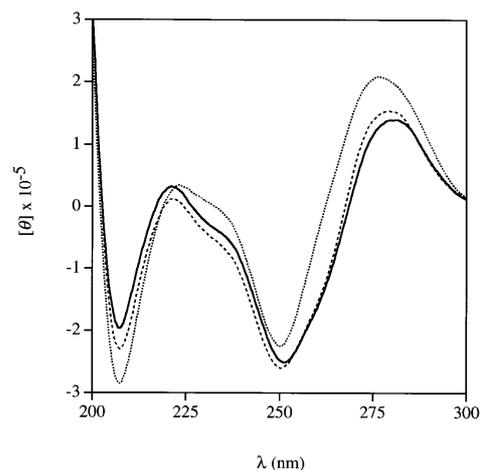


Figure 4. CD spectra of ODNs 1–3. ODN 1 (TT) —; ODN 2 (NN); ODN 3 (SS) - - -.

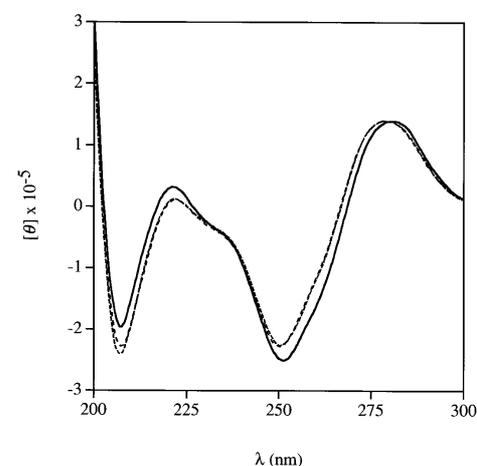


Figure 5. CD spectra of ODNs 1, 4 and 5. ODN 1 (TT) —; ODN 4 (NS); ODN 5 (SN) - - -.

also provided additional proof of sample purity. Standard two-dimensional (2D) experiments (double quantum filtered COSY, TOCSY and NOESY) allowed the assignment of virtually all non-exchangeable protons (48). Spectra in H₂O also yielded information on the exchangeable imino protons. These were assigned by the well-known connectivities between H3 of thymidine and H2 of adenine in AT base pairs, and between H1 of guanosine and the amino protons of cytosine in CG base pairs (48). The fluorinated sugar rings provided an expeditious starting point in the resonance assignment strategy owing to the distinct coupling constant and chemical shift information observed for the 2'/2'' protons on the carbons bearing fluorine. Identification of the residue C1 allowed for complete assignment of both furanosyl and aromatic base protons via intraregion [di(X,Y)] and sequential [ds(X,Y)] NOEs to requisite base protons. Cross validation of assignments were made from the expeditious assignment of the T7 and T8 protons.

Comparison of the NMR data of ODN 2 and ODN 3 revealed local structural variations in the vicinity of the fluorinated residues. A qualitative analysis suggested that the overall conformation of ODN 3 was typical B-type. Cross-peak intensities

for scalar coupling interactions between H1'-H2'/H2'' appeared much greater than those for H3'-H2'/H2'', and in most cases the H3'-H2'' cross peaks were extremely weak or absent. This is consistent with the presence of 2'-endo (south) sugar puckers. For ODN 2, half of the base pairs were also characteristic B-type. However, several changes in residues G4-C9 suggested that the *ribo* (north) configuration induced by fluorine in T7 and T8 had a significant influence on the surrounding topology. In contrast to the lack of intensities for the H3'-H2'' cross-peaks in G4, A5 and A6 in ODN 3, there was a moderate intensity correlation observed in ODN 2 for the same residues. Furthermore, the H3'-H2'' correlation for C9 which appeared strong in ODN 2 was rather weak in ODN 3. These distinctions argue that the pucker of the sugar rings of G4, A5, A6 and C9 contained a higher percentage of a 3'-endo (north) conformation due to the influence of the middle fluorinated *ribo* thymidines.

Intraresidue and sequential NOE cross peaks for ODN 3 (Fig. 6a) and ODN 2 (Fig. 6b) also corroborated the aforementioned differences in conformation between these two oligomers. In general, a B-type conformation is expected to display strong intraresidue [di(2',6/8)] and sequential [ds(2'',6/8)] correlations, whereas an A-type conformation is characterized by weak intraresidue [di(2',6/8)] correlations. For ODN 3, di(2',6/8) correlations were consistently strong (note that T7 and T8 do not have H2' protons in ODN 3), but appeared weaker for the modified T7 and T8 residues in ODN 2. Also, uniformly strong ds(2'',6/8) cross-peak intensities were observed for ODN 3, while cross peaks in this region weakened in the vicinity of T7 and T8 in ODN 2. These differences, along with the increased intensity of both di(3',6/8) and ds(3',6/8) within and surrounding T7 and T8 in ODN 2 strongly suggest a more 3'-endo (north) type of pucker for the sugar rings in this region of the DNA. The fine details of the NMR structure determination of these two oligomers will be published elsewhere.

CONCLUSION

Our results confirm that the presence of two rigid 2'-endo (south) thymidines in the middle of a Dickerson-Drew duplex increase the stability of the already favorable B-type conformation. On the contrary, two 3'-endo thymidines biased toward the antipodal north pucker destabilize the B-type conformation and appear to induce a local A-like structural perturbation according to CD and NMR spectroscopies. It is reasonable to expect that the microstructure of the modified thymidines contributes to the forces that induce the stabilizing or destabilizing polymorphism in these ODNs. The enthalpy and entropy contributions to the N \rightleftharpoons S equilibrium of the fluorinated thymidines can stabilize or destabilize the duplex in the same direction as their individual preference for either extreme of the pseudorotational cycle. For example, the *gauche* effect of the O4'-C1'-C2'-F and F-C2'-C1'-N fragments of the 2'-fluoro-2'-deoxyarabinofuranosyl thymine (2) units is expected to drive N \rightleftharpoons S equilibrium to the south, prevailing over the anomeric effect which pushes the equilibrium in the opposite direction (49,50). This means that the conformational enthalpy introduced into the biopolymer by these south pseudorotamer units is negative, and should favor a more stable B-type duplex. The reverse is true for the case of 2'-fluoro-2'-deoxyribofuranosyl thymine (1) units which are biased toward north pseudorotamers, and hence their conformational enthalpy contribution is positive. The conformational preferences shown

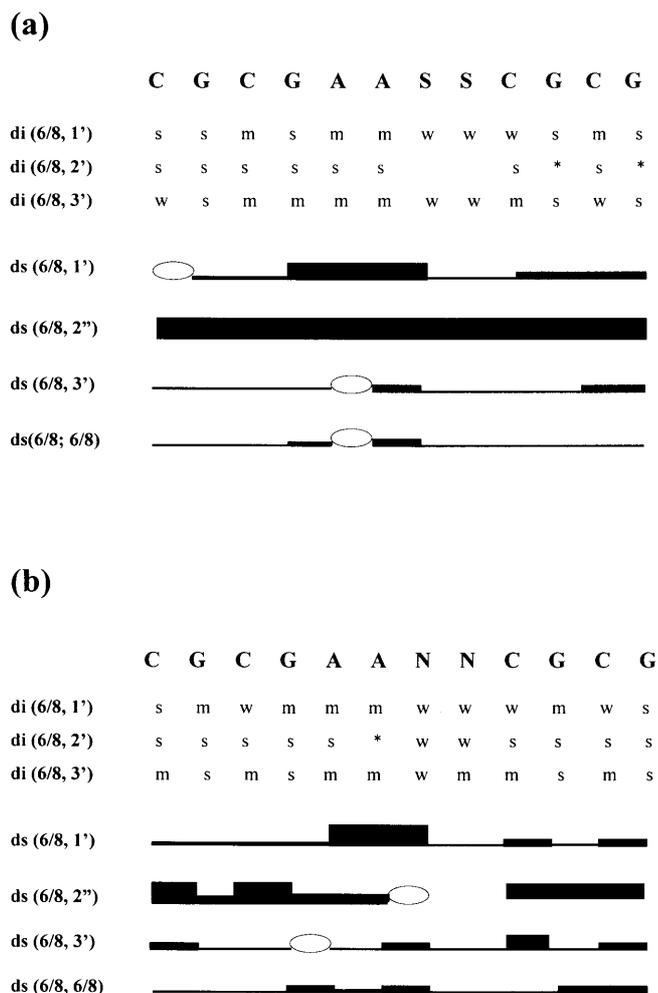


Figure 6. Schematic representation of intraresidue [di(X,Y)] and sequential [ds(X,Y)] NOE intensities for ODN 3 (a) and ODN 2 (b). Intraresidue peak intensities are described as strong (s), medium (m) or weak (w). The thickness of the bars for the sequential correlations directly relates to peak intensities. A * (top) or an empty circle (bottom) represents a peak that was not unequivocally determined due to partial overlap.

by these individual fluorothymidine moieties are obviously moderated by other forces in an oligomeric structure, but their tendency to stabilize or destabilize a B-type conformation appears to be dictated by their individual preference for a south or north sugar pucker. Not all the bases on a similar fluorinated sugar moiety are expected to behave comparably to the fluorothymidines because the stereoelectronic *gauche* and anomeric effects are counteractive and differ according to the type of base (49). Specifically for the 2'-fluoro-2'-deoxyarabinofuranosyl thymine units, the prevalence of the *gauche* effect over the anomeric effect results in an enthalpy driven stabilization of the south furanose pseudorotamer. However, as the anomeric effect increases, this stabilizing effect is expected to diminish. The combined stereoelectronic and steric contributions of the nucleobases to the anomeric effect decreases in the following order: cytosine > uracil > thymine > guanine \approx adenine (50), which would explain why in a related study (30) 2'-fluoro-2'-deoxyarabinofuranosyl uracil and cytosine units did not have the same stabilizing effect on the Dickerson-Drew duplex as the thymine analogue.

The recently solved X-ray structure of the most stable duplex, ODN 3, showed that the modified 2'-fluoro-2'-deoxyarabino-furanosyl thymine residues adopt a unique O4'-endo (east) conformation. In the crystal lattice, this conformation helps to avoid intraresidue steric clashes between the fluorine and the C6 carbon of the base, which would result from a pure 2'-endo (north) conformation that still maintains a glycosyl torsion angle compatible with a B-form geometry (52). Remarkably, the O4'-endo residues can be accommodated without significantly altering the global geometry of the B-DNA duplex (52). According to this X-ray analysis, the increased stability of ODN 3 could originate from the conformational preorganization of the sugar moieties and the ordering of the water structure around the fluorine atoms. Our NMR data for ODN 3 also agrees with the existence of a B-type duplex, but containing instead fluorothymidine units closer to a 2'-endo conformation. It is possible, however, that these fluorosugar moieties exhibit different degrees of flexibility in the two environments. In the absence of an X-ray structure for ODN 2, spectral evidence suggests that there is a measurable deviation from the canonical B-form in the middle of the duplex due to the preference of the furanose rings to deviate from the 2'-endo envelope pucker in the vicinity of the fluorinated base pairs. Refinement of the NMR data with molecular dynamics simulations is in progress to determine the preferred pseudorotational parameters in solution for the modified sugar rings of ODN 2 and ODN 3.

Supplementary material

2-D NOESY NMR spectra for ODN 3 (south DNA, NOESY, D₂O, 298 K) and ODN 2 (north DNA, NOESY, D₂O, 298 K) are available as supplementary material via NAR Online (<http://www.oup.co.uk/nar>).

ACKNOWLEDGEMENT

The authors wish to express their sincerest thanks to Dr Serge L. Beaucage of the Division of Hematologic Products, Center for Biologics Evaluation and Research, FDA, for his support.

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