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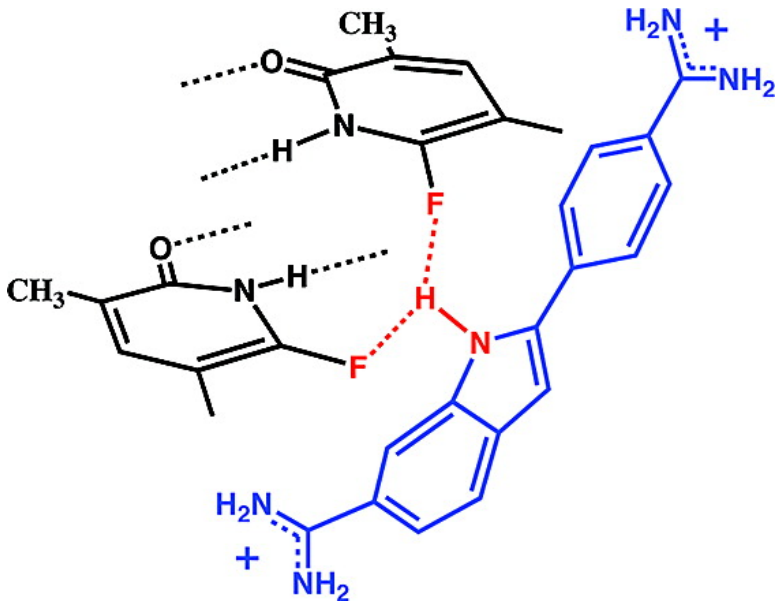

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Probing the Nature of Three-Centered Hydrogen Bonds in Minor-Groove Ligand−DNA Interactions: The Contribution of Fluorine Hydrogen Bonds to Complex Stability

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Abstract: Double-stranded DNA sequences have been prepared in which single atoms (the O2-carbonyls of selected thymines) have been replaced by fluorine or methyl. To maintain normal Watson−Crick hydrogen bonding with the complementary purines, these analogue derivatives have been prepared as C-nucleosides. The O2-carbonyls of interest for this study are those involved in a bifurcated (or three-centered) hydrogen bond with the minor groove binding ligand 4′,6-diamidino-2-phenylindole (DAPI). T_M studies of the duplexes illustrate that the DNA duplexes are destabilized when fluorine or methyl replaces one or both of the minor groove O2-carbonyls, which can in part be explained by changes in minor groove hydration. In the presence of DAPI, most of the duplexes exhibit an increased T_M due to the presence of DAPI bound in the minor groove. The extent of helix overstabilization negatively correlates with the presence of one or both methyl groups in the minor groove, suggesting that ligand binding is weakened in the presence of the non-carbonyl functional groups. The presence of single fluorine appears to promote helix stabilization, and native-like stabilization occurs when both fluorines are present. K_D values quantitate binding effects between DAPI and the native and analogue sequences. Sequences with one or both methyl groups exhibit very poor binding with DAPI, while those containing a single fluorine behave essentially like native carbonyl-containing sequences. With both fluorines present, K_D values were observed to increase by a moderate 3-fold at 100 mM NaCl and somewhat more at 200 mM NaCl. Binding affinities with both methyl groups present were 500−1000-fold weaker than native. The results suggest that organofluorines can function as hydrogen-bond acceptors, at least in the bifurcated interaction that contributes to minor groove binding by DAPI.

Introduction

There are a number of reported DNA-ligand crystal structures in which the ligands bind to double-stranded DNA in the minor groove of dA−dT rich sequences.[1−6] In these structures, water seems to be largely excluded from the minor groove at the binding site, including the specifically bound water molecules of the minor-groove spine of hydration[7−9] commonly found in dA−dT sequences. These complexes appear to be stabilized by specific hydrogen-bonding interactions between the ligands and functional groups located in the DNA minor groove. Among the contacts observed to stabilize such complexes are three-centered or bifurcated hydrogen-bonding interactions in which two carbonyls, functioning as hydrogen-bond acceptors, share a hydrogen that is formally bound to an electronegative heteroatom such as nitrogen. Many DNA minor groove binding ligands such as distamycin,[3,4] netropsin,[5,6] the Hoechst bisbenzimidazoles,[2] and DAPI (4′,6-diamidino-2-phenylindole)[1] all exhibit this type of bifurcated hydrogen-bonding interaction(s) when bound in the DNA minor groove.

We have measured the strength of these types of binding interactions using analogue sequences in which one or both of the hydrogen-bond partners have been removed.[10] The subsequent binding can be characterized and quantified by thermodynamic parameters and the $\Delta\Delta G$ for the specific interaction extracted from the data. In previous work,[10] a study was performed for a thymine derivative in which the O2-carbonyl was replaced by hydrogen. Each of the two hydrogen-bonding interactions present in the bifurcated hydrogen bond was measured to contribute approximately 2 kcal/mol (total value: 4 kcal/mol) to the binding affinity of DAPI for the dA−dT rich minor groove.

Here, we describe an alternative approach in which the O2-carbonyl is replaced by methyl or fluorine. Both of these

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derivatives have molecular sizes that better mimic the carbonyl oxygen relative to hydrogen, and both have been used in a variety of isosteric hydrophobic analogues.[11,12] The analogue sequences containing one or two methyl groups still should interrupt hydrogen-bonding interactions, much as the hydrogen-containing sequence did, but may also provide either a compensating hydrophobic effect or additional unfavorable steric effects. The analogue sequences containing fluorine(s) may still form bifurcated hydrogen bonds depending on the ability of aryl-fluorides to act as hydrogen-bonding acceptors, and they may or may not provide a hydrophobic effect. Organofluorines of the type C−F···H−X, where X is an electronegative heteroatom, do not generally form strong hydrogen bonds, in part due to their poor polarizability.[13−16] Here, we describe the effects of these substitutions on the binding of DAPI to the DNA minor groove.

Experimental Section

Materials. Oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. 2′-Deoxynucleotide phosphoramidites and 3′-terminal nucleoside controlled pore glass support (CPG) were purchased from Glenn Research (Sterling, VA). High performance liquid chromatography (HPLC) was performed on a Waters HPLC system using C-18 reversed-phase columns (ODS-Hypersil, 5 μm-particle size, and 120 Å pore) with detection at 260 nm. Oligonucleotides were desalted with Econo-Pac 10DG disposable chromatography columns (Bio Rad, Hercules, CA). Fluorescence measurements and titration experiments were performed on a Jobin-Yvon FluoroLog spectrofluorimeter.

Methods. Nucleoside Phosphoramidite Syntheses. 6-[2-(4-Nitrophenyl)ethoxy]-3-[2-deoxy-5-*O*-(4,4-dimethoxytrityl)-*β*-D-*erythro*-pentafuranosyl]-2-methylpyridine. To 6-[2-(4-nitrophenyl)ethoxy]-3-(*β*-D-2-deoxy-*erythro*-pentafuranosyl)-2-methylpyridine[17] (100 mg, 0.27 mmol, 1.0 equiv) dissolved in anhydrous pyridine (10 mL) and coevaporated twice from pyridine and finally dissolved in 10 mL of anhydrous pyridine and triethylamine (0.2 mL, 0.14 mmol, 0.5 equiv) were added dimethylaminopyridine (10 mg, 0.08 mmol, 0.3 equiv) and 4,4′-dimethoxytrityl chloride (100 mg, 0.29 mmol, 1.1 equiv). The reaction mixture was stirred at ambient temperature and was complete in 12 h, as revealed by TLC analysis. Pyridine was removed under high vacuum rotary evaporation, and the residue was dissolved in dichloromethane (30 mL) and washed with saturated $NaHCO_3$ solution (3 × 20 mL). The organic phase was collected, dried over Na_2SO_4, and concentrated to dryness. The residue was purified by flash column chromatography eluting with 2% methanol in dichloromethane to give product (162 mg, 0.24 mmol, 90%) as a white foam. mp 68−69 °C, TLC in 1% methanol in dichloromethane. R_f = 0.2; ^{1}H NMR (500 MHz, DMSO-d_6) δ = 8.17 (d, J = 8.6 Hz, 2H), 7.67−7.20 (m, 12H), 6.88 (d, J = 8.9 Hz, 4H), 6.55 (d, J = 8.5 Hz, 1H), 5.17 (dd, J = 5.6, 9.7 Hz, 1H), 5.14 (d, J = 4.2 Hz, 1H), 4.51 (t, J = 6.5 Hz, 2H), 4.15 (m, 1H), 3.91 (m, 1H), 3.73 (s, 6H), 3.17 (t, J = 6.5 Hz, 2H), 3.17 (m, 2H), 2.38 (s, 3H), 2.16−2.12 (m, 1H), 1.74−1.71 (m, 1H) ppm; ^{13}C NMR (125 MHz, DMSO-d_6) δ = 161.9, 158.8, 152.7, 148.0, 146.9, 145.6, 137.3, 136.4, 136.3, 130.9, 130.4, 130.4, 129.2, 128.5, 128.4, 127.3, 124.1, 113.8, 108.2, 86.4, 86.1, 76.1, 73.0, 65.8, 65.0, 55.7, 42.9, 35.2, 22.3 ppm; exact mass calculated for $[C_{40}H_{41}N_2O_8]^+$ requires m/z 677.2863; found, 677.2857 (ESI+).

6-[2-(4-Nitrophenyl)ethoxy]-3-[2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-*β*-D-*erythro*-pentafuranosyl]-2-fluoropyridine. To 6-[2-(4-nitrophenylethoxy)]-3-(*β*-D-2-deoxy-*erythro*-pentafuranosyl)-2-fluoropyridine[17] (100 mg, 0.27 mmol, 1.0 equiv) dissolved in anhydrous DMF (2 mL) and anhydrous dichloromethane (2 mL) were added 2,6-lutidine (0.15 mL, 1.35 mmol, 5 equiv), silver trifluoromethanesulfonate (100 mg, 0.38 mmol, 1.4 equiv), and dimethoxytrityl chloride (109 mg, 0.32 mmol, 1.2 equiv). The reaction mixture was stirred at ambient temperature and was complete in 24 h as revealed by TLC analysis. DMF was removed completely under high vacuum rotary evaporation; the residue was dissolved in dichloromethane (10 mL) and washed with saturated $NaHCO_3$ solution (2 × 10 mL). The organic phase was collected, dried over Na_2SO_4, and concentrated to dryness. The residue was purified by flash column chromatography eluted with 1:1 ethyl acetate:petroleum ether to give product (140 mg, 79%) as a white foam. mp 55−56 °C, TLC in 1:1 ethyl acetate:petroleum ether. R_f = 0.2; ^{1}H NMR (500 MHz, $CDCl_3$ + pyridine-d_5) δ = 8.05 (d, J = 8.7 Hz, 2H), 7.78 (t, J = 7.8 Hz, 1H), 7.38−7.09 (m, 11H), 6.70−6.72 (m, 4H), 6.44 (d, J = 8.2 Hz, 1H), 5.28 (m, 1H), 4.43−4.38 (m, 3H), 4.09 (m, 1H), 3.67 (s, 6H), 3.22 (d, J = 4.6 Hz, 2H), 3.06 (t, J = 6.5 Hz), 2.29−2.25 (m, 1H), 1.90−1.85 (m, 1H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$ + pyridine-d_5) δ = 161.6, 161.4, 159.6, 158.6, 157.7, 146.9, 146.4, 145.1, 140.9, 140.9, 136.2, 130.3, 130.0, 129.4, 128.3, 127.9, 126.9, 123.8, 115.3, 115.1, 113.2, 113.1, 107.6, 107.5, 86.7, 86.3, 73.6, 66.2, 64.6, 55.3, 42.9, 35.3 ppm; exact mass calculated for $[C_{39}H_{37}FN_2O_8Na]^+$ requires m/z 703.2432; found, 703.2435 (ESI+).

3-[2-Deoxy-3-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphino)-5-*O*-(4,4′-dimethoxytrityl)-*β*-D-*erythro*-pentafuranosyl]-2-fluoro-6-[2-(4-nitrophenyl)ethoxy]pyridine. To a solution of 3-[2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-*β*-D-*erythro*-pentafuranosyl]-2-fluoro-6-[2-(4-nitrophenyl)ethoxy]pyridine (100 mg, 0.15 mmol) and *N*,*N*-diisopropylethylamine (128.9 μL, 0.18 mmol, 5 equiv) in anhydrous dichloromethane (10 mL) was added dropwise 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (39.6 μL, 0.18 mmol, 1.2 equiv) at 0 °C. The reaction mixture was stirred at ambient temperature for 2 h, and then methanol (1 mL) was added and stirring was continued for 20 min. The mixture was washed with saturated $NaHCO_3$ water solution (2 × 10 mL) and water (2 × 10 mL) and dried over Na_2SO_4 overnight. The drying reagent was removed by filtration, and the solution was concentrated to 0.5 mL in vacuo. The residue was purified by precipitation from hexane to give product (104 mg, 80%) as a colorless foam consisting of a 1:1 mixture of diastereoisomers; TLC in ethyl acetate:petroleum ether (1:10) (R_f = 0.5); ^{31}P NMR (162 MHz, $CDCl_3$) δ = 148.6, 148.2 ppm; exact mass calculated for $[C_{48}H_{55}FN_4O_9P]^+$ requires m/z 881.3691; found, 881.3692 (ESI+).

3-[2-Deoxy-3-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphino)-5-*O*-(4,4′-dimethoxytrityl)-*β*-D-*erythro*-pentafuranosyl]-2-methyl-6-[2-(4-nitrophenyl)ethoxy]pyridine. To a solution of 3-[2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-*β*-D-*erythro*-pentafuranosyl]-2-methyl-6-[2-(4-nitrophenyl)ethoxy]pyridine (100 mg, 0.15 mmol) and *N*,*N*-diisopropylethylamine (129 μL, 0.74 mmol, 5 equiv) in anhydrous dichloromethane (10 mL) was added dropwise 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (40 μL, 0.18 mmol, 1.2 equiv) at 0 °C. The reaction mixture was stirred at ambient temperature for 2 h, and then methanol (1 mL) was added and stirring was continued for 20 min. The mixture was washed with saturated $NaHCO_3$ water solution (2 × 10 mL) and water (2 × 10 mL) and dried over Na_2SO_4 overnight. The drying reagent was removed by filtration, and the solution was concentrated to 0.5 mL in vacuo. The residue was purified by precipitation from hexane to give product (95 mg, 73%) as a colorless foam consisting of a 1:1 mixture of diastereoisomers; TLC in ethyl acetate:petroleum ether (1:10) (R_f = 0.5); ^{31}P NMR (162 MHz, $CDCl_3$) δ = 148.6, 148.1 ppm; exact mass calculated for $[C_{49}H_{58}N_4O_9P]^+$ requires m/z 877.3941; found, 877.3939 (ESI+).

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DNA Synthesis. Oligonucleotides were prepared by solid-phase synthesis techniques. The two analogue 2′-deoxynucleoside phosphoramidites, one containing a protected 6-methyl-2-pyridone nucleobase and the second containing a protected 6-fluoro-2-pyridone nucleobase, exhibited coupling efficiencies that were indistinguishable from those of the common nucleoside phosphoramidites. For deprotection of the assembled sequences, the *p*-nitrophenylethyl protecting groups were removed as the first step by treating the solid support with a solution of 1.0 M 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) in anhydrous acetonitrile for 8−48 h, and finally by washing with acetonitrile three times. Ammonia treatment then followed in the normal manner.

Purification of the oligonucleotides was accomplished by fast flow HPLC (4.6 × 120 mm, reverse phase C18 column, trityl on), starting with 100% A for 1 min, then using a linear gradient 0−50% B over 4.5 min (A, 50 mM triethylammonium acetate, adjusted to pH 7.0 with glacial acetic acid; B, 50 mM triethylammonium acetate in 70% acetonitrile, pH 7.0). The DMT protected 12-mers had retention times of about 5.0 min. The collected oligonucleotides were reduced in volume and detritylated with 80% aqueous acetic acid (60 min, on ice). The resulting oligonucleotides were then desalted (Sephadex G-10) and stored at −20 °C. The unnatural nucleotides could be incorporated into the DNA strands with essentially the same coupling efficiency as the common nucleotides.

After HPLC isolation MALDI-TOF analyses were performed to confirm the identity of the oligos: 5′-d[CCGGAATTCGCC]-3′ calcd MS 3604. Found: 3600. 5′-d[GGCGAATTCCGG]-3 calcd MS 3685. Found: 3683. 5′-d[GGCGAA(m^{6}2P)TCCGG]-3′ calcd MS 3668. Found: 3690. 5′-d[CCGGAA(m^{6}2P)TCGCC]-3′ calcd MS 3587. Found: 3582. 5′-d[GGCGAA(F^{6}2P)TCCGG]-3′ calcd MS 3672. Found: 3670. 5′-d[CCGGAA(F^{6}2P)TCGCC]-3′ calcd MS 3591. Found: 3593

Thermal Denaturation Studies. Thermal denaturation studies were performed in 20 mM NaH_2PO_4, 1.0 M NaCl, pH 7.0 solution. Absorbance and temperature values were measured with an AVIV 14DS UV/visible spectrophotometer equipped with digital temperature control. The temperature of the cell compartment increased in 0.5 °C steps (from 0 to 95 °C), and when the equilibrium was reached temperature and absorbance data were collected. The T_M values were determined manually from the absorbance versus temperature plots and by first derivative analysis.

Fluorescence Spectroscopy. Fluorescence spectra were obtained on a Jobin-Yvon FluoroLog spectrofluorimeter equipped with a constant temperature water bath. All measurements were taken at 10 °C using matched quartz cuvettes. All buffers and solutions used were filtered with a 0.2 μm filter and degassed by being bubbling nitrogen for 30 min. All measurements were taken in 0.02 M phosphate, 0.2 M NaCl, pH 7.0 buffer. Solutions were mixed by turning the cuvette upside down several times and then allowing the solution to equilibrate for 1 min. The solutions were excited at 355 nm. Fluorescence measurements were obtained by titrating a constant concentration of ligand (between 1 and 24 nM), with a relatively concentrated solution of dsDNA (between 1 and 30 μM). Emission spectra were obtained by scanning the region from 400 to 500 nm for each data point. The background fluorescence intensity (buffer and ligand) was always subtracted from each data point. The dissociation constant (K_D) for ligand and dsDNA was determined using eq 1, where $\sum\Phi_f$ is the total fluorescence intensity upon saturation of ligand by dsDNA, K is the equilibrium association constant (K_A), and [dsDNA] is the concentration of free dsDNA in solution.

$$F = \sum \Phi_f(K[\text{dsDNA}]/(1 + K[\text{dsDNA}])) \qquad (1)$$

Results and Discussion

The structure of DAPI (4′,6-diamidino-2-phenylindole) bound to double-stranded DNA has been solved by crystal structure analysis[1] using a double-stranded DNA dodecamer as a target.

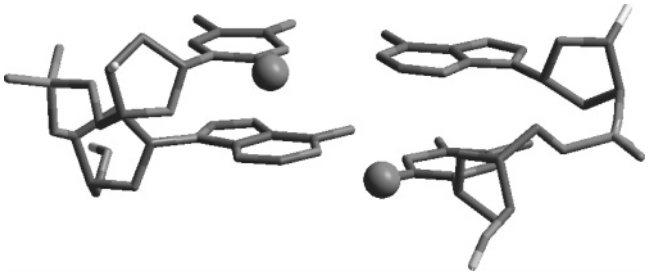


Figure 1. View into the minor groove of the central d(A−T, T−A) sequence. The two thymidine O2-carbonyls are highlighted as spheres.

The reported structure indicates the presence of a three-centered hydrogen bond involving the indole NH of DAPI and the two minor groove thymine carbonyls of the centrally located dA−dT base pairs. These two carbonyls (see Figure 1) in the unliganded state would host at least one water molecule as an integral part of the minor groove spine of hydration. In the liganded state, the indole NH is present in place of that water molecule and forms a bifurcated hydrogen bond involving the indole nitrogen and the two minor groove carbonyls.

We have previously described[10] sequences in which these two thymine carbonyls were removed and replaced by hydrogens. Although the modified residue forms a Watson−Crick base pair,[18] these duplexes are destabilized,[19] in part because of hydration effects. The sequences exhibit relatively poor binding by DAPI, and those experiments suggested that the three-centered bond in the native complex contributed as much as 4 kcal/mol of binding energy in the formation of the ligand DNA complex. To further probe the nature of this hydrogen-bonding interaction involving the ligand DAPI, we have prepared two additional types of derivatives, those in which the O2-carbonyl of each thymine-like residue has been replaced by either fluorine or methyl (Figure 2). (For synthetic accessibility, we have used modified uracils in place of modified thymines.) The derivatives prepared are pyridine C-nucleosides in place of native pyrimidine N-nucleosides. These analogues were chosen to maintain the possibility of "normal" Watson−Crick interbase hydrogen bonding. Changing the O2-functionality as described would otherwise alter the tautomeric nature of the nucleobase, and thereby hydrogen-bonding patterns. In each of the modified pairs, a bidentate Watson−Crick base pair should form; for a related derivative, in which the O2-carbonyl was replaced by hydrogen, crystallographic data[18] confirm that expectation. The averaged C−N bond distance in DNA nucleosides of the C2′-endo conformation has been reported from a series of examples to be 1.474 Å.[26] To date we have obtained[27] the crystal structure of a C-nucleoside related to those described here. In that structure the measured C−C bond distance was 1.495 Å. The difference between these two bond lengths is 0.021 Å, which represents less than a 2% difference in length.

***T_M* Data.** We prepared a series of non-self-complementary dodecamers so that we could alter the nature of the minor groove

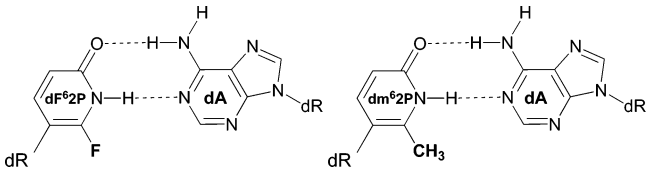
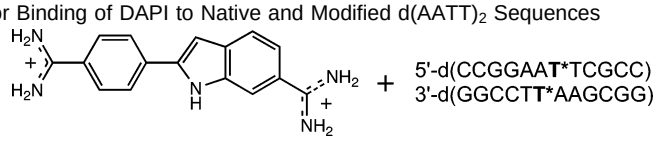


Figure 2. Bidentate base pairs formed from dA and either analogue pyrimidine derivative: a pyridine C-nucleoside with either fluorine (dF^{6}2P) or methyl (dm^{6}2P) replacing the thymine O2-carbonyl.

Table 1. T_M, K_D, and ΔG Values for Binding of DAPI to Native and Modified d(AATT)$_2$ Sequences



entry	central T's	T_M °C	T_M (with DAPI) °C	ΔT_M °C	K_D nM	ΔG kcal/mol	$\Delta\Delta G$ kcal/mol
			[NaCl] = 200 mM				
1	T−T	62.8	71.4	8.6	8.6 ± 1.3	−11.0	
2	T−dm^{6}2P	54.1	58.4	4.3	312.0 ± 25	−8.8	2.2
3	dm^{6}2P−T	53.9	58.0	4.1	301.0 ± 37	−8.9	2.1
4	dm^{6}2P−dm^{6}2P	50.3	51.3	1.0	4207 ± 138	−7.3	3.7
			[NaCl] = 200 mM				
1	T−T	62.8[a]	71.4[a]	8.6	8.6 ± 1.3	−11.0	
5	T−dF^{6}2P	60.4[a]	69.2[a]	8.8	8.4 ± 0.9	−11.0	0.0
6	dF^{6}2P−T	60.2[a]	68.6[a]	8.4	8.8 ± 2.1	−10.9	0.1
7	dF^{6}2P−dF^{6}2P	55.1[a]	60.0[a]	4.9	145 ± 35	−9.3	1.7
			[NaCl] = 100 mM				
8	T−T	60.0	70.5	10.5	4.2 ± 0.3	−11.4	
9	T−dm^{6}2P	51.9	58.4	6.5	21.6 ± 2.1	−10.4	1.0
10	dm^{6}2P−T	51.8	58.6	6.8	16.7 ± 1.8	−10.6	0.9
11	dm^{6}2P−dm^{6}2P	47.3	49.2	1.9	2103 ± 138	−7.7	3.7
			[NaCl] = 100 mM				
8	T−T	60.0	70.5	10.5	4.2 ± 0.2	−11.4	
12	T−dF^{6}2P	57.6	69.6	12.0	2.3 ± 0.1	−11.7	−0.3
13	dF^{6}2P−T	56.1	69.1	13.0	1.2 ± 0.2	−12.1	−0.7
14	dF^{6}2P−dF^{6}2P	52.0	59.6	7.6	14 ± 1.1	−10.7	0.8

[a] T_M values could be reproduced ±0.5 °C.

functional groups by making changes in one strand of the DNA or the other. The central core of the duplex formed from these two strands remains as the self-complementary sequence d(GAATTC)$_2$. The T_M for the native dodecamer at 200 mM NaCl is 62.8 °C; at 100 mM NaCl the T_M dropped slightly to 60.0 °C (Table 1).

Substitution of either central T residue in the d(GAATTC)$_2$ duplex target site by a 3-(β-D-2-deoxy-*erythro*-pentafuranosyl)-2-methyl-6-pyridone (dm^{6}2P)[28] analogue replaces either of the central thymine O2-carbonyls with methyl group, and these sequences resulted in decreases in T_M values by about 9 °C. Replacement of both O2-carbonyls with methyl groups lowered the T_M by 12.5 °C, consistent with a simple steric effect in the minor groove or changes in hydration effects (Table 1). A reduction in the salt concentration resulted in corresponding moderate decreases in duplex T_M values. Corresponding transitions for the 3-(β-D-2-deoxy-*erythro*-pentafuranosyl)-2-fluoro-6-pyridone (dF^{6}2P)[28]-substituted duplexes were quite different. Very minor changes in T_M values were observed between the native duplex and the monosubstituted duplex (<3 °C at 100 and 200 mM salt). A more significant change in T_M was observed with both analogues present (ΔT_M = 7.7 and 8 °C at the two salt concentrations). These smaller values, relative to the methyl substitutions, may reflect the smaller atomic radius of fluorine resulting in less disruptive steric effects, or its more electronegative character that might permit it to take part in minor groove hydration effects.

T_M values for the duplexes in the presence of DAPI all increased relative to those in the absence of DAPI. We have previously reported[10] the use of JOB plot analyses to confirm that a 1:1 complex is formed between DAPI and this double-stranded 12-mer. For the native duplexes, addition of DAPI under either concentration of salt (100 or 200 mM) increased the T_M value by 8.6 and 10.5 °C, respectively. In the presence of 1 equiv of DAPI ([DNA] = [DAPI] = 3 μM), a variety of stabilization effects were observed. With sequences containing one dm^{6}2P analogue for either central T residue, the presence of DAPI increased the T_M by 4 °C, roughly one-half of that observed of the native sequence. When both methyl analogues were present, barely a 1 °C increase in T_M was observed. Both results suggest that the DAPI binds less effectively to any of the sequences containing minor groove methyl groups, relative to the native sequences. Salt effects are minor with slightly greater T_M values obtained at the lower salt concentration. By comparison, complexes involving the DNA sequences with a single fluorine residue and DAPI are at least as stable as the native sequence−DAPI complex at 200 mM salt, and at lower

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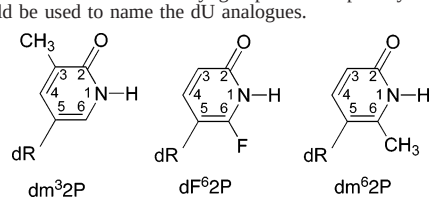
(28) The nucleoside abbreviations for the dU analogues use locants that correspond with those used for the dT analogue dm^{3}2P (far left), even though the absence of the C3 methyl group alters the priority of the locants that should be used to name the dU analogues.



salt concentration are actually slightly more stable ($\Delta T_M = 12.0$ and 13.0 °C, respectively). With two fluorines present at the center of the sequence, a reduction in T_M relative to the native sequence was observed at both salt concentrations, but the fluorine-containing DAPI-duplexes were significantly more stable than the corresponding methyl-containing complexes.

Binding Studies. Equilibrium dissociation constants (K_D values) for DAPI were obtained for all native and modified sequences (Table 1) using the inherent fluorescent properties of the bound DAPI ligand.[10] As with the T_M studies, we examined DAPI binding under two different salt concentrations, 100 and 200 mM. The native sequence exhibited a K_D value of 4.2 and 8.6 nM at those two salt concentrations, respectively. At the higher salt concentration, replacement of either O2-carbonyl by a methyl group results in a roughly 35-fold increase in the K_D value, and when both sites contained methyl groups the K_D value increased some 500-fold. These values correspond to changes in binding energy of 2.1−2.2 kcal/mol for the single site analogues and 3.7 kcal/mol when both methyl groups are present. The results from binding assays at the lower salt concentration were generally similar, although the single-site analogues differed from the native complex by only 1 kcal/mol; with both analogues present the $\Delta\Delta G$ value was 3.7 kcal/mol. These values are generally consistent with those we reported[10] for sequences containing the 5-(*β*-D-2-deoxy-*erythro*-pentafuranosyl)-3-methyl-2-pyridone ($dm^3$2P) analogues in which the O2-carbonyls of the T residues were replaced with hydrogens. The results suggest a similar loss in binding affinity regardless of whether the C2 sites of the thymine(s) contain hydrogens or the sterically larger methyl groups. It also suggests that either type of analogue can be used to probe for stabilizing hydrogen-bonding interactions in the minor groove.

We then examined similar analogue sequences in which the same O2-carbonyls were replaced by one or two fluorines. Surprisingly, with one fluorine atom at either site, binding affinities as measured by K_D values were essentially identical to that of the native sequence at 200 mM salt. At the lower salt concentration, the measured K_D values for the analogue sequences were lower than that of the native sequence suggesting a tighter binding by DAPI when one fluorine atom was present in place of the O2-carbonyl hydrogen-bond acceptor. However, with both sites containing fluorine atoms the K_D values rose moderately by 17-fold (200 mM salt) and 3-fold (100 mM salt). These values reflect DAPI binding that is more similar to that of the native sequence than either the methyl- or the hydrogen-containing analogue sequences. While the double-substitution analogues were not as effective in binding DAPI as the native sequence, they were much more effective than those sequences having hydrogens or methyl groups in place of the native carbonyls.

An estimation for the energetic contribution of the F···H hydrogen bond in the DNA−DAPI complex can be obtained from the difference in the methyl-substituted and fluorine-substituted complexes. At 200 mM salt this value is approximately 2.0 kcal/mol, or 1.0 kcal/mol for each F···H bond of the three-centered interaction. At 100 mM salt this value increases to 2.9 kcal/mol or approximately 1.5 kcal/mol for each fluorine hydrogen bond. More difficult to interpret are the results for complexes that contain one carbonyl acceptor and one fluorine acceptor. The free energy values in these cases are as good or slightly better than that of the native complex. One could interpret those results as implying that hydrogen bonding to fluorine is equivalent to that of a carbonyl, but DAPI is clearly not bound as well to the sequence with two fluorines. More likely is that with one fluorine and one carbonyl present there is some asymmetry in the binding; perhaps the hydrogen bond to the carbonyl in these complexes is optimized in terms of angle and distance. So, in this study we cannot separate the binding contributions for fluorine and carbonyl acceptors in these mixed sequences.

A final set of experiments was performed with the methyl analogues to determine if binding by DAPI could be improved at even lower salt concentrations. At 10 mM salt the K_D for the native sequence was observed to be 0.8 nM; the single site methyl analogues exhibited K_D values of 4.9 and 3.7 nM, and the double site analogue had a K_D value of 140 nM. These values correspond to $\Delta\Delta G$ values of 1.1, 1.0, and 3.1 kcal/mol, respectively. While the complexes all exhibited tighter binding, there are still substantial differences in binding affinities suggesting that loss of the O2-carbonyl hydrogen-bond acceptors and their replacement with hydrophobic hydrogen or methyl groups negatively impacts ligand binding. The simplest interpretation of these results is that the three-centered hydrogen-bonding interaction in the DAPI−DNA complex is worth about 3.7 kcal/mol in binding energy (slightly less at very low salt concentrations). This value is consistent with that reported[10] previously for the hydrogen-containing analogue sequences.

The observations for the methyl-substituted sequences are relatively straightforward to interpret. The methyl groups should fully disrupt the bifurcated hydrogen bond present in the DAPI−DNA complex, and there is a corresponding loss of binding energy that can be quantified by thermodynamic analysis. It is conceivable that more dramatic steric interactions might result, but the measured values seem quite close to those obtained for DNA containing the $dm^3$2P analogue, which replaces each carbonyl with the smaller hydrogen atom. At 200 mM salt a $\Delta\Delta G$ value of about 2 kcal/mol for each "arm" of the bifurcated bond using the methyl-substituted analogue corresponds favorably with similar values observed in our previous study[10] when the target O2 carbonyls were deleted in favor of the sterically smaller hydrogen atom(s). These values are moderated somewhat by the salt concentration, but with both hydrogen-bond acceptors absent there is a 3.7 kcal/mol loss in binding energy regardless of which salt concentration is used.

The results for the fluorine-containing sequences cannot be interpreted in a similar fashion. Replacement of one minor-groove carbonyl by fluorine results in a sequence that binds DAPI as well as the native sequence or better than the native sequence at l00 mM salt. One interpretation is that organofluorines are effective hydrogen-bond acceptors. However, this view is at best controversial. Examination of the Cambridge Structural Database by Howard and co-workers[20] revealed that a hydrogen bond to aliphatic organofluorine derivatives has at best one-half the binding energy of a hydrogen bond to oxygen. Additionally, shorter donor−acceptor bonds were observed[20] for $C(sp^3)$−F acceptors in comparison to $C(sp^2)$−F acceptors, an observation more recently confirmed,[21] suggesting that aliphatic fluorine derivatives are better acceptors than aromatic fluorines. Acidic hydrogens appear to prefer to bind oxygen or nitrogen relative to fluorine.[22] Although fluorine is highly

electronegative, it has poor polarizability evidenced by the fact that polyfluorinated compounds tend to be more hydrophobic in nature than the corresponding hydrogen derivatives. Polarizability is a necessary characteristic to function as an effective hydrogen-bond acceptor. Nonetheless, the introduction of fluorine−hydrogen bonds has been observed to improve protein−ligand interactions.[23] Therefore, the results for the fluorine-substituted sequences are more challenging to interpret.

The observation that replacement of either carbonyl with fluorine has little effect upon K_D values or corresponding ΔG values at 200 mM salt, and at 100 mM salt the binding actually improves with fluorine, suggests a favorable fluorine hydrogen-bonding interaction. This portion of the study suggests that fluorine−hydrogen bonds contribute about the same as carbonyl−hydrogen bonds (∼2 kcal/mol) to complex stability because there is no loss of complex stability in the presence of a single fluorine. On the other hand, replacement of both carbonyls with fluorines results in substantially weaker binding with $\Delta\Delta G$ values varying from 0.4 to 0.8 kcal/mol for each fluorine−hydrogen interaction (depending on salt concentration), values less than those measured for the corresponding carbonyl−hydrogen bond (∼2 kcal/mol), but values that still suggest a moderate energetic contribution by these interactions to complex stability.

A recent analysis[23] of a number of protein structures where fluorine is present has concluded that the strength of the fluorine−hydrogen bond is more dependent upon its directionality with C(sp^3)−F acceptors than with C(sp^2)−F acceptors. The gas-phase energetics of the two different acceptors have been variously determined,[15] and the aliphatic system with the shorter hydrogen bond also exhibits the greater bond energy (e.g., −2.38 kcal/mol vs −1.48 kcal/mol). Two examples of bifurcated bonds of the type N−H····$(F-R)_2$ have also been reported.[15,24,25] In one case, the crystallized compound had H····F distances determined to be 2.15 and 2.18 Å; the N−H····F bond angles were both 122°.[24] In the second case, the H····F distances were measured at 1.935 and 2.184 Å with a bond angles of 107° and 135°.[25]

Fluorine- and methyl-substituted aromatic nucleobases have been described by Kool and colleagues,[26] and both contribute significantly to base stacking effects as measured by the "dangling end"[27] approach. Similar enhanced base stacking effects for the fluorine-containing duplexes could explain the very similar T_M values for the native and fluorine-containing duplexes. By comparison, the presence of the minor groove methyl group in the present sequences destabilizes the duplexes quite significantly, while in the Kool study enhanced base stacking was observed (for 4-methylindole). These differences might reflect the locations of the methyl groups, major groove versus minor groove. In the latter location, steric effects may predominate as the smaller minor groove accommodates the pyridine methyl substitution.

Dipole−dipole or dipole−quadrupole interactions may also contribute to the observed stabilities. We have determined the dipoles of 2-fluoropyridine and 2-pyridone to be 3.145 and 3.919 D, respectively. Dipole−dipole interactions between the pyrrole subunit of DAPI and the target minor-groove functional groups could explain the similarities in binding between the native sequence and the two monofluoro-sequences. In those cases, the pyrrole dipole may be able to orient itself most favorably with the remaining O2-carbonyl such that the observed binding by the ligand is largely unaltered. The dipole−quadrupole interaction may also contribute to complex stability, particularly if the interaction with the O2-carbonyl is favored.

We have also considered the possibility that the fluorine(s) might introduce new hydrophobic interactions to account for enhanced binding effects. However, neither the sequences reported here with methyl groups, nor the sequences reported previously with hydrogens, at these sites appear to provide any added binding effects due to the hydrophobic effect, and the presence of methyl(s) or hydrogen(s) might well contribute in this manner even better than fluorine(s). Comparatively, a sequence used in a previous study[10] containing 3-deazaadenine resulted in a more tightly bound DAPI complex despite the absence of adenine N3-nitrogen hydrogen-bond acceptor. Here, we were compelled to suggest the possibility that the analogue base had unmasked site for the hydrophobic effect that contributed to overall ligand binding affinity. While we cannot completely exclude the possibility of fluorine hydrophobic effects for the present complexes, it is difficult to invoke that explanation in light of the data for related hydrophobic target sites.

Conclusions

We have described the syntheses of DNA sequences with analogue nucleosides, those in which the O2-carbonyls of thymine residues have been replaced with methyl or fluorine. Aside from these functional group modifications, the nucleosides were prepared as C-nucleosides to ensure normal Watson−Crick base pairing. These oligonucleotides complement previous sequences[10] prepared with hydrogen in place of the thymine carbonyl. These atomic level changes in the DNA minor groove can be used to probe the strength of the interactions made to the native minor-groove carbonyls. Disruption of the bifurcated minor groove hydrogen bond leads to a change in T_M values for the duplexes in the presence of DAPI. Measurements of dissociation constants (K_D) have permitted the quantitation of the DAPI−DNA interaction. The latter can be used to obtain a thermodynamic characterization of the interaction. Surprisingly, the presence of fluorine atoms in place of carbonyls results in a stabilizing effect of 0.4−0.8 kcal/mol for each interaction depending on salt concentration. These interactions are likely the sum of both hydrogen-bonding effects and dipole−dipole or dipole−quadrupole interactions.

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Supporting Information Available: Supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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