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A Series of Nonpolar Thymidine Analogues of Increasing Size: DNA Base Pairing and Stacking Properties

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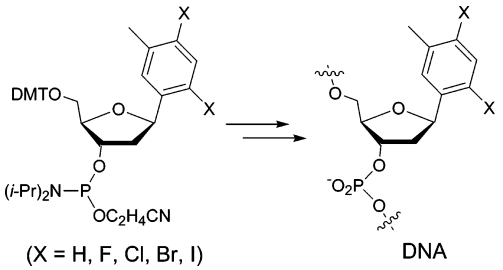

We describe the properties in DNA of a set of five nonpolar nucleoside mimics in which shape is similar but size is increased gradually. The compounds vary in the size of their exocyclic substituents, which range from hydrogen to iodine, and are designed to test the steric effects of nucleosides, nucleotides, and DNA in biological systems in a systematic way. We describe the conversion of toluene, 2,4-difluorotoluene, 2,4-dichlorotoluene, 2,4-dibromotoluene, and 2,4-diiodotoluene deoxyribosides into suitably protected phosphoramidite derivatives and their incorporation into synthetic DNAs. Studies of their behavior in the context of hexamer and dodecamer duplexes were carried out, with comparison to natural thymine. Thermal melting data with compounds in 5′ dangling positions showed that all five compounds stack more strongly than thymine, and all the dihalo-substituted cases stack more strongly than the unsubstituted toluene case. Stacking correlated with surface area and hydrophobicity, both of which increase across the series. In base-pairing studies, all five compounds showed destabilized pairing opposite natural bases (relative to thymine-adenine pairing), as expected. Notably, pairing among the nonpolar base analogues was considerably more stable, and some of the pairs involving the largest analogues showed stability equal to that of a natural thymine−adenine pair. The results establish the base pairing properties of a potentially useful new series of biochemical probes for DNA−protein interactions and also identify a set of new, stable hydrophobic base pairs for designed genetic pairing systems.

Introduction

Nucleoside analogues that lack specific hydrogen-bonding groups have proven useful in a number of biological contexts for probing the physical and chemical importance of such electrostatically charged moieties. For example, Strazewski and Tamm reported over two decades ago the synthesis of pyrimidine analogues lacking one of three hydrogen bonding groups and investigated their substrate abilities with DNA polymerase enzymes.[1] In another example, McLaughlin reported nucleobases with single functional groups deleted and described their properties in pairing in DNA.[2] Taking this approach to its logical limit, we described the strategy of removing *all* Watson−Crick hydrogen-bonding groups in nucleoside analogues, preparing several "nonpolar nucleoside isosteres", which maintain the steric size and shapes of natural nucleobases but lack polar functionality.[3,4] Examples included 4-methylazabenzimidazole, an adenine mimic,[5] and 2,4-difluorotoluene, a thymine mimic.[6]

Nonpolar nucleoside isosteres have proven useful in probing the recognition of DNA by other nucleic acids[3]

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and in studies of the physical origins of DNA curvature.[7] Biophysical studies have shown that thymine and adenine isosteres destabilize DNAs in which they are substituted, unless they are in a terminal position, in which case they can be strongly stabilizing, due to their avid stacking with natural DNA bases.[4,8] Structural studies have shown that, despite the destabilization when present at nonterminal locations, thymine and adenine mimics show essentially the same structures as the natural congeners. For example, difluorotoluene was shown to occupy a Watson−Crick-like position opposite adenine in a 12mer DNA duplex,[9] even though several lines of evidence showed that the fluoroaromatic compound does not measurably form hydrogen bonds in aqueous solution.[4]

Nonpolar nucleoside mimics have also been increasingly useful of late in the study of protein−DNA and enzyme−DNA recognition. Studies have been reported with purine and pyrimidine mimics in a number of DNA repair enzymes, including MutY,[10] fpg,[11] MutS, and homologues,[12] and in polypurine tract recognition by HIV reverse transcriptase.[13] Those studies have shed light on the relative importance of hydrogen bonding and steric interactions to these enzymes' biochemical activities. In addition to this, nonpolar nucleoside isosteres have proven broadly useful in the study of DNA replication by a wide variety of polymerase enzymes. Such nonpolar analogues were first reported in 1997 to act as surprisingly strong substrates for DNA polymerase I,[14,15] leading to the conclusion that at least some replicative DNA polymerases function well in the synthesis of a base pair without Watson−Crick hydrogen bonds. This has since been confirmed by a number of studies of varied polymerase enzymes in vitro[5, 14−16] and recently in living bacterial cells as well.[17] The discovery of the lack of a hydrogen-bonding requirement in replication has led to the design of other nonisosteric DNA base pairs for expansion of the genetic information-encoding system.[18,19]

The results of replication with isosteres have led to the hypothesis that, for many high-fidelity enzymes, steric exclusion effects may be dominant in the selective replication of matched base pairs at the exclusion of mismatched ones.[20] In such a model, high fidelity requires a tight active site to reject sterically mismatched nucleotides and to enforce correct overall Watson−Crick-like geometry in the incipient pair.[21] Interestingly, recent studies of sterically altered deoxyribose have added support to this idea at the level of the DNA backbone.[22]

Since steric effects are widely believed to be crucial to biological selectivity in enzymatic systems, it would be useful to have chemical tools to probe such effects in a systematic way. With this in mind, we conceived a new series of thymine analogues; 2,4-di*H*ydrogentoluene (**H**), di*F*luorotoluene (**F**), 2,4-dich*L*orotoluene (**L**), 2,4-di*B*romotoluene (**B**), and 2,4-di*I*odotoluene (**I**) in which the size is varied systematically by replacing the oxygen nucleobase substituents with hydrogen, fluorine, chlorine, bromine, and iodine (Figure 1). Since the oxygens are the main protruding groups of thymine on its Watson−Crick edge, this replacement has the effect of maintaining an approximate shape of T while gradually increasing size by about one Angstrom across the series. An early report described the synthesis of the five deoxyribosides in this series, and established their sugar ring conformational preferences, which are virtually the same as those of thymidine.[23] For applications in biological recognition studies, it is important to evaluate the behavior of these molecular probes in DNA, in the absence of enzymes. Here we describe the derivatization of these compounds for incorporation into oligonucleotides, the characterization of DNA strands containing them, and the evaluation of their pairing and stacking properties in the double helix.

Experimental Section

Synthesis of Modified Nucleoside Phosphoramidites. The C-glycoside series (**dH**, **dL**, **dB**, **dI**) was prepared as described previously,[23] while one compound (**dF**) is now commercially available as the phosphoramidite derivative. 5′-Tritylation of the other four com-

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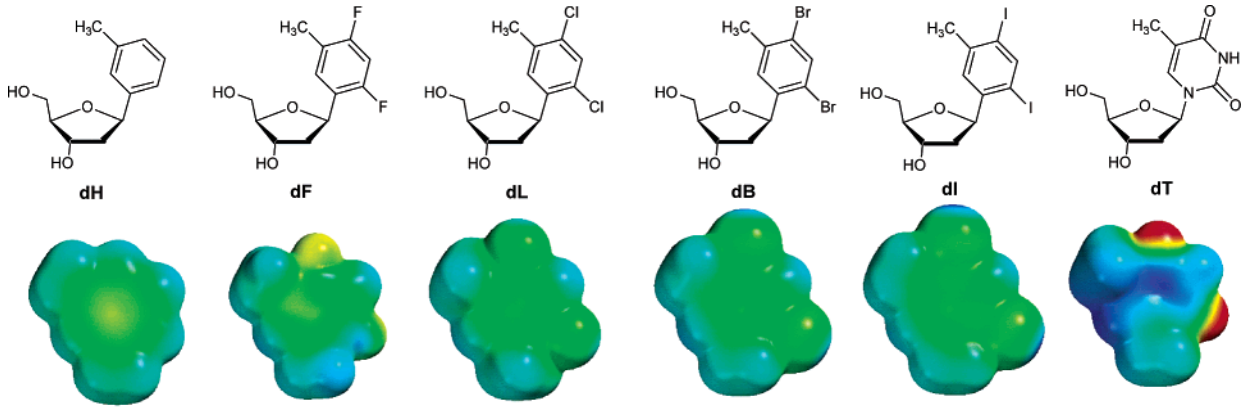
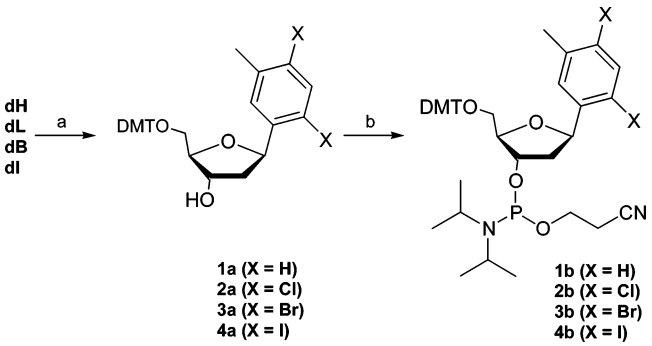


FIGURE 1. Structures of the thymidine nucleoside analogues (top), designed to have gradually increasing steric demand, compared with thymidine at right. Below them are space-filling models of the base analogues with methyl groups at the point of attachment to deoxyribose, with PM3-calculated electrostatic potentials (Spartan ′02, Wavefunction, Inc.) mapped onto the van der Waals surfaces (electrostatic scale: −50 to +30).

SCHEME 1[a]



[a] Conditions: (a) 4,4′-dimethoxytrityl chloride, DIPEA, pyridine; (b) 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, DIPEA, CH_2Cl_2.

pounds was carried out in pyridine in the presence of *N,N*-diisopropylethylamine, giving the corresponding 5′-ethers (**1a**−**4a**) in yields of 45−51%. The 3′-phosphoramidite species were prepared using standard methods, yielding the desired phosphorylated species (**1b**−**4b**) in 50−74% (Scheme 1).

Oligodeoxynucleotide Synthesis. Phosphoramidite derivatives we prepared as described above. The phosphoramidite derivative of analogue **dF** was purchased from Glen Research. DNA oligonucleotides were synthesized on an Applied Biosystems 394 synthesizer using standard β-cyanoethylphosphoramidite chemistry. Self-complementary oligomers with 5′-dangling ends (X) were synthesized in DMT-off mode and purified by reversed-phase HPLC (SB-C18 column, 0 to *n* %, 30 min gradient CH_3CN/ 50 mM triethylammonium acetate (TEAA) (pH 7.5), *n* = 18, 20, 25, 28, 33, 37, 45 for X = none, **T**, **H**, **F**, **L**, **B**, **I**). Oligomers for base pairing were synthesized in DMT-on mode. Sequences containing only natural bases were purified by Poly-Pack II (Glen Research, cat. no. 60-3100-10), and the sequences with a nonnatural base were purified by reversed-phase HPLC (SB-C18 column, 0−50%, 20 min gradient CH_3CN/50 mM TEAA (pH 7.5)). The post-HPLC detritylation and precipitation followed the standard protocol.[24] All oligonucleotides with a nonnatural base were characterized by MALDI mass spectrometry (Supporting Information, Table S4), and their purities were checked by analytical reversed-phase HPLC (Supporting Information, Figure S3).

The oligomers were quantitated by absorbance at 260 nm. Molar extinction coefficients were calculated by the nearest neighbor method.[25] Values for oligonucleotides containing nonnatural residues were obtained in the following way: The molar extinction coefficients for each of the new nucleosides were measured at 260 nm in methanol because of their low water solubility. The molar extinction coefficients for **dH**, **dF**, **dL**, **dB**, and **dI** were found to be 250, 1000, 250, 500, and 3900 M^{-1} cm^{-1}, respectively. The individual extinction coefficients for all the bases in a given oligomer were summed. Since in the most cases the content of nonnatural residues in the sequences is low, this estimation method is unlikely to generate large errors in concentration.

Thermal Denaturation Studies. After the samples were prepared in melting buffer (1 M NaCl, 10 mM phosphate buffer (pH 7.0) with 0.1 mM EDTA), they were heated to 95 °C and allowed to slowly cool at a rate of 1 °C/min to 5 °C. The melting studies were carried out in Teflon-stoppered 1 cm path length quartz cells on a Varian Cary 1 UV−vis spectophotometer equipped with thermoprogrammer. Absorbance was monitored at 280 nm for stacking and at 260 nm for base pairing. In all cases the complexes displayed sharp, apparently two-state transitions. The data were analyzed by the melt curve processing program, MeltWin v. 3.0. Melting temperatures (T_m) were determined by computer-fit of the first derivative of absorbance with respect to 1/*T*. Uncertainty in T_m is estimated at ±0.5 °C based on repetitions of experiments. Free-energy values were derived by two methods: (1) The denaturation data was computer-fitted with an algorithm employing linear sloping baselines, using the two-state approximation for melting. Fits were excellent, with χ^{-2} values of 10^{-6} or better. (2) Van't Hoff thermodynamic parameters were derived from linear plots of $1/T_m$ vs $\ln[C_T]$ by measuring T_m as a function of concentration. Close agreement was seen with

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TABLE 1. Melting Data and Thermodynamic Parameters for Stacking for Thymine and Nonpolar Thymine Analogues, As Measured by Dangling End Thermal Denaturation Studies with Self-Complementary Strands (5′-XCGCGCG)[a]

dangling residue (X)	T_m[b] (°C)	ΔT_m (°C)	$\Delta H°$ (kcal/mol, van't Hoff)	$\Delta S°$ (cal/K·mol, van't Hoff)	$\Delta G°_{37}$ (kcal/mol, van't Hoff)	$\Delta G°_{37}$ (kcal/mol, fits)	$\Delta\Delta G°$ stacking per base (kcal/mol)
none	38.6	–	−41.6 ± 5.0	−109 ± 16	−7.8 ± 0.2	−7.8 ± 0.1	–
T	46.0	7.4	−54.4 ± 4.1	−146 ± 13	−9.1 ± 0.1	−9.1 ± 0.1	0.65
H	49.7	11.1	−55.4 ± 2.0	−147 ± 6	−9.7 ± 0.1	−9.7 ± 0.0	0.95
F	52.4	13.8	−54.5 ± 0.9	−143 ± 3	−10.1 ± 0.0	−10.1 ± 0.0	1.15
L	52.3	13.7	−52.7 ± 1.2	−138 ± 4	−10.0 ± 0.1	−10.1 ± 0.0	1.10
B	54.3	15.7	−62.0 ± 2.1	−165 ± 7	−10.8 ± 0.1	−10.2 ± 0.0	1.50
I	53.8	15.2	−55.7 ± 4.4	−146 ± 14	−10.4 ± 0.2	−10.2 ± 0.1	1.30

[a] Conditions: 1 M NaCl, 10 mM phosphate buffer (pH 7.0) with 0.1 mM EDTA, monitored at 280 nm. [b] 5.0 μM DNA strand concentration for T_m value shown.

the results from curve-fitting, indicating that the two-state approximation is a reasonable one for these two sequences.

Results

Sequence Design. We incorporated all five phosphoramidites into three new sequence contexts each for further study of pairing and stacking properties. The first is a self-complementary sequence designed to form a hexamer duplex, with the nonnatural base analogue overhanging (dangling) at the 5′ ends (Table 1). This same sequence context has been used previously in several studies of DNA base stacking.[8] The second and third contexts comprise a pair of 12mer sequences that form duplexes with well-behaved two-state behavior.[3b] One strand is pyrimidine-rich, while the other is purine-rich; we substituted each analogue at a central position of each strand to test two widely varied sequence contexts for pairing effects.

Base Stacking. DNA base stacking stability has been correlated with the size of compounds when mono-, bi-, tri-, and tetracyclic base analogues were compared.[8] To test this with the present series, which varies in size by a smaller amount, we examined the short self-complementary oligonucleotides by thermal denaturation experiments. The self-complementary duplexes contained the modified nucleosides as a 5′ overhanging base. The data are given in Table 1, and examples of denaturation curves and van't Hoff plots are given in Supporting Information, Figure S1. The results show that all five compounds stabilized the duplex more than natural thymidine does in the same position. The least stabilizing of the series was the smallest, the toluene case (**H**). Interestingly, the remaining four larger compounds (**F**, **L**, **B**, **I**) showed only small differences in their stabilization of this duplex, with a small increase in stabilization with the increase in size overall.

Because stacking propensities of DNA bases and analogues have been most strongly correlated with surface area and hydrophobicity,[8] we calculated these parameters for the base analogues and their deoxyriboside derivatives. The data are shown in Supporting Information, Table S1. Results showed that the surface area of the base analogues increased in a regular way from 154 to 201 Å^2 as the series proceeded from the smallest substituents (**H**) to the largest (**I**). Calculated log P (octanol−water) values were determined for the free deoxyribosides of the series; these also increased monotonically as size increased. The smallest log P value (for **dH**) was 1.25, and the largest (for **dI**) was 3.97. Thus these nucleosides are expected to be strongly hydrophobic compared to thymidine nucleoside (calculated log P = −1.29), which is a relatively polar, water-soluble compound.

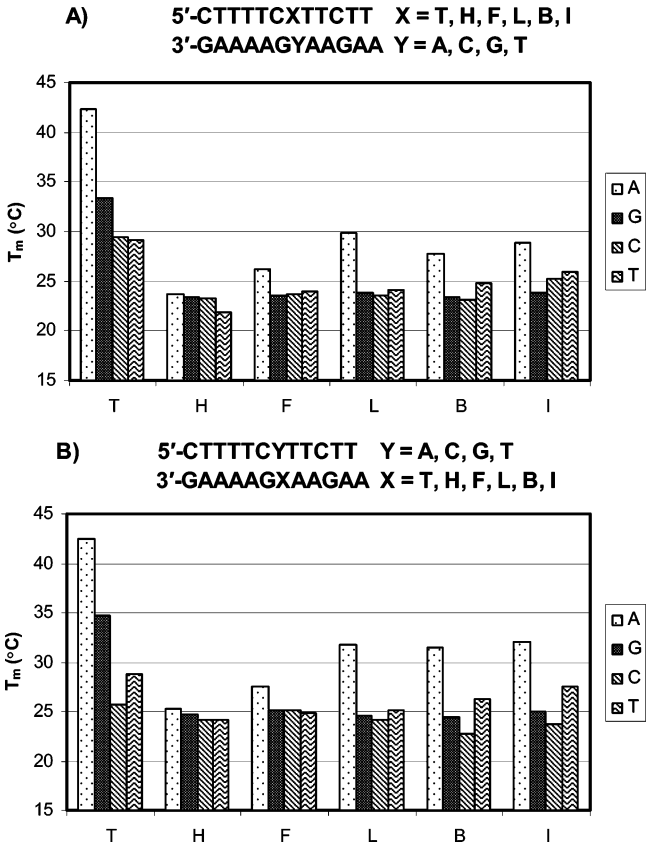

FIGURE 2. Base pairing of nonpolar thymine analogues (**H**, **F**, **L**, **B**, **I**) opposite the natural bases in the center of a 12-base pair duplex, evaluated by thermal melting temperature (T_m). Nonnatural base analogues were substituted in a pyrimidine-rich strand (A) or a purine-rich strand (B).

Base Pairing. Pairing studies of the nonpolar series were then carried out in a 12 base pair duplex; results are summarized in Figure 2 (see also the Supporting Information, Table S2). The data showed that all five compounds are quite destabilizing (compared to **T**) when paired opposite natural bases. Results were the same regardless of whether the analogues were in the pyrimidine- or purine-rich strand (Figure 2A,B). For each analogue, pairing stability was nearly the same regardless of which natural base was opposite it; however, all five compounds showed a small but significant preference

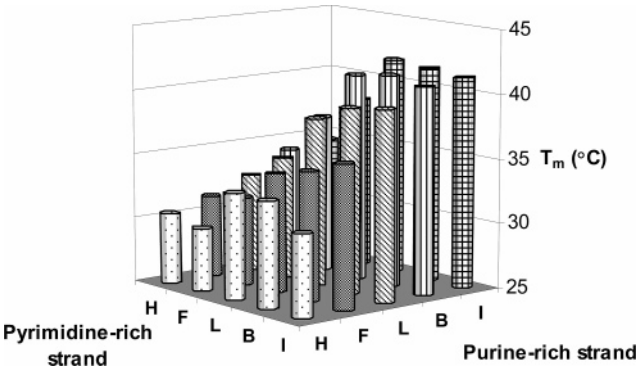

FIGURE 3. Pairing of hydrophobic nucleosides with themselves near the center of a 12-base pair duplex, as measured by thermal melting temperature.

for adenine as partner. The data also showed that, although all analogues were destabilizing opposite the natural bases, there was a general lessening of the destabilization as the analogue increased in size. Of the five nonpolar compounds, the diiodo case was the least destabilizing when paired opposite natural bases.

Finally, the entire set of nonnatural pairs was tested, evaluating various combinations of compounds (**H**, **F**, **L**, **B**, **I**) paired against each other. The data showed a wide range of thermal stabilities for the duplexes containing nonpolar pairs (Figure 3 and Supporting Information, Table S3). Most of the nonnatural pairs were destabilizing relative to a **T**−**A** base pair in the same context; the most destabilizing nonpolar pairs involved the smallest base analogues. The **H**−**H** and **F**−**H** pairs were among these (Figure 3). Notably, however, some pairs, involving **L**, **B**, **I** in the pyrimidine strand and **B**, **I** in the purine strand, were not as destabilizing, and a few (**L**−**I**, **B**−**I**, and **I**−**I** in particular) were equally as stabilizing as a thymine−adenine pair in this context.

A more detailed evaluation of the thermodynamics of duplexes containing the smaller set of self-pairs was carried out by combined use of melt curve fitting and van't Hoff analysis (Table 2 and Supporting Information, Figure S2). The data revealed the **I**−**I** self-pair to be the most stable, and the **H**−**H** pair to be the least stable, with a large difference of 13 °C in T_m and 2.7 kcal/mol between these dodecamer duplexes containing only a single pair difference. Both the T_m and free energy values for the **I**−**I**-containing duplex were identical to the values for the same duplex containing a **T**−**A** pair at that site (Supporting Information, Table S2).

Discussion

Since these nonpolar analogues lack the ability to undergo Watson−Crick hydrogen bonding, their stabilization of DNA (in cases where stabilization does occur) must arise instead from favorable stacking and/or the associated changes in solvation. The current data show that the halogenated compounds increase in their stacking ability by a small amount as size increases, a trend that is generally expected since surface area and hydrophobicity—which are strong predictors of stacking in DNA[8]— increase as well. However, since the size change is relatively small across the series, the difference in stacking is moderate. A similar magnitude of stacking across the series is probably a desirable property for a number of biophysical and mechanistic applications, such as probing protein−DNA interactions and enzyme active sites. This keeps nonsteric properties as equal as possible as the effects of these compounds are compared, allowing for more confident conclusions about steric influences alone.

With natural bases as partners, the members of this series behaved remarkably similarly in pairing ability despite the changes in size. Stability was nearly the same regardless of which natural base was opposite the compounds. It is interesting, however, that all five compounds showed a small but significant preference for adenine as partner. We attribute this to two known properties of adenine: first, it is the strongest stacking of the four natural bases.[8−a,26] Probably for the same reason, adenine is known to be the most stabilizing of the natural bases when the partner lacks a base altogether (an abasic site).[27] The second property is the relatively weak solvation of adenine compared to the other three bases.[28] If a nonpolar base is placed opposite adenine, partially desolvating it, this would have less energetic cost than if any other base were desolvated. Looking at the trend in the series, there was a slightly more pronounced preference for adenine exhibited by the largest members of the series. One possible explanation for this is the greater hydrophobicity of the larger analogues, which might lead to a stronger preference for the least well solvated natural base.

The earliest studies of nonpolar-nonpolar base analogue pairs reported that nonpolar−nonpolar pairs were less destabilizing than pairs of nonpolar bases opposite the polar natural bases,[3b] and this same effect is observed here as well. This selective pairing of nonpolar compounds can be attributed to avoidance of desolvation costs when pairing opposite polar compounds, as well as to increased surface area of hydrophobic contact. Notably, some of the pairs in this series are not destabilizing to DNA, and a few of the largest pairs, such as **B**−**I** and **I**−**I**, were as stabilizing as a natural base pair. Thus, these last pairs can be added to a small but growing list of nonpolar aromatic DNA base replacements that are stable and selective in DNA, but which are also orthogonal, pairing poorly with natural bases.[18,19,29] Such properties may one day find use in designed genetic pairing systems.

Experimental Section

General Procedure for Preparation of 5′-*O*-Tritylated *β*-C-Nucleosides. 1′,2′-Dideoxy-*β*-1′-(3-methylphenyl)-D-ribofuranose (**1a**) (0.5 mmol) was coevaporated with dry pyridine twice and dissolved in pyridine (3 mL). To the solution were

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TABLE 2. Stabilities of X−X Self-Pairs of Nonpolar Thymidine Analogues (H, F, L, B, I), As Measured by Melting Temperature (T_m) and Free Energy ($\Delta G°$) for the Duplexes[a]

5′-CTTTTC**X**TTCTT 3′-GAAAAG**X**AAGAA	T_m[b] (°C)	$\Delta H°$ (kcal/mol, van't Hoff)	$\Delta S°$ (cal/K·mol, van't Hoff)	$\Delta G°_{37}$ (kcal/mol, van't Hoff)	$-\Delta G°_{37}$ (kcal/mol, fits)
H·H	29.4	−54.3 ± 4.6	−152 ± 15	−7.1 ± 0.1	−7.0 ± 0.1
F·F	31.9	−65.4 ± 7.8	−187 ± 26	−7.2 ± 0.2	−7.3 ± 0.1
L·L	38.0	−73.3 ± 11.2	−208 ± 36	−8.5 ± 0.3	−8.5 ± 0.1
B·B	40.8	−73.0 ± 3.5	−205 ± 11	−9.3 ± 0.1	−9.3 ± 0.1
I·I	42.1	−69.0 ± 3.5	−192 ± 6	−9.5 ± 0.0	−9.7 ± 0.1

[a] Conditions: 1 M NaCl, 10 mM phosphate buffer (pH 7.0) with 0.1 mM EDTA, monitored at 260 nm. [b] 2.5 μM concentration for each DNA strand for T_m value. For reference, T_m = 42.4 °C for X_1 = **T**, X_2 = **A** and T_m = 42.4 °C for X_1 = **A**, X_2 = **T**.

added diisopropylethylamine (0.13 mL, 1.5 equiv) and 4,4′-dimethoxytrityl (DMT) chloride (170 mg, 1.0 equiv). The mixture was stirred at room temperature for 2 h, and then an additional portion of DMTCl (42 mg, 0.25 equiv) was added to the solution. After 1 h, the reaction mixture was quenched with methanol (5 mL) and volatiles were removed in vacuo. The residue was loaded onto a silica gel column (preequilibrated with 5% triethylamine in hexane) and eluted (30:10:1 hexane/ethyl acetate/triethylamine).

1′,2′-Dideoxy-β-1′-(3-methylphenyl)-5′-*O*-(4,4′-dimethoxytrityl)-D-ribofuranose (1a): ^{1}H NMR ($CDCl_3$, ppm) 7.47 (d, 2H, J = 7.2 Hz), 7.36 (dd, 4H, J = 8.8, 2.4 Hz), 7.29−7.15 (m, 6H), 7.08 (d, 1H, J = 7.2 Hz), 6.82 (d, 4H, J = 8.8 Hz), 5.14 (dd, 1H, J = 10, 5.6 Hz), 4.42 (br, 1H), 4.07−4.04 (m, 1H), 3.78 (s, 6H), 3.37−3.34 (m, 1H), 3.29−3.25 (m, 1H), 2.30 (s, 3H), 2.23 (ddd, 1H, J = 13, 5.6, 2.0 Hz), 2.10−2.03 (m, 1H); ^{13}C NMR ($CDCl_3$, ppm) 158.6, 145.1, 141.96, 138.2, 136.3, 130.3, 128.4, 128.1, 127.0, 126.9, 123.4, 113.3, 86.5, 86.4, 80.3, 74.9, 64.7, 55.4, 44.1, 21.7; HRMS (FAB+, NBA matrix) calcd mass 533.2303 for [$C_{33}H_{34}O_5$ + Na], found 533.2288.

General Procedure for Preparation of 3′-*O*-Phosphoramidites. 1′,2′-Dideoxy-β-1′-(3-methylphenyl)-5′-*O*-(4,4′-dimethoxytrityl)-D-ribofuranose (0.4 mmol) was dissolved in dry dichloromethane (5 mL), and to this were added diisopropylethylamine (0.28 mL, 4 equiv) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.13 mL, 1.5 equiv). The reaction mixture was stirred at room temperature for 3 h, and then all volatiles were removed in vacuo. The residue was loaded onto a silica gel column (preequilibrated with 5% triethylamine in hexane) and eluted (60:20:1 hexane/ethyl acetate/triethylamine).

1′,2′-Dideoxy-β-1′-(3-methylphenyl)-5′-*O*-(4,4′-dimethoxytrityl)-D-ribofuranose cyanoethyl *N*,*N*-diisopropylphosphoramidite (1b): ^{1}H NMR ($CDCl_3$, ppm) 7.50−7.47 (m, 2H), 7.39−7.35 (m, 4H), 7.28−7.18 (m, 6H), 7.09−7.07 (m, 1H), 6.82−6.79 (m, 4H), 5.13 (app quintet, 1H, J = 6.0 Hz), 4.53−4.50 (m, 1H), 4.23 (br, 1H), 3.85−3.66 (m, 2H), 3.78 (app d, 6H, J = 3.0 Hz, OCH_3), 3.63−3.58 (m, 2H), 3.35−3.29 (m, 1H), 3.26−3.22 (m, 1H), 2.61 (app t, 1H, J = 6.5 Hz), 2.45 (app t + d, 1H, J = 7.0 (for triplet), 2.0 (for doublet) Hz), 2.41−2.31 (m, 1H), 2.31 (s, 3H, $ArCH_3$), 2.08−2.01 (m, 1H), 1.20−1.15 (m, 8H), 1.08 (app d, 4H, J = 6.5 Hz); ^{13}C NMR ($CDCl_3$, ppm) 158.7, 145.2, 141.9, 138.2 (d), 136.4 (m), 130.4 (d), 128.6 (d), 128.0, 127.0 (t), 123.5, 117.8 (d), 113.3, 86.3 (d), 85.9 (d),80.6 (d), 76.5 (d), 76.2 (d), 64.5 (d), 58.6 (d), 55.5 (d), 43.5 (m), 31.9, 24.8 (m), 22.9, 21.7, 20.6 (m), 14.4; ^{31}P NMR ($CDCl_3$, ppm, external H_3PO_4 standard) 148.2, 148.5; HRMS (FAB+, NBA matrix) calcd mass 733.3382 for [$C_{42}H_{51}N_2O_6P$ + Na], found 733.3378.

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Supporting Information Available: Thermodynamic data (melting curves, van't Hoff plots, T_m data) and calculated physical properties. Characterization of compounds **2a**,**b**, **3a**,**b** and **4a**,**b** and ^{1}H NMR spectra of 5′-*O*-tritylated β-C-nucleosides and 3′-*O*-phosphoramidites. HPLC chromatograms and MALDI MS of unnatural oligonucleotides. This material is available free of charge via the Internet at http://pubs.acs.org.

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