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Fluorous Base-Pairing Effects in a DNA Polymerase Active Site

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Abstract: We describe selective "fluorous" effects in the active site of a DNA polymerase, by using nucleotide analogues whose pairing edges are perfluorinated. The 5′-triphosphate deoxynucleotide derivatives of DNA base analogues 2,3,4,5-tetrafluorobenzene (^{F}**B**) and 4,5,6,7-tetrafluoroindole (^{F}**I**), as well as hydrocarbon controls benzene (**B**) and indole (**I**), were synthesized and studied as substrates for the DNA Polymerase I Klenow fragment (KF exo−). Modified nucleotides were present in the DNA template or were supplied as nucleoside triphosphates in studies of the steady-state kinetics of single nucleotide insertion. When supplied opposite the non-natural bases in the template strand, the hydrophobic nucleoside triphosphates were incorporated by up to two orders of magnitude more efficiently than the natural deoxynucleoside triphosphates. The purine-like fluorinated indole nucleotide (^{F}**I**) was the most efficiently inserted of the four hydrophobic analogues, with the most effective incorporation occurring opposite the pyrimidine-like tetrafluorobenzene (^{F}**B**). In all cases, the polyfluorinated base pairs were more efficiently processed than the analogous hydrocarbon pairs. A preliminary test of polymerase extension beyond these pairs showed that only the ^{F}**B** base is appreciably extended; the inefficient extension is consistent with recently published data regarding other nonpolar base pairs. These results suggest the importance of hydrophobicity, stacking, and steric interactions in the polymerase-mediated replication of DNA base pairs that lack hydrogen bonds. These findings further suggest that the enhanced hydrophobicity of polyfluoroaromatic bases could be employed in the design of new, selective base pairs that are orthogonal to the natural Watson–Crick pairs used in replication.

Keywords: DNA replication · fluorous · hydrophobic effect · Klenow fragment · stacking interactions

Introduction

Several studies with modified DNAs have established that it is possible to design nonpolar nucleobase analogues that exhibit selective and stable pairing in the absence of hydrogen bonding. Original designs focused on nonpolar shape mimics of natural bases, thereby conserving base pair size.[1] These hydrophobic compounds exhibited selective pairing with one another rather than with natural nucleobases, presumably because of the energetic cost of desolvating the polar partners. Later experiments showed that larger aromatic species can exhibit even stronger base stacking,[2] leading to pairs that are as stable as, or more stable than, natural base pairs.[3,4] Subsequently, a wide variety of non-hydrogen-bonding base pairs have been examined as substrates for DNA replication by polymerases, and some of these pairs were found to be efficiently processed by some enzymes.[5] The potential of this research is two-fold. Firstly, some of the nonpolar analogues can be useful as biophysical probes of nucleic acid structures and enzymatic mechanisms.[6] Secondly, non-natural base pairs that act orthogonally to the natural pairs might be useful as biotechnological tools,[7] and could expand the natural genetic alphabet.[8]

If the solvophobic effect influences the assembly of DNA base pairs, then it may be possible to improve the performance and selectivity of assembly by enhancing the hydrophobicity to a greater degree than is possible with hydrocarbons. Perfluorinated ("fluorous") hydrocarbons are considerably more hydrophobic than standard hydrocarbons.[9] This effect has been used extensively for separations in synthetic chemistry,[10] and recently, in the stabilization of biological macromolecules. For example, the selective pairing of peptides containing perfluorinated side-chains has been demonstrated,[11] and perfluorinated amino acid side-chains have been shown to stabilize the hydrophobic-driven folding of proteins.[12] Therefore, we[13] and others[14,15] are now investi-

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gating whether related effects might be observed in the context of nucleic acids.

Recent work has described pentafluorobenzene as a nucleobase analogue. This was initially conceived as a useful perfluorinated derivative for studying hydrophobic and stacking effects in nucleic acids.[14,15] However, the bis-*ortho*-difluoro substitution causes destabilization, and a tetrafluorobenzene variant was shown to have more favorable properties.[16]

More recent studies have shown that hydrophobic base analogues, in which the entire Watson–Crick edges are fluorinated, can selectively pair with one another in duplex DNA (Figure 1a).[13] For example, results of thermal denaturation studies of synthetic DNAs revealed that a tetrafluoroindole analogue could exhibit selective pairing opposite other highly fluorinated bases in preference to hydrocarbon species. Results of solvent partitioning studies confirmed that deoxyribosides with polyfluorinated base moieties were considerably more hydrophobic than the deoxyribosides of analogous hydrocarbon aromatic compounds.[13] Thus, the pairing selectivity was ascribed to the favorable solvation effect of burying the highly hydrophobic polyfluorinated surfaces within the helical core. However, these effects were not tested with enzymes that replicate DNA.

Although compounds containing one or two fluorines have been studied by using DNA polymerases,[5,17] there are no reports of base analogues in which the entire pairing edges were completely fluorinated. Here, we describe the first attempts to apply such "fluorous" nucleotide analogues to DNA replication, by using DNA Polymerase I Klenow fragment (KF exo−). Our aim was to investigate whether the unusually high hydrophobicity of the analogues would exert any selectivity effects in the enzyme's active site, or whether the large departure from natural nucleotide properties would inhibit their ability to act as substrates. Thus, we investigated the efficiency of polymerase processing for combinations of the polyfluorinated nucleotides, their standard hydrocarbon analogues, and the four natural bases. The data show evidence for a selective "fluorous" effect in DNA replication.

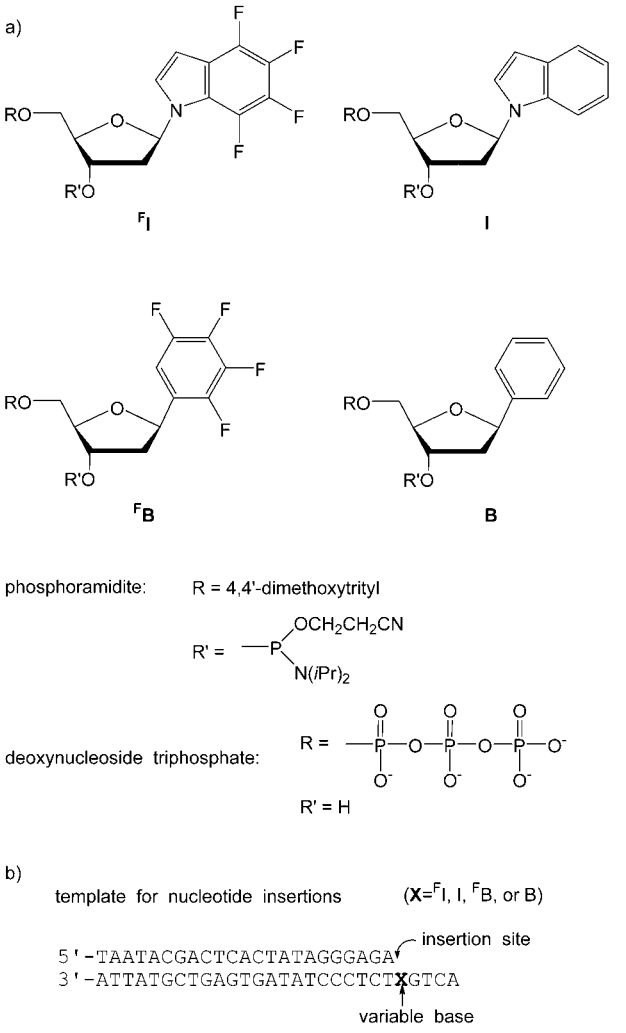

Figure 1. a) Chemical structures of nucleosides ^{F}**I**, **I**, ^{F}**B**, and **B**. b) Sequences of template–primer duplexes used in the steady-state studies.

Results

We measured the ability of DNA Polymerase I Klenow fragment (KF exo−) to process base pairs involving all possible combinations of hydrocarbon and fluorocarbon deoxyglycosides paired opposite each other, and opposite natural nucleobases. Structures of the modified nucleotide analogues and the substrate DNAs are given in Figure 1. A 23 nucleotide (nt) primer bound to a 28 nt template was used as the substrate duplex. Qualitative data showing products at early time points of single reactions are shown in Figure 2, and quantitative kinetics data are listed in Tables 1–3.

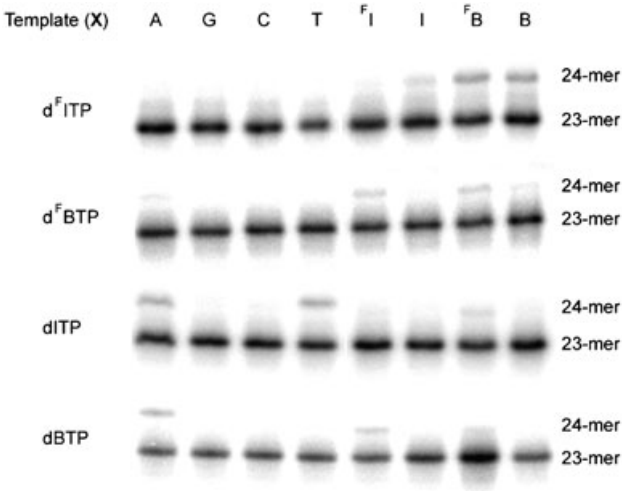

Figure 2. Autoradiograms showing single-nucleotide insertions by the KF (exo^{-}) enzyme, including all possible cases of hydrophobic bases in the template and as a dNTP. The data represent reactions with 25 μM dNTP, and the reactions were stopped after the following durations: d ^{F}**I**TP, 2 min; d ^{F}**B**TP, 5 min; d**I**TP, 20 min; d**B**TP, 20 min. The template–primer sequences are shown in Figure 1b.

Table 1. Steady-state kinetic parameters for the incorporation of single hydrophobic nucleotides into a template–primer duplex containing variable base **X** by the KF (exo$^-$) polymerase.[a]

	Template	K_m		V_{max}		Efficiency
d**N**TP	**X**	[μM]	error	[% min^{-1}]	error	[V_{max}/K_m]
^{F}**I**	^{F}**I**	16.0	6.7	2.77	0.29	1.7×10^{5}
I	^{F}**I**	44.0	3.6	2.22	0.09	5.1×10^{4}
^{F}**B**	^{F}**I**	23.5	7.9	1.64	1.06	7.0×10^{4}
B	^{F}**I**	72.9	7.4	5.85	0.06	8.0×10^{4}
^{F}**I**	**I**	12.5	1.8	5.73	0.58	4.6×10^{5}
I	**I**	61.5	19.2	1.02	0.05	1.7×10^{4}
^{F}**B**	**I**	6.1	5.4	0.14	0.06	2.3×10^{4}
B	**I**	84.1	0.0	0.66	0.00	7.8×10^{3}
^{F}**I**	^{F}**B**	45.8	4.0	52.74	5.20	1.2×10^{6}
I	^{F}**B**	99.2	9.3	3.00	0.08	3.0×10^{4}
^{F}**B**	^{F}**B**	64.3	9.9	3.58	0.89	5.6×10^{4}
B	^{F}**B**	59.3	4.9	2.66	0.05	4.5×10^{4}
^{F}**I**	**B**	38.1	19.9	25.56	1.52	6.7×10^{5}
I	**B**	103.9	5.3	1.70	0.02	1.6×10^{4}
^{F}**B**	**B**	24.8	8.6	0.92	0.12	3.7×10^{4}
B	**B**	52.4	8.7	0.44	0.02	8.3×10^{3}

[a] Conditions: 5 μM template–primer duplex (Figure 1b), 0.025 u μL^{-1} enzyme, 50 mM Tris·HCl (pH 7.0), 10 mM $MgCl_2$, 1 mM DTT, and 0.1 mg mL^{-1} BSA, incubated for 1–20 min at 37 °C in a reaction volume of 10 μL.

Table 2. Steady-state kinetic parameters for the incorporation of single hydrophobic nucleotides into a template–primer duplex containing variable base **X** by the KF (exo$^-$) polymerase.[a]

	Template	K_m		V_{max}		Efficiency
d**N**TP	**X**	[μM]	error	[% min^{-1}]	error	[V_{max}/K_m]
^{F}**I**	**A**	22.5	4.7	1.52	0.32	6.7×10^{4}
I	**A**	71.1	9.3	0.65	0.03	9.1×10^{3}
^{F}**B**	**A**	27.6	25.1	0.10	0.03	3.5×10^{3}
B	**A**	61.5	13.2	0.80	0.03	1.3×10^{4}
^{F}**I**	**G**	11.0	5.9	0.53	0.06	4.9×10^{4}
I	**G**	22.7	14.2	0.11	0.02	4.9×10^{3}
^{F}**B**	**G**	24.1	19.0	0.07	0.03	3.0×10^{3}
B	**G**	21.7	12.5	0.10	0.01	4.5×10^{3}
^{F}**I**	**C**	56.9	32.0	0.97	0.14	1.7×10^{4}
I	**C**	20.0	0.0	0.10	0.00	5.2×10^{3}
^{F}**B**	**C**	3.7	3.1	0.07	0.03	1.8×10^{4}
B	**C**	11.9	0.0	0.26	0.00	2.2×10^{4}
^{F}**I**	**T**	14.6	8.8	2.90	0.72	2.0×10^{5}
I	**T**	66.1	13.3	0.18	0.03	2.7×10^{3}
^{F}**B**	**T**	5.7	3.1	0.07	0.04	1.3×10^{4}
B	**T**	6.1	0.0	0.26	0.00	4.3×10^{4}

[a] Conditions: see footnotes to Table 1.

Table 3. Steady-state kinetic parameters for the incorporation of natural nucleotides into a template–primer duplex containing variable unnatural base **X** by the KF (exo$^-$) polymerase.[a] Data for a natural pair (first entry) are given as a positive control.

	Template	K_m		V_{max}		Efficiency
d**N**TP	**X**	[μM]	error	[% min^{-1}]	error	[V_{max}/K_m]
A	**T**	3.4	1.4	36.0	4.0	1.1×10^{7}
A	^{F}**I**	47.3	47.7	0.66	0.36	1.4×10^{4}
G	^{F}**I**	158.9	80.6	0.18	0.04	1.1×10^{3}
C	^{F}**I**	125.2	53.8	0.25	0.08	2.0×10^{3}
T	^{F}**I**	8.1	1.5	1.12	0.08	1.4×10^{5}
A	**I**	36.5	3.2	1.18	0.02	3.2×10^{4}
G	**I**	27.5	15.1	0.51	0.06	1.8×10^{4}
C	**I**	39.1	7.8	0.78	0.06	2.0×10^{4}
T	**I**	89.5	10.4	0.75	0.06	8.4×10^{3}
A	^{F}**B**	32.6	0.8	3.42	0.03	1.0×10^{5}
G	^{F}**B**	70.6	22.1	0.50	0.25	7.0×10^{3}
C	^{F}**B**	41.1	6.0	4.02	0.24	9.8×10^{4}
T	^{F}**B**	21.3	17.0	0.93	0.24	4.4×10^{4}
A	**B**	25.7	1.9	3.41	0.08	1.3×10^{5}
G	**B**	77.8	54.2	0.30	0.13	3.9×10^{3}
C	**B**	85.5	14.4	0.73	0.06	8.5×10^{3}
T	**B**	26.2	2.8	0.70	0.04	2.7×10^{4}

[a] Conditions: see footnotes to Table 1.

The data revealed a relatively broad range of efficiencies for the 16 nonpolar–nonpolar pairs, which do not differ greatly in terms of structure (Table 1). Efficiencies were measured as V_{max}/K_m from the steady-state kinetics experiments. The least efficient pairing was the insertion of benzene (**B**) opposite indole (**I**), denoted here as **B**→**I**. This insertion was approximately 150-fold less efficient than the most efficient pairing, which involved the pairing of two highly fluorinated base analogues, ^{F}**I**→^{F}**B** (Figure 2 and Table 1). On the whole, the efficiency of the polyfluorinated base pairings was higher than that reported for non-natural pairs. The ^{F}**I**→^{F}**B** pairing approached the efficiency of natural base pairs; for example, insertion of A opposite T by this polymerase occurs with a V_{max}/K_m only 9-fold higher than for ^{F}**I**→^{F}**B** (see Table 3, first entry).

In general, the polyfluorinated bases showed greater polymerase activity than the analogous hydrocarbons. As triphosphate derivatives, the fluorinated nucleotides were more active than their nonfluorinated versions by factors of 1.2 to 42 (measured as ratios of V_{max}/K_m). The one exception of the eight comparisons involved d^{F}**B**TP, which was inserted opposite ^{F}**I** in the template almost as efficiently as d**B**TP. Conversely, the fluorinated bases acted as generally better templates. Insertions of hydrophobic dNTPs across fluorinated bases showed 3–10-fold greater efficiency for indole template variants (except one) and 1.5–5.4-fold greater efficiency for phenyl template variants. Again, there was only one exception out of eight, namely, the insertion of d^{F}**I**TP opposite indole species.

The examination of data for hydrocarbon–hydrocarbon pairing compared to fluorocarbon–fluorocarbon pairing revealed significant selectivity for "fluorous" base pairs. For example, a comparison of **B**→**B** to ^{F}**B**→^{F}**B** showed a sevenfold advantage for the fluorous pair. For the insertion of indole analogues opposite indole, the fluorous advantage was tenfold. For the pairings of benzene opposite indole, the advantage was ninefold; however, for the converse case—insertion of indole opposite benzene—the advantage was considerably larger: 75-fold.

To test whether there was a preference for the insertion of hydrophobic nucleotides opposite hydrophobic partners, we measured efficiencies for the insertion of the non-natural nucleotides opposite the natural DNA bases (Table 2). In general, the fluorinated compounds did show this orthogonal behavior; the ^{F}**B** dNTP analogue was more efficiently inserted opposite ^{F}**I**, **I**, ^{F}**B**, or **B** than opposite any of the four

natural bases, and ^{F}**I** nucleoside triphosphate was inserted more efficiently opposite **I**, ^{F}**B**, or **B** than opposite A, C, G, or T. The indole nucleotide showed similar orthogonality, but benzene dNTP did not demonstrate this hydrophobic selectivity, being inserted approximately equally well opposite all eight bases used in the study.

The converse experiments were also performed (see data in Table 3), testing insertion of natural nucleoside triphosphates opposite hydrophobic bases in the template. Natural dNTPs were inserted opposite the hydrophobic bases with variable efficiency (Table 3), and on average, were more poorly inserted opposite hydrophobic template bases than were the nonpolar dNTPs, demonstrating the orthogonal properties of the non-natural analogues. For a given nonpolar template base, the insertion efficiencies for the natural dNTPs were lower than those for the most effective hydrophobic dNTPs by factors of 1.2–14. For **I**, ^{F}**B**, and **B** templates, the orthogonalities were generally large, at five orders of magnitude or greater. One exception, the ^{F}**I** template, showed little or no nonpolar–nonpolar selectivity: natural dTTP was inserted opposite ^{F}**I** with an efficiency only ~ 20% lower than that for ^{F}**I** triphosphate.

Although a number of nonpolar nucleotide analogues have been shown to incorporate with high efficiency into a primer–template terminus, most nonpolar nucleotide analogues display low efficiency (see Discussion). Thus, we carried out an initial survey of the ability of KF exo− to elongate primers after the nonpolar nucleotides were successfully incorporated. This was done by first adding the non-natural nucleotide and incubating under conditions shown previously to allow for full incorporation. The set of four natural nucleoside triphosphates was then added and the results evaluated by performing gel electrophoresis to check for further elongation past the initial single elongation. A sample set of the gel data is shown in Figure 3. As a whole, these four nucleotides at the primer terminus were observed to be poorly extended by KF. A control assay incorporating a mixture of all four natural bases showed observable extension to the end of the template, even with the inefficient incorporation of natural nucleotides opposite hydrophobic bases in the template. However, nearly all of the hydrophobic–hydrophobic pairs showed little or no significant extension. One exception was the ^{F}**B**→^{F}**I** fluorous pair, in which the ^{F}**B** primer terminus appeared to be extended to a small extent (Figure 3).

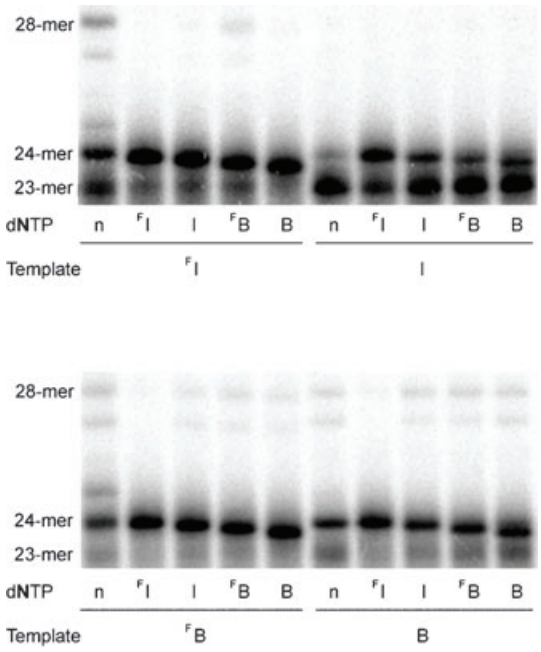

Figure 3. Autoradiograms showing primer elongations up to and beyond the 24th template base. Reactions were performed with 125 μM of each hydrophobic dNTP (or no hydrophobic dNTP, lanes labeled n) and 25 μM of a mixture of all four natural triphosphates (dATP, dGTP, dCTP, dTTP).

Discussion

The results suggest that polyfluorination can generally increase polymerase activity of nonpolar nucleotide derivatives. We hypothesize that this is best explained by the increased hydrophobicity contributed by the fluorinated surfaces. For the hydrophobic template bases described here, insertion efficiency of a hydrophobic dNTP correlates with measured hydrophobicity from partitioning experiments;[13] the order from most to least hydrophobic is ^{F}**I** > ^{F}**B** > **I** > **B**. Significantly, this is also the order of measured stacking affinities of the four base analogues against an adjacent adenine. It has been shown previously that stacking of aromatic species with neighboring DNA bases correlates strongly with hydrophobicity.[18] In general, this notion is consistent with the closing of the polymerase around the incipient pair to successfully process it,[19] and this should lead to the entropically favorable desolvation of these nonpolar surfaces. The observed selectivity of polyfluorinated bases for other polyfluorinated bases is consistent with hydrophobic effects, in which the most hydrophobic surfaces are expected to be buried together more favorably than surfaces of lower hydrophobicity. Similarly, the positioning of hydrophobic surfaces against polar functionality is expected to be energetically unfavorable, due to the high cost of desolvating the polar surfaces. This explains why the insertion of nonpolar dNTPs opposite polar natural bases, and vice versa, is unfavorable.

Interestingly, we observed that the selectivity of polyfluorinated bases for other polyfluorinated bases was greatest for the insertion of the fluorinated indole opposite the fluorinated benzene (75-fold advantage of fluorination), compared to the converse case of fluorinated benzene opposite fluorinated indole (9-fold). We hypothesize that this difference arises, at least in part, from the exceptionally strong stacking of tetrafluoroindole (in its dNTP form) on the primer–template terminus. In previous dangling-end experiments,[13] tetrafluoroindole stacked with an affinity of 3.1 kcal mol^{-1}, considerably higher than fluorobenzene (2.1 kcal mol^{-1}). Without fluorine, indole stacks considerably

less well (1.9 kcal mol^{-1}). This may partly explain the strong preference for the incorporation of the fluorinated nucleotide over the nonfluorinated one.

Although steric effects in polymerase active sites can sometimes lead to dramatic selectivity, even in the absence of Watson–Crick hydrogen bonds,[6b] in the present hydrophobic–hydrophobic pairs it appears that the steric selectivity is relatively low. Most of the pairs are probably small enough to fit within the steric footprint of a Watson–Crick pair, thus presenting no strong steric clashes as the enzyme closes around the incipient pairs. For example, both benzene and fluorinated benzene should be accommodated opposite one another without strong steric exclusion. The main exception in this study could be the examples of indole–indole pairing. Models suggest that the indole–indole pairs are too large to fit in the *anti–anti* pairing mode. We suggest that these pairs are probably processed in one of two alternative geometries: either they orient *syn–anti*, or the incoming indole nucleotide stacks partially on top of the template indole in an intercalated mode. Because the indole nucleotides stack strongly, this favorable energy can compensate for the cost of the less favorable geometry.

One of the general aims of base pair design has been the development of pairing that is orthogonal to the natural bases and pairs.[8] Some of the nonpolar fluorous pairs presented here show many of the features of useful orthogonal enzymatic replication, the most promising of which may be the ^{F}**I**–^{F}**B** pair. The processing of this pair is among the most efficient of the current group. Most importantly, the hydrophobic–hydrophobic selectivity is high, with natural dNTPs being inserted opposite natural bases 1000-fold more efficiently than opposite the hydrophobic ^{F}**I** or ^{F}**B**. The converse situation, with d^{F}**I**TP or d^{F}**B**TP as the incoming nucleotide, also shows hydrophobic selectivity: the efficiency of insertion of d^{F}**I**TP is greatest opposite ^{F}**B**, and an order of magnitude less if inserted opposite natural bases. Similarly, d^{F}**B**TP is inserted opposite ^{F}**I** at least four times more efficiently than opposite natural bases. Thus, the pair operates with good orthogonality with respect to the natural nucleotides and natural templates bases.

As for the selectivity for each other, the ^{F}**B** template displays a selectivity for the incorporation of d^{F}**I**TP that is more than one order of magnitude higher than that for d^{F}**B**TP. However, in the converse case (the ^{F}**I** template), this selectivity does not apply; d^{F}**I**TP is inserted with somewhat greater efficiency than d^{F}**B**TP. This complicating factor for this base pair would not be problematic for single-stranded replication, but would cause interference in double-stranded replications, such as those mediated by PCR.

In DNA alone in the absence of enzymes, ^{F}**I** and ^{F}**B** have been shown to pair with each other quite selectively over natural bases. Thus, this self-pair joins other hydrophobic compounds as orthogonal pairs that increase the number of functioning base pairs for DNA.[8d,17] The widespread application of this orthogonal pair is, however, limited by its low polymerase extension efficiency, which is also a problem for other hydrophobic pairs.[5c,8d,17,20]

Finally, it is interesting to compare the present results involving the polymerase replication of highly fluorinated nucleobase analogues to those reported earlier for other fluorinated base analogues. The earliest example was 2,4-difluorotoluene (**F**), which is less highly fluorinated and was designed as a nearly perfect isostere of thymine.[1a] The structure of the present tetrafluorobenzene base analogue (^{F}**B**) appears to differ only subtly from that of **F**, with fluorine atoms replacing the smaller H-3 hydrogen atom and the C-5 methyl group of **F**. Notably, in the polymerase active site, ^{F}**B** behaves very differently to difluorotoluene. For example, the insertion of dFTP opposite A is at least two orders of magnitude more efficient than the insertion of d^{F}**B**TP.[5b] In addition, the insertion of d^{F}**B**TP is fairly nonselective, whereas that of dFTP is highly selective, showing a selectivity for insertion opposite A that is 3–5 orders of magnitude greater than for insertion opposite T, C, or G.[5b] One possible explanation for these marked differences is the somewhat larger steric size and different shape of ^{F}**B**, which presents a fluorine rather than hydrogen at the 3′-position of the base. This might exclude ^{F}**B** from being paired opposite to A or G. In addition, the notably high hydrophobicity of ^{F}**B** might also inhibit its pairing opposite the polar T and C template bases, which require to be desolvated to be paired. Another interesting comparison for ^{F}**B** is a previously reported 3-fluorobenzene analogue.[17] As with ^{F}**B**, this compound is incorporated better opposite itself than opposite natural bases. However, its self-pair appears to be more efficiently processed than the ^{F}**B**→^{F}**B** pair. This may due to the former compound adopting a conformation that places at least one of the fluorines away from the center of the pair,[17] thereby providing more steric room for inclusion of both fluoroaromatics.

Future experiments will focus on larger polyfluorinated compounds, with the aim of further enhancing pairing stability. Hopefully, this will improve the understanding of selective interactions involved in base pairing, base stacking, and replication by DNA polymerases.

Experimental Section

Synthesis of 5′-triphosphate derivatives: The tetrafluoroindole, tetrafluorobenzene, indole, and phenyl deoxyribosides were prepared as described previously.[13,16] The 5′-triphosphate derivatives were synthesized according to literature procedures.[21] Briefly: the free nucleoside (0.20 mmol) was dissolved in trimethylphosphate (1.0 mL), and the solution was cooled to 0 °C. Proton Sponge (64 mg 1,8-bis(dimethylamino) naphthalene, (Aldrich)) and phosphorous oxychloride (21 μL) were added, and the solution was stirred for 3 h at 0 °C. Tributylamine (0.30 mL) and tributylammonium pyrophosphate (173 mg) were added, and the solution was stirred for 1–5 min before adding 1 M triethylammonium bicarbonate (6 mL, pH 8.0) to quench the reaction. After stirring for 20 min at room temperature, the reaction mixture was concentrated to a volume of 1–2 mL by means of lyophilization. The triphosphate was purified by subjecting it to anion exchange HPLC using a Waters Protein-Pak DEAE-8HR column with a 0.1–1.0 M gradient of triethylammonium bicarbonate (pH 8.0). The appropriate fractions were then concentrated and further purified by performing reverse-phase HPLC with a C18 column and a buffer solution of 5–25% acetonitrile in 50 mM triethylacetic acid

(pH 7.0). The concentrations were determined by using extinction coefficients for the free nucleosides.[13,16] Compounds were characterized by conducting proton, fluorine, and phosphorous NMR spectroscopy and by MALDI-TOF mass spectrometric analysis (see Supporting Information).

Oligodeoxynucleotide synthesis: Primer and template DNA oligonucleotides (Figure 1b) were synthesized by using an Applied Biosystems 392 synthesizer and standard β-cyanoethylphosphoramidite chemistry. Sequences containing unnatural nucleotide derivatives **B**, **I**, ^{F}**B**, and ^{F}**I** were prepared from cyanoethylphosphoramidite derivatives, as described previously.[13,16] Oligonucleotides were purified and quantified as described.[13]

Single nucleotide insertion reactions: The final concentrations used for single nucleotide insertions were 5 μM primer/template, 25 u μL^{-1} Klenow fragment (exo−, Amersham), and 25 μM triphosphate (dNTP). Primer 5′-termini were labeled by using [γ-^{32}P]ATP and T4 polynucleotide kinase. Labeled primer (20–25 nM) and unlabeled primer (20 μM) were annealed to the template (20 μM) in an "annealing buffer" (100 mM Tris·HCl (pH 7.0), 20 mM $MgCl_2$, 2 mM DTT, and 0.1 mg mL^{-1} BSA) by heating to 95 °C for 3 min and cooling to room temperature over 1 h. Solution A was produced by adding KF (0.1 u mL^{-1}), diluted to a 1:1 solution in annealing buffer, to the annealed duplex DNA, followed by incubation at 37 °C for 2 min. Solution B contained dNTP (50 μM) in a buffer of Tris·HCl (200 mM pH 7.0), $MgCl_2$ (20 mM), and mercaptoethanol (6 mM). Polymerase reactions were started by mixing equal volumes of solution A containing the DNA–enzyme complex and solution B containing dNTP substrates. The reaction mixture was incubated at 37 °C and terminated by adding 1.5 volumes of stop buffer (95% formamide, 20 mM EDTA, 0.025% xylene cyanol, and 0.025% bromophenol blue). The reactions were incubated for different times depending on the base used, and the extent of the reaction was determined by running the quenched reaction samples on a 20% denaturing polyacrylamide gel. The percentage of incorporation was determined by performing scanning phosphorimagery and quantization by using ImageQuant (Amersham).

Steady-state kinetics: Steady-state kinetics for single nucleotide insertions were performed as described above. The final concentrations for insertion were 5 μM primer/template, 0.025 u mL^{-1} Klenow fragment (exo−), and various concentrations of dNTP from 5–500 μM. Reaction times were adjusted so that the extent of the reaction within 1 h was 1–20%. The reaction extents were determined by running quenched reaction samples on 20% denaturing polyacrylamide gels to separate unreacted primer from insertion products; relative velocities were calculated as the extent of the reaction divided by the reaction time.

Bypass reactions: Processivity reactions for the hydrophobic nucleotides were performed. Final concentrations for insertion were 5 μM primer/template, 0.1 u mL^{-1} Klenow fragment (exo−), 125 μM of hydrophobic dNTPs, and 25 μM of natural dNTPs. A single, unnatural dNTP was allowed to insert for 60 min before a mixture of natural dNTPs was added and allowed to react for 18 min. Products obtained before and after the addition of natural dNTP were analyzed by using 5′-^{32}P-end-labeled primers and autoradiography.

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