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Minor Groove Hydrogen Bonds and the Replication of Unnatural Base Pairs

Shigeo Matsuda, Aaron M. Leconte, and Floyd E. Romesberg*

Contribution from the Department of Chemistry, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, California 92037

Received November 19, 2006; E-mail: floyd@scripps.edu

Abstract: As part of an effort to expand the genetic alphabet, we examined the synthesis of DNA with six different unnatural nucleotides bearing methoxy-derivatized nucleobase analogues. Different nucleobase substitution patterns were used to systematically alter the nucleobase electronics, sterics, and hydrogen-bonding potential. We determined the ability of the Klenow fragment of *E. coli* DNA polymerase I to synthesize and extend the different unnatural base pairs and mispairs under steady-state conditions. Unlike other hydrogen-bond acceptors examined in the past, the methoxy groups do not facilitate mispairing, implying that they are not recognized by any of the hydrogen-bond donors of the natural nucleobases; however, they do facilitate replication. The more efficient replication results largely from an increase in the rate of extension of primers terminating at the unnatural base pair and, interestingly, requires that the methoxy group be at the *ortho* position where it is positioned in the developing minor groove and can form a functionally important hydrogen bond with the polymerase. Thus, *ortho* methoxy groups should be generally useful for the effort to expand the genetic alphabet.

1. Introduction

An unnatural base pair that is stable and replicable would increase the biotechnological utility and information storage potential of DNA.[1−5] Toward this goal, we have examined unnatural nucleotides that bear predominantly hydrophobic nucleobase analogues.[6−14] These analogues are expected to pair with each other within duplex DNA via hydrophobic and packing interactions but not with the natural nucleotides, due to forced desolvation of the more hydrophilic natural nucleobases. Our initial efforts focused on nucleobase analogues with large aromatic surface areas and, along with other studies from the Hirao,[4,5,15] Kuchta,[16,17] Kool,[18,19] and Berdis[20,21] labs, revealed that hydrophobic and packing forces were well suited to mediate base pair stability and polymerase-mediated synthesis (by insertion of the unnatural triphosphate opposite the unnatural nucleotide in the template).[10−12] However, the utility of these unnatural base pairs has been consistently limited by insertion of the next correct dNTP (i.e., extension).

More recently, a significant improvement in extension rate has been achieved with several nucleobase analogues that have relatively little aromatic surface area.[6,9,22,23,25] Presumably, these pairs form a more natural-like primer terminus, as opposed to larger analogues that are likely to distort the primer terminus. While the **BEN** self pair (formed between two identical **BEN** analogues, Figure 1a) is not particularly stable[24] or well recognized by DNA polymerases,[9] several derivatives have been identified that form self pairs or heteropairs (formed between two different analogues) with significantly improved properties. For example, the **3FB**,[6] **DM5**,[9,24] and **TM**[9,11,12,24] nucleotides (Figure 1a) form pairs that are reasonably stable and efficiently synthesized by the exonuclease deficient Klenow fragment of

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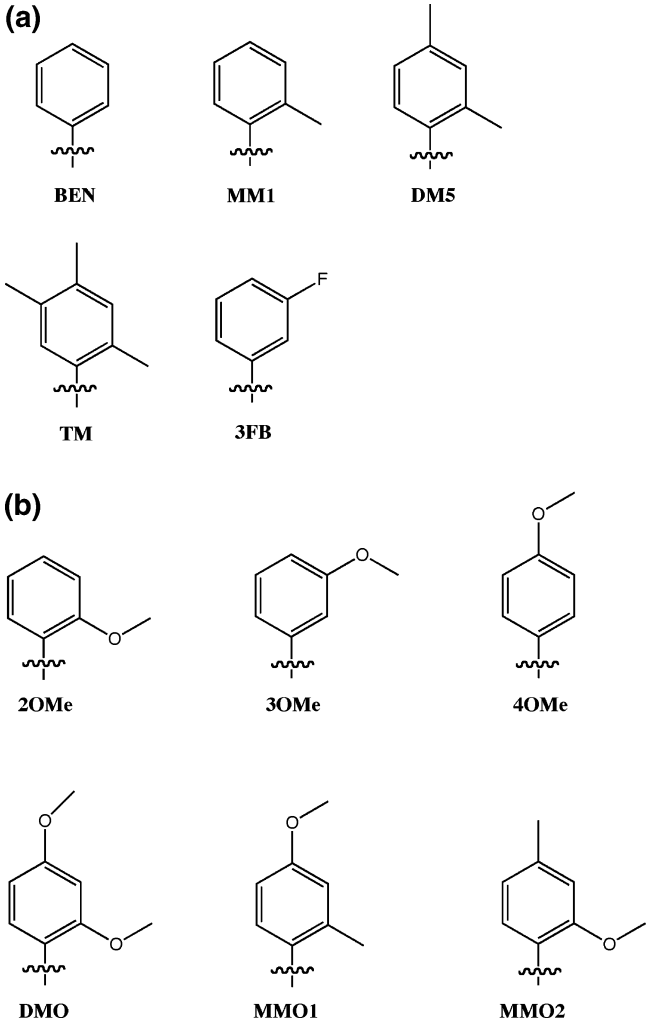

Figure 1. (a) Unnatural nucleobases previously reported. (b) Methoxy substituted benzene analogues used in this study.

E. coli DNA polymerase I (Kf) but that are also extended with increased efficiency. Nonetheless, the extension of these pairs remains significantly less efficient than that of a natural base pair, and modifications that further increase extension rates are still required.

One possible reason for the poor extension of the predominantly hydrophobic base pairs is that, while optimized interbase packing may obviate the need for interbase hydrogen bonds (H-bonds),[26] efficient extension may require H-bonding between the nucleobase at the primer terminus and the polymerase.[19,27,28] Indeed, the natural nucleobases all have an H-bond acceptor oriented into the developing minor groove, and structural studies have revealed a conserved H-bond between these acceptors at the primer terminus and polymerase based H-bond donors,[29] such as Arg668 in Kf. Biochemical studies have shown that extension is significantly reduced when these H-bonds are disrupted.[27,30] Thus, we have explored the derivatization of the phenyl-based nucleosides with either minor groove carbonyl groups[23] (by changing the *C*-nucleoside to an *N*-nucleoside) or minor groove nitrogen substituents that may act as H-bond acceptors.[22] However, while these modifications increase extension rates, they also destabilize the base pair and appear to be recognized by the H-bond donor of dG, resulting in reduced thermal and replication fidelity.

To identify H-bond acceptors that may not be recognized by the natural nucleobases but that could mediate important primer−polymerase interactions, we have begun to characterize phenyl-based nucleotides derivatized with methoxy groups. Based on both theory[31,32] and experiment,[31−34] aryl methoxy groups should be capable of forming H-bonds, although strong H-bonds will only be possible when the methyl group is rotated out of the plane of the aryl ring, localizing electron density at the oxygen and making it a better H-bond acceptor. Indeed, thermodynamic studies of the same derivatives suggest that the methoxy groups are prone to desolvate upon duplex formation (S.M. and F.R. unpublished results). Thus, it is unclear whether these methoxy groups would engage the polymerase H-bond donors and align the primer terminus for efficient extension.

We now report a detailed characterization of the Kf-mediated synthesis and extension of the methoxy derivatized base pairs. The analogues characterized were designed to systematically explore the effects of methoxy groups at the *ortho*, *meta*, and/or *para*-positions (Figure 1b). Generally, we find that the addition of methoxy groups increases the selectivity of unnatural base pair synthesis, and when the groups are present at the *ortho* position, they also have a selective and significant effect on extension, with some pairs being extended only ∼100-fold less efficiently than a natural base pair. Using the R668A mutant of Kf, we show that this efficient extension requires the presence of the polymerase-based H-bond donor. The data suggest that an appropriately positioned methoxy group can productively engage the H-bond donor of the DNA polymerase, which facilitates extension, but not the H-bond donors of any natural nucleobase, which would stabilize or facilitate mispair formation.

2. Results

2.1. Unnatural Base Pair Synthesis Efficiency. The unnatural nucleotides were synthesized and converted into the corresponding phosphoramidites or triphosphates as described in the Supporting Information. The phosphoramidites were used to synthesize template DNA containing the unnatural nucleotides at a single defined position. To begin to examine how methoxy groups impact polymerase-mediated replication, we determined the steady-state rates with which Kf extends a primer terminating immediately 5′ to the unnatural base in the template by insertion of an unnatural triphosphate (Table 1). For reference, Kf inserts a natural dATP opposite dT with a second-order rate constant (i.e., efficiency or k_{cat}/K_M) of 1.7×10^8 M^{-1} min^{-1}. To compare the ability of nucleobases with either an *ortho* methyl or an *ortho* methoxy group to direct triphosphate insertion, we first examined the insertion of the unnatural triphosphates opposite either **MM1** (Table 1, **X** = **MM1**) or **2OMe** (Table 1, **X** =

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Table 1. Rates of Unnatural Base Pair Synthesis[a]

5′-d(TAATACGACTCACTATAGGGAGA)
3′-d(ATTATGCTGAGTGATATCCCTCT**X**GCTAGGTTACGGCAGGATCGC

X	triphosphate	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} min^{-1})
2OMe	**2OMe**	0.69 ± 0.16	104 ± 32	6.6 × 10^3
	DMO	3.31 ± 0.73	85 ± 4	3.9 × 10^4
	MMO2	3.47 ± 0.57	72 ± 2	4.8 × 10^4
	MM1	1.73 ± 0.16	117 ± 16	1.5 × 10^4
	DM5	14.0 ± 0.60	103 ± 11	1.4 × 10^5
	TM	18.2 ± 1.3	26 ± 4	7.0 × 10^5
MMO1	**2OMe**	2.06 ± 0.51	109 ± 8	1.9 × 10^4
	DMO	6.16 ± 0.57	42 ± 10	1.5 × 10^5
	MMO2	4.34 ± 1.1	39 ± 6	1.1 × 10^5
	MM1	2.89 ± 0.67	78 ± 4	3.7 × 10^4
	DM5	11.5 ± 1.30	62 ± 17	1.9 × 10^5
	TM	7.18 ± 0.81	15.4 ± 1.5	4.7 × 10^5
MMO2	**2OMe**	1.50 ± 0.35	120 ± 20	1.3 × 10^4
	DMO	5.14 ± 1.37	51 ± 5	1.0 × 10^5
	MMO2	5.13 ± 0.67	44 ± 4	1.2 × 10^5
	MM1	4.38 ± 0.36	109 ± 17	4.0 × 10^4
	DM5	10.5 ± 2.8	48 ± 15	2.2 × 10^5
	TM	22.0 ± 1.7	13.8 ± 3.2	1.6 × 10^6
DMO	**2OMe**	0.76 ± 0.20	104 ± 29	7.3 × 10^3
	DMO	1.85 ± 0.17	26 ± 3	7.1 × 10^4
	MMO2	1.85 ± 0.38	38 ± 2	4.9 × 10^4
	MM1	1.68 ± 0.29	46 ± 10	3.7 × 10^4
	DM5	3.30 ± 0.29	28 ± 9	1.2 × 10^5
	TM	4.17 ± 1.04	9.3 ± 0.3	4.5 × 10^5
MM1	**2OMe**	1.12 ± 0.28	128 ± 26	8.8 × 10^3
	DMO	4.05 ± 0.42	52 ± 13	7.8 × 10^4
	MMO2	5.24 ± 1.22	39 ± 2	1.3 × 10^5
	MM1	0.93 ± 0.31	108 ± 11	8.6 × 10^3
	DM5	7.11 ± 1.67	82 ± 7	8.7 × 10^4
	TM	5.50 ± 0.91	15.7 ± 2.6	3.5 × 10^5
DM5	**2OMe**	2.54 ± 0.31	89 ± 4	2.9 × 10^4
	DMO	7.05 ± 0.45	52 ± 6	1.4 × 10^5
	MMO2	9.63 ± 0.81	28 ± 3	3.4 × 10^5
	MM1	2.28 ± 0.34	102 ± 12	2.2 × 10^4
	DM5	50 ± 4.6	25 ± 6	2.0 × 10^6
	TM	10.8 ± 1.5	12.6 ± 2.2	8.6 × 10^5
TM	**2OMe**	4.24 ± 0.76	39 ± 5	1.1 × 10^5
	DMO	7.63 ± 1.30	24 ± 6	3.2 × 10^5
	MMO2	9.47 ± 1.68	11.7 ± 1.8	8.1 × 10^5
	MM1	3.19 ± 0.09	55 ± 18	5.8 × 10^4
	DM5	18.1 ± 1.0	29 ± 6	6.2 × 10^5
	TM	31 ± 1.8	14 ± 3	2.2 × 10^6

[a] See Experimental Section for details.

2OMe). **MM1** in the template directs unnatural base pair synthesis with second-order rate constants (i.e., efficiency, k_{cat}/K_M) between 8.6 × 10^3 and 3.5 × 10^5 M^{-1} min^{-1}. Interestingly, methoxy substitution at the *ortho* position slightly decreases the rate of insertion of the more hydrophilic triphosphates but increases the rate of insertion of the more hydrophobic triphosphates. The most efficiently synthesized pair results from the insertion of d**TM**TP opposite **2OMe**, which proceeds with a second-order rate constant of 7.0 × 10^5 M^{-1} min^{-1}. Because methoxy substitution increases the polarity of the nucleobase, the data suggest that, in addition to hydrophobic forces, specific structural and/or electrostatic interactions must contribute to base pair synthesis.

We next examined the effect of substituting methoxy groups for methyl groups within the **DM5** scaffold in the template. This scaffold was chosen because of the relative stability and efficient replication of the **DM5** self pair. The **DM5** analogue

Table 2. Incorporation of Unnatural and Natural Triphosphates Opposite Unnatural Bases in the Template[a]

5′-d(TAATACGACTCACTATAGGGAGA)
3′-d(ATTATGCTGAGTGATATCCCTCT**X**GCTAGGTTACGGCAGGATCGC)

X	triphosphate	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} min^{-1})
2OMe	A	3.78 ± 0.17	44 ± 11	8.6 × 10^4
	C	nd[b]	nd[b]	<1.0 × 10^3
	G	nd[b]	nd[b]	<1.0 × 10^3
	T	nd[b]	nd[b]	<1.0 × 10^3
3OMe	A	1.77 ± 0.22	32 ± 10	5.5 × 10^4
	C	0.29 ± 0.04	168 ± 28	1.7 × 10^3
	G	nd[b]	nd[b]	<1.0 × 10^3
	T	0.33 ± 0.08	130 ± 12	2.5 × 10^3
4OMe	A	2.96 ± 0.25	25 ± 7	1.2 × 10^5
	C	nd[b]	nd[b]	<1.0 × 10^3
	G	nd[b]	nd[b]	<1.0 × 10^3
	T	3.03 ± 0.23	95 ± 18	3.2 × 10^4
MMO1	A	4.50 ± 0.40	25 ± 8	1.8 × 10^5
	C	nd[b]	nd[b]	<1.0 × 10^3
	G	nd[b]	nd[b]	<1.0 × 10^3
	T	0.28 ± 0.02	128 ± 6	2.2 × 10^3
MMO2	A	3.27 ± 0.33	32 ± 4	1.0 × 10^5
	C	nd[b]	nd[b]	<1.0 × 10^3
	G	nd[b]	nd[b]	<1.0 × 10^3
	T	nd[b]	nd[b]	<1.0 × 10^3
DMO	A	1.08 ± 0.15	13 ± 1.3	8.3 × 10^4
	C	nd[b]	nd[b]	<1.0 × 10^3
	G	nd[b]	nd[b]	<1.0 × 10^3
	T	0.14 ± 0.03	57 ± 15	2.5 × 10^3

[a] See Experimental Section for details. [b]Reaction was too inefficient for k_{cat} and K_M to be determined independently.

directs the insertion of the unnatural triphosphates with second-order rate constants between 2.2 × 10^4 and 2.0 × 10^6 M^{-1} min^{-1} (Table 1, **X = DM5**). Methoxy substitution of the template base (i.e., Table 1, **X = MMO1** or **MMO2**) generally decreases the rates of unnatural triphosphate insertion, most so when the templating analogue has two methoxy substituents (i.e., Table 1, **X = DMO**). With d**MMO2**TP the effects are slightly larger, and they are largest with d**DM5**TP. However, the opposite effect was observed with both d**MM1**TP and d**TM**TP, where changing the *ortho* substituent of the template analogue from a methyl to a methoxy group increases the rate of unnatural triphosphate insertion. These data reinforce the conclusion that specific structural and/or electrostatic effects contribute to efficient unnatural base pair synthesis.

2.2. Unnatural Base Pair Synthesis Fidelity. To examine the synthesis of mispairs we determined the efficiencies with which Kf inserts natural dNTPs opposite the unnatural bases in the template (Table 2). dATP is consistently the most efficiently inserted natural triphosphate, with k_{cat}/K_M values falling between 5.5 × 10^4 M^{-1} min^{-1} and 1.8 × 10^5 M^{-1} min^{-1}. While this is more efficient than mispair synthesis among the natural nucleotides,[35] it is significantly less efficient than dATP insertion opposite any of the carbocyclic analogue **MM1**, **MM2**, **MM3**, or **DM5** (which template dATP insertion with second-order rate constants of 3.9 × 10^5 to 2.9 × 10^6 M^{-1} min^{-1}).[9] After dATP, the next most efficiently inserted triphosphate is dTTP, with rates ranging from too slow to detect (<10^3 M^{-1}min^{-1}) to 2 ×

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Table 3. Rates of Correct Extension of Unnatural Base Pairs[a]

5′-d(TAATACGACTCACTATAGGGAGA**Y**)
3′-d(ATTATGCTGAGTGATATCCCTCT**X**GCTAGGTTACGGCAGGATCGC)

X	Y	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} min^{-1})	X	Y	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} min^{-1})
2OMe	**2OMe**	nd[b]	nd[b]	$<1.0 \times 10^3$	**DMO**	**2OMe**	nd[b]	nd[b]	$<1.0 \times 10^3$
	3OMe	0.27 ± 0.02	173 ± 11	1.6×10^3		**3OMe**	0.35 ± 0.08	152 ± 62	2.3×10^3
	4OMe	0.96 ± 0.24	209 ± 61	4.6×10^3		**4OMe**	0.37 ± 0.04	163 ± 42	2.3×10^3
	DMO	0.30 ± 0.10	178 ± 77	1.7×10^3		**DMO**	nd[b]	nd[b]	$<1.0 \times 10^3$
	MMO1	0.21 ± 0.03	165 ± 51	1.3×10^3		**MMO1**	0.17 ± 0.04	143 ± 33	1.2×10^3
	MMO2	0.38 ± 0.14	170 ± 39	2.2×10^3		**MMO2**	0.47 ± 0.11	158 ± 22	3.0×10^3
	MM1	nd[b]	nd[b]	$<1.0 \times 10^3$		**MM1**	nd[b]	nd[b]	$<1.0 \times 10^3$
	DM5	0.47 ± 0.15	231 ± 48	2.0×10^3		**DM5**	0.35 ± 0.03	124 ± 13	2.8×10^3
	TM	0.40 ± 0.09	173 ± 40	2.3×10^3		**TM**	0.34 ± 0.02	99 ± 14	3.4×10^3
3OMe	**2OMe**	3.12 ± 0.82	112 ± 29	2.8×10^4	**MM1**	**2OMe**	9.34 ± 1.48	124 ± 14	7.5×10^4
	3OMe	nd[b]	nd[b]	$<1.0 \times 10^3$		**3OMe**	0.40 ± 0.02	153 ± 13	2.6×10^3
	4OMe	0.79 ± 0.15	218 ± 53	3.6×10^3		**4OMe**	0.92 ± 0.05	153 ± 25	6.0×10^3
	DMO	3.14 ± 0.31	132 ± 21	2.4×10^4		**DMO**	5.90 ± 1.19	86 ± 3	6.9×10^4
	MMO1	0.22 ± 0.08	175 ± 74	1.3×10^3		**MMO1**	0.25 ± 0.06	92 ± 10	2.7×10^3
	MMO2	4.95 ± 1.39	160 ± 53	3.1×10^4		**MMO2**	7.18 ± 0.39	50 ± 10	1.4×10^5
	MM1	nd[b]	nd[b]	$<1.0 \times 10^3$		**MM1**	0.25 ± 0.07	173 ± 100	1.4×10^3
	DM5	0.32 ± 0.07	138 ± 43	2.3×10^3		**DM5**	0.35 ± 0.08	163 ± 17	2.1×10^3
	TM	0.18 ± 0.05	144 ± 25	1.3×10^3		**TM**	0.54 ± 0.07	168 ± 46	3.2×10^3
4OMe	**2OMe**	2.49 ± 0.83	162 ± 11	1.5×10^4	**DM5**	**2OMe**	7.30 ± 0.54	57 ± 13	1.3×10^5
	3OMe	nd[b]	nd[b]	$<1.0 \times 10^3$		**3OMe**	1.23 ± 0.33	121 ± 30	1.0×10^4
	4OMe	nd[b]	nd[b]	$<1.0 \times 10^3$		**4OMe**	1.29 ± 0.36	104 ± 35	1.2×10^4
	DMO	3.08 ± 0.73	147 ± 41	2.1×10^4		**DMO**	5.44 ± 0.49	54 ± 5	1.0×10^5
	MMO1	nd[b]	nd[b]	$<1.0 \times 10^3$		**MMO1**	0.48 ± 0.13	121 ± 10	4.0×10^3
	MMO2	6.04 ± 0.55	127 ± 30	4.8×10^4		**MMO2**	6.07 ± 0.85	18.6 ± 2.5	3.3×10^5
	MM1	nd[b]	nd[b]	$<1.0 \times 10^3$		**MM1**	0.23 ± 0.09	220 ± 91	1.1×10^3
	DM5	nd[b]	nd[b]	$<1.0 \times 10^3$		**DM5**	6.5 ± 1.1	161 ± 17	4.0×10^4
	TM	nd[b]	nd[b]	$<1.0 \times 10^3$		**TM**	1.04 ± 0.18	95 ± 10	1.1×10^4
MMO1	**2OMe**	9.19 ± 1.24	86 ± 3	1.1×10^5	**TM**	**2OMe**	12.0 ± 2.7	12.4 ± 1.5	9.7×10^5
	3OMe	0.68 ± 0.12	195 ± 74	3.5×10^3		**3OMe**	3.79 ± 0.09	92 ± 17	4.1×10^4
	4OMe	0.64 ± 0.14	172 ± 39	3.7×10^3		**4OMe**	3.37 ± 0.30	89 ± 19	3.8×10^4
	DMO	6.52 ± 0.77	60 ± 18	1.1×10^5		**DMO**	4.13 ± 0.60	13.1 ± 2.9	3.2×10^5
	MMO1	0.27 ± 0.04	125 ± 35	2.2×10^3		**MMO1**	1.51 ± 0.27	85 ± 24	1.8×10^4
	MMO2	6.88 ± 1.24	46 ± 11	1.5×10^5		**MMO2**	10.1 ± 1.4	7.9 ± 0.97	1.3×10^6
	MM1	0.17 ± 0.07	168 ± 43	1.0×10^3		**MM1**	0.62 ± 0.11	108 ± 3	5.7×10^3
	DM5	0.56 ± 0.14	132 ± 16	4.2×10^3		**DM5**	2.74 ± 0.54	92 ± 18	3.0×10^4
	TM	0.39 ± 0.04	144 ± 34	2.7×10^3		**TM**	7.9 ± 1.4	152 ± 32	5.2×10^4
MMO2	**2OMe**	0.44 ± 0.11	201 ± 51	2.2×10^3	**BEN**	**2OMe**	1.49 ± 0.35	272 ± 28	5.5×10^3
	3OMe	1.07 ± 0.14	243 ± 37	4.4×10^3		**3OMe**	nd[b]	nd[b]	$<1.0 \times 10^3$
	4OMe	1.83 ± 0.66	184 ± 34	9.9×10^3		**4OMe**	nd[b]	nd[b]	$<1.0 \times 10^3$
	DMO	0.55 ± 0.12	147 ± 24	3.7×10^3		**DMO**	2.42 ± 0.41	191 ± 44	1.3×10^4
	MMO1	0.50 ± 0.13	247 ± 15	2.0×10^3		**MMO1**	nd[b]	nd[b]	$<1.0 \times 10^3$
	MMO2	0.87 ± 0.28	165 ± 47	5.3×10^3		**MMO2**	4.05 ± 0.65	233 ± 21	1.7×10^4
	MM1	0.36 ± 0.20	184 ± 95	2.0×10^3		**MM1**	nd[b]	nd[b]	$<1.0 \times 10^3$
	DM5	1.48 ± 0.08	148 ± 24	1.0×10^4		**DM5**	nd[b]	nd[b]	$<1.0 \times 10^3$
	TM	0.98 ± 0.23	155 ± 33	6.3×10^3		**TM**	nd[b]	nd[b]	$<1.0 \times 10^3$

[a] See Experimental Section for details. [b] Reaction was too inefficient for k_{cat} and K_M to be determined independently.

10^3 $M^{-1}min^{-1}$, for insertion opposite **3OMe**, **MMO1**, and **DMO**, or 3.2×10^4 M^{-1} min^{-1}, for insertion opposite **4OMe**. Thus, dTTP insertion is strongly favored by *para* methoxy substitution and disfavored by *ortho* methoxy substitution. dCTP is only inserted with a detectable rate opposite **3OMe**, and dGTP is not detectably inserted opposite any of the analogues. Of particular interest is the selectivity against mispairing of the analogues, especially those with *ortho* methoxy H-bond acceptors (**2OMe**, **MMO2**, and **DMO**). The generally decreased rates with which the natural triphosphates are inserted opposite the methoxy derivatized analogues, relative to their fully carbocyclic counterparts, are surprising considering their decreased hydrophobicity, and this again suggests that specific structural and/or electrostatic effects are important.

2.3. Unnatural Base Pair Extension Efficiency. To examine the contribution of methoxy groups to extension, we first examined the **BEN** nucleotide and nucleotides bearing single substituents at the *ortho*, *meta*, or *para* position of the phenyl nucleobase scaffold (Table 3). These analogues were incorporated into oligonucleotide templates and annealed to primers containing one of the analogues shown in Figure 1, resulting in the formation of an unnatural self-pair or heteropair at the primer terminus. We determined the ability of Kf to extend each primer by incorporation of the next correct triphosphate (dCTP). For reference, a dA:dT pair is extended in the same sequence context with a k_{cat}/K_M of 1.7×10^8 M^{-1} min^{-1}. Pairs with **BEN** in the template were recognized poorly by Kf (Table 3, **X** = **BEN**). Only primers terminating with **2OMe**, **DMO**, or **MMO2** are extended with a detectable rate ($k_{cat}/K_M > 1 \times 10^3$ M^{-1} min^{-1}). Pairs formed with **2OMe** in the template are extended poorly (Table 3, **X** = **2OMe**), while pairs with either **3OMe** or **4OMe** in the template are extended between 1.5×10^4 and 4.8×10^4

M^{-1} min^{-1}, but again only when paired opposite **2OMe**, **DMO**, or **MMO2** at the primer terminus (Table 3, **X** = **3OMe** or **4OMe**). These data suggest that an *ortho* methoxy group at the primer terminus facilitates extension.

We next examined the *ortho*, *para* di-substituted analogues **MMO1**, **MMO2**, and **DMO** in the template (Table 3). Kf does not efficiently recognize primers that terminate opposite **MMO2** or **DMO**, regardless of the analogue at the primer terminus (Table 3, **X** = **MMO2** or **DMO**). In each case the second-order rate constants are less than or equal to 1×10^4 M^{-1} min^{-1}. Also, most pairs involving **MMO1** in the template are not well extended (Table 3, **X** = **MMO1**). However, the pairs formed with **2OMe**, **MMO2**, or **DMO** in the primer and **MMO1** in the template are again extended more efficiently, with second-order rate constants of $\sim 1 \times 10^5$ M^{-1} min^{-1}. These data reinforce the idea that *ortho* positioned methoxy groups at the primer terminus increase the rates of extension but also suggest that the *ortho* methyl group of **MMO1** in the template contributes to efficient extension as well.

To further explore the effect of *ortho* methyl substituents in the template, the nucleobase analogue **MM1**, **DM5**, or **TM** was incorporated into the template strand (Table 3, **X** = **MM1**, **DM5**, or **TM**). Generally, the pairs formed with each of the primers are extended slightly better than the pairs without *ortho* methyl groups in the template analogue, and the rates parallel the extent of methyl group substitution (**TM** > **DM5** > **MM1**). Again, as with the monosubstituted analogues, the pairs formed with **2OMe**, **DMO**, or **MMO2** in the primer are consistently extended more efficiently. In fact, the pairs formed between **2OMe**, **DMO**, or **MMO2** at the primer terminus and **TM** in the template are extended with rates between 3.2×10^5 M^{-1} min^{-1} and 1.3×10^6 M^{-1} min^{-1}. The **MMO2:TM** heteropair is extended ~1000-fold faster than the unsubstituted **BEN** self pair (which is extended with a rate of 1.6×10^3 M^{-1} min^{-1})[9] and only ~100-fold less efficiently than a natural base pair. The increased rates of extension of the **2OMe:TM**, **DMO:TM**, and **MMO2:TM** heteropairs, relative to the **BEN** self pair, result from both increases in the apparent k_{cat} and decreases in the apparent K_M. The data clearly reveal that unnatural base pair extension is facilitated by both an *ortho* methoxy group in the primer nucleobase and an *ortho* methyl group in the template nucleobase.

2.4. Unnatural Base Pair Extension Fidelity. To examine extension fidelity, we first characterized the rate at which Kf extends a primer terminating with dA paired opposite **MMO1** or **MMO2** (Table 4). To further elucidate the effect of the methoxy group substituent, primers terminating with dA paired opposite **MM1**, **DM5**, or **TM** were also characterized. The mispairs with dA at the primer terminus were examined because, in each case, they are the most efficiently synthesized (see above). All mispairs except dA:**MMO2** are extended by dCTP insertion with rates between 1.9×10^5 and 5.2×10^5 M^{-1} min^{-1}. In contrast, the dA:**MMO2** mispair is extended less efficiently, with a k_{cat}/K_M of 4.6×10^4 M^{-1} min^{-1}. This rate is only marginally more efficient than the extension of a natural mispair[35] and demonstrates that the *ortho* methoxy group decreases the rate of mispair extension.

To further explore whether *ortho* methoxy groups at the primer terminus form mispairs with natural nucleotides that are recognized and extended by Kf, we paired **2OMe**, **DMO**, or **MMO2** opposite each natural nucleotide in the template and determined the efficiency of mispair extension (Table 4). None of the mispairs are extended efficiently, with rates varying from too low to detect ($<10^3$ M^{-1} min^{-1}) to 2.6×10^4 M^{-1} min^{-1}. Interestingly, extension of the mispairs with dG is very inefficient. These results clearly demonstrate that an H-bond is not formed between guanine and the *ortho* methoxy substituent or that if an H-bond is formed, it does not contribute to a structure at the primer terminus that is recognized by Kf. In fact, the mispairs between either purine and the unnatural analogues are extended less efficiently than those with either pyrimidine. Because the purine mispairs are more structurally similar to a natural base pair, the more efficient extension of the pyrimidine mispairs is surprising.

Table 4. Rates of Mispair Extension[a]

```
5′-d(TAATACGACTCACTATAGGGAGAY)
3′-d(ATTATGCTGAGTGATATCCCTCTXGCTAGGTTACGGCAGGATCGC)
```

X	Y	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} min^{-1})
MM1	A	5.61 ± 0.38	29 ± 6	1.9×10^5
DM5	A	7.29 ± 1.75	27 ± 6	2.7×10^5
MMO1	A	9.48 ± 0.69	23 ± 2	4.1×10^5
MMO2	A	8.65 ± 0.89	187 ± 17	4.6×10^4
TM	A	8.13 ± 1.59	15.5 ± 2.2	5.2×10^5
A	**2OMe**	nd[b]	nd[b]	$<1.0 \times 10^3$
G	**2OMe**	nd[b]	nd[b]	$<1.0 \times 10^3$
C	**2OMe**	0.40 ± 0.15	111 ± 37	3.6×10^3
T	**2OMe**	0.39 ± 0.09	87 ± 13	4.5×10^3
A	**DMO**	nd[b]	nd[b]	$<1.0 \times 10^3$
G	**DMO**	nd[b]	nd[b]	$<1.0 \times 10^3$
C	**DMO**	1.31 ± 0.23	69 ± 11	1.9×10^4
T	**DMO**	0.92 ± 0.04	59 ± 6	1.6×10^4
A	**MMO2**	nd[b]	nd[b]	$<1.0 \times 10^3$
G	**MMO2**	nd[b]	nd[b]	$<1.0 \times 10^3$
C	**MMO2**	0.67 ± 0.25	134 ± 32	5.0×10^3
T	**MMO2**	2.11 ± 0.63	80 ± 13	2.6×10^4

[a] See Experimental Section for details. [b] Reaction was too inefficient for k_{cat} and K_M to be determined independently.

Table 5. Rates of Correct Extension by R668A Kf Mutant[a]

```
5′-d(TAATACGACTCACTATAGGGAGAY)
3′-d(ATTATGCTGAGTGATATCCCTCTXGCTAGGTTACGGCAGGATCGC)
```

X	Y	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} min^{-1})
T	A	9.09 ± 1.69	29 ± 6	3.1×10^5
TM	**MMO2**	2.31 ± 0.79	63 ± 9	3.7×10^4
TM	**DM5**	0.60 ± 0.09	44 ± 3	1.4×10^4

[a] See Experimental Section for details.

2.5. Primer−Polymerase Interactions and Unnatural Base Pair Extension. To determine whether the minor groove methoxy groups facilitate unnatural base pair extension through formation of an H-bond with the polymerase, we examined extension rates with the R668A mutant of Kf[36] (Table 5). While other residues are also important for minor groove recognition of the primer terminus,[36,37] Arg668 is the most thoroughly characterized, and among the identified residues, it has the

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largest effect during extension. Previous studies have shown that either removal of the H-bond donor from the polymerase (by mutation of Arg668 to Ala) or removal of the hydrogen bond acceptor from a natural nucleobase at the primer terminus (by substituting 3-deaza-dG for dG) significantly decreases the rate of primer extension.[27,30] Additionally, this mutant has been used to show that the difference in extension rates of two natural nucleobase analogues is due to H-bonding with the DNA polymerase.[38] Kf R668A extends the **DM5:TM** heteropair with a rate only 2-fold reduced from that for wild-type Kf, demonstrating that Arg668 does not form a functionally important H-bond with **DM5**. However, extension of the **MMO2:TM** heteropair is reduced 35-fold, to essentially the same rate as that of the **DM5:TM** heteropair. The apparent K_M increased almost 8-fold with **MMO2** at the primer terminus, while it decreased 2-fold with **DM5** at the primer terminus. In both cases, k_{cat} decreased by approximately 4-fold. While it is difficult to interpret these changes mechanistically, as the kinetics are run under steady-state conditions, it is clear that removing the H-bond donor from the polymerase selectively decreases the extension efficiency of the pair with the minor groove methoxy group at the primer terminus. Thus, the *ortho* methoxy group of **MMO2** appears to favorably interact with Arg668, presumably via H-bonding.

Discussion

Extension of unnatural base pairs by DNA polymerases generally limits their replication, and thus understanding how to facilitate extension is critical for developing viable unnatural base pairs.[1] Previously, we examined phenyl-based nucleotides bearing H-bond acceptors that, when at the primer terminus, are expected to be oriented in the developing minor groove where they might engage a conserved polymerase-based H-bond donor. Specifically, we examined both minor groove carbonyl groups[23] and nitrogen substituents.[22] In addition, Hirao and coworkers have positioned an aldehyde group in the minor groove.[5,39,40] While these modifications facilitate extension, they also destabilize the base pair and, at least with the pyridone and pyridine nucleobases, appear to be recognized by the H-bond donor of dG, resulting in poor thermal and replication fidelity.

We are interested in nucleobase modifications that might facilitate H-bonding with the polymerase but not with a natural nucleobase. Thermodynamic studies have indicated that, when positioned *ortho* to the glycosidic linkage, methoxy groups are desolvated upon duplex formation (S.M. and F.R. unpublished results). This suggests that the H-bonding strength of the minor groove methoxy groups is insufficient to maintain solvation. Nonetheless, we were interested in examining whether these minor groove methoxy groups can engage the "preordered" H-bond donor of polymerases that is known to be required to align the primer terminus for efficient extension. Thus, we examined the effects of methoxy group substituents on polymerase-mediated replication.

Many previous studies have identified interbase hydrophobic interactions as a major force underlying the synthesis of pairs between different predominantly hydrophobic nucleobase analogues.[4−6,10,11] Interestingly, the effects of substituting methoxy groups for methyl groups in unnatural base pair and mispair synthesis is not consistent with simple changes in hydrophobicity. For example, substitution of the *ortho* methyl group of **MM1** with a methoxy group, to give **2OMe**, results in the more efficient insertion of more hydrophobic triphosphate analogues but less efficient insertion of more hydrophilic triphosphate analogues. In addition, **2OMe** in the template is more selective against insertion of the more hydrophilic natural triphosphates than **MM1**. The effects can also be complex; for example, within the **DM5** scaffold, a minor groove methoxy group slightly increases the rate of d**MM1**TP and d**TM**TP insertion but significantly decreases the rate of d**DM5**TP insertion. These triphosphate analogues differ only by single methyl substituents, and the rates do not parallel the extent of substitution. In total, these results suggest that the methoxy substituents are capable of mediating specific structural and/or electrostatic effects that contribute to efficient unnatural base pair synthesis.

As discussed above, continued primer extension after synthesis of the unnatural base pair has traditionally limited the replication of DNA containing unnatural base pairs. We find that primers that terminate with a minor groove methoxy group paired opposite a template analogue with a minor groove methyl group are efficiently extended. Remarkably, the **MMO2:TM** (primer:template) pair is extended only 100-fold slower than a natural base pair in the same sequence context. It is not obvious how the methyl group in the developing minor groove of the template analogue contributes to efficient extension. However, the fact that mispairs with natural pyrimidines in the template are extended more efficiently than mispairs with purines suggests that the role of both the methyl group and the carbonyl group may be structural, perhaps helping to form a primer terminus that is optimally packed and structured for continued extension.

The effect of a minor groove methoxy group at the primer terminus is consistent and significant. For example, when paired opposite **TM**, **MMO2** is extended more than 40-fold more efficiently than its carbocyclic analogue **DM5**. Although this difference is less than the approximately 3500-fold difference observed with dG and 3-deazaG,[30] it is similar to that observed with other unnatural nucleobases where H-bond acceptors have been introduced.[4,19,22,23,39,40] This suggests that while H-bonding between the primer terminus and the polymarase is important, other factors also contribute.

Methoxy groups of anisole are typically only moderate H-bond acceptors, as suggested by their pK_a of −6.5.[41] However, both experimental and theoretical studies indicate that this is due in part to conjugation of the lone pairs of electrons on oxygen into the aromatic ring, which requires the methyl group to be in the plane of the ring.[31−34] The same studies also indicate that when the methyl group rotates out of the plane, the electrons localize on the oxygen atom and it becomes a significantly better H-bond acceptor. Interestingly, molecular dynamics simulations suggest that the minor groove methyl groups of the unnatural base pairs rotate out of the plane of the phenyl ring to optimize packing interactions with flanking nucleobases (S.M and F.R. unpublished results). This, along with the "preordered" positioning of the polymerase-based H-bond donor, Arg668, appears

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(40) Mitsui, T.; Kitamura, A.; Kimoto, M.; To, T.; Sato, A.; Hirao, I.; Yokoyama, S. *J. Am. Chem. Soc.* **2003**, *125*, 5298−5307.
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to be sufficient to form a functional H-bond between the primer terminus and the polymerase. This conclusion is strongly supported by the selective decrease in R668A Kf-mediated extension efficiency of the **MMO2:TM** pair relative to the **DM5:TM** pair. The "preordering" of the Arg668 donor within the polymerase−DNA complex likely underlies the ability of the minor groove methoxy group to act as an H-bond acceptor despite its reduced ability to H-bond with minor groove waters of solvation.

It appears that an *ortho* positioned methoxy group is able to form a productive H-bond with the polymerase that appropriately aligns the primer terminus for continued extension. This, and the observation that these substituents do not increase mispair recognition or stability, as has been observed with other H-bond acceptors,[22,23] suggests that suitably positioned methoxy groups will be of great value in the design of new unnatural base pairs.

Experimental Section

General Methods. Chemical reagents were purchased from Sigma-Aldrich and used without further purification, unless otherwise stated. All unnatural nucleosides and nucleotides used in this study were synthesized as described in the Supporting Information. All reagents for oligonucleotide synthesis were purchased from Glen Research. Oligonucleotides were synthesized using an Applied Biosystems Inc. 392 DNA/RNA synthesizer. ^{31}P NMR spectra were recorded on a Bruker AMX-400 spectrometer. Coupling constants (J values) are reported in Hz. The chemical shifts are given in δ (ppm) using 85% H_3PO_4 in D_2O for ^{31}P NMR as an external standard. T4 polynucleotide kinase and Klenow fragment exo- were purchased from New England Biolabs. [γ-^{33}P]-ATP was purchased from Amersham Biosciences. The R668A/D424A double mutant of Kf was a generous gift from Catherine M. Joyce (Yale University). The D424A mutation renders the polymerase exonuclease deficient. For simplicity, the double mutant is referred to as the R668A mutant of the exonuclease deficient polymerase.

Synthesis of Oligonucleotides. Oligonucleotides were prepared by the β-cyanoethylphosphoramidite method on controlled pore glass supports (1 μmol) using an Applied Biosystems Inc. 392 DNA/RNA synthesizer as the standard method. After automated synthesis, the oligonucleotides were cleaved from the support by concd aqueous ammonia for 1 h at room temperature, deprotected by heating at 55 °C for 12 h, and purified by denaturing polyacrylamide gel electrophoresis (12−20%, 8 M urea). The primer oligonucleotides containing unnatural bases at the 3′-end were obtained using a Universal Support, or 3′-phosphate CPG, which was treated with alkaline phosphatase after deprotection according to manufacturer's protocols.

General Triphosphate Synthesis Procedure. Proton sponge (1.5 equiv) and nucleoside (1 equiv) were dissolved in trimethylphosphate (final concentration ~0.3 M) and cooled to 0 °C. $POCl_3$ (1.05 equiv) was added dropwise, and the mixture was stirred at 0 °C for 2 h. Tributylamine (5 equiv) was added, followed by a solution of tributylammonium pyrophosphate (5 equiv) in DMF (final concentration ~0.15 M). After 3 min, the reaction was quenched by addition of 1 M aqueous triethylammonium bicarbonate (10 vol equiv). The resulting crude solution was stirred for 30 min at 0 °C and then lyophilized. The crude material was purified by reversed phase HPLC (C18 column, 1−35% CH_3CN in 0.1 M NEt_3−HCO_3, pH 7.5) followed by lyophilization to afford the triphosphate as a white solid. **TM** and **DM5** triphosphates were synthesized as described previously.[9,12]

2OMe triphosphate: ^{31}P NMR (162 MHz, D_2O) δ −5.90 (d, J = 21.2 Hz), −10.55 (d, J = 19.8 Hz), −22.16 (t, J = 20.7 Hz). **3OMe** triphosphate: ^{31}P NMR (162 MHz, D_2O) δ −10.32 (d, J = 19.9 Hz), −10.76 (d, J = 20.4 Hz), −22.83 (t, J = 19.9 Hz). **4OMe** triphosphate: ^{31}P NMR (162 MHz, D_2O) δ −10.39 (d, J = 19.9 Hz), −10.71 (d, J = 20.1 Hz), −22.82 (t, J = 19.9 Hz). **DMO** triphosphate: ^{31}P NMR (162 MHz, D_2O) δ −6.01 (d, J = 21.2 Hz), −10.52 (d, J = 19.9 Hz), −22.21 (t, J = 20.6 Hz). **MMO1** triphosphate: ^{31}P NMR (162 MHz, D_2O) δ −9.35 (d, J = 19.3 Hz), −10.67 (d, J = 20.1 Hz), −22.68 (t, J = 20.1 Hz). **MMO2** triphosphate: ^{31}P NMR (162 MHz, D_2O) δ −5.91 (d, J = 21.2 Hz), −10.56 (d, J = 19.6 Hz), −22.17 (t, J = 20.6 Hz). **MM1** triphosphate: ^{31}P NMR (162 MHz, D_2O) δ −8.03 (bs), −10.63 (d, J = 19.9 Hz), −22.48 (t, J = 20.4 Hz).

Gel-Based Kinetic Assay. Primer oligonucleotides were 5′-radiolabeled with T4 polynucleotide kinase (New England Biolabs) and [γ-^{33}P]-ATP (GE Biosciences). Primers were annealed to template oligonucleotides in the reaction buffer by heating to 90 °C followed by slow cooling to ambient temperature. Assay conditions included 40 nM primer/template, 0.1−1.3 nM enzyme (either Kf or Kf R668A), 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, and 50 μg/mL acetylated BSA. The reactions were carried out by combining the DNA−enzyme mixture with an equal volume (5 μL) of 2 × dNTP stock solution, incubating at 25 °C for 1−10 min, and quenching by the addition of 20 μL of loading dye (95% formamide, 20 mM EDTA, and sufficient amounts of bromophenol blue and xylene cyanole). The reaction mixtures were resolved by 15% polyacrylamide and 8 M urea denaturing gel electrophoresis, and radioactivity was quantified using a PhosphorImager (Molecular Dynamics) and ImageQuant software. The Michaelis−Menten equation was fit to a plot k_{obsd} versus triphosphate concentration using the program Kaleidagraph (Synergy Software). The data presented are averages of three independent determinations.

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Supporting Information Available: Nucleoside and nucleotide synthesis and characterization. Representative kinetics plots for heteropair synthesis, misincorporation, correct pair extension, and mispair extension. This material is available free of charge via the Internet at http://pubs.acs.org.

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