Dual Specificity of the Pyrimidine Analogue, 4-Methylpyridin-2-one, in DNA Replication

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Received September 13, 1999

Studies of the enzymatic incorporation of modified nucleotides into DNA provide valuable information about the interactions among triphosphates, templates, and polymerases, thus allowing one to understand the fidelity of replication. Experiments using unnatural bases that have different H-bonding patterns from Watson−Crick types,[1−5] such as isoguanine and isocytosine,[4,5] have indicated that correct H-bond formation between bases is important for accurate replication. On the other hand, recent studies using non-H-bonded base analogues have shown the importance of shape complementarity between bases.[6−11] For example, a deoxynucleoside 5′-triphosphate derivative of difluorotoluene that is isosteric with T is incorporated opposite both A and non-H-bonding analogues of A by the Klenow fragment (KF) of *Escherichia coli* DNA polymerase I.[6−10] Thus, on the basis of the concepts of H-bonding and shape fitting,[12−14] novel unnatural bases that pair specifically with natural or unnatural base(s) could be designed.

To create a novel base analogue with unique base-pairing specificity, we modified 2-(1H)-pyridinone (**1** in Figure 1) and examined its enzymatic incorporation into DNA. This pyrimidine analogue **1** in a template leads to nonspecific incorporation of natural bases.[14,15] However, the 4-amino substitution, 3-deazacytidine (**2**), in a template specifically incorporates either dCTP or dTTP, and forms the pyrimidine−pyrimidine base pairs.[14,15] To further change the selectivity of **1**, we newly designed 4-methylpyridin-2-one (**3**), in which the 4-amino group of **2** is substituted with a methyl group. In contrast to **2**, the hydrophobicity of **3** may prevent base pairing with the natural pyrimidines, C and T, which are associated with water molecules.[12] In addition, the 4-methyl group of **3** may sterically clash with the 6-amino group of A and may eliminate base pairing with A. While the 3-hydrogen of **3** collides with the 1-imino proton of G, the 2-keto group of **3** forms a hydrogen bond with the 2-amino group of G. Thus, as a C-analog the unnatural hydrophobic nucleobase **3** was expected to be incorporated opposite G selectively.

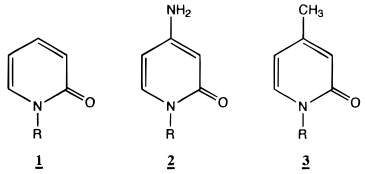

Figure 1. Structures of unnatural pyrimidine nucleoside analogues. R = 2-deoxy-β-D-ribofuranosyl

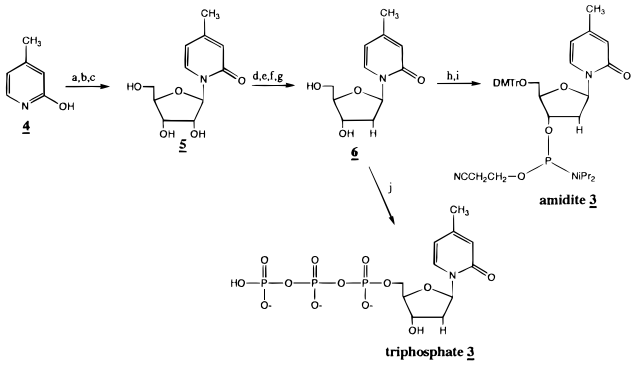

Figure 2. A scheme for the synthesis of amidite **3** and triphosphate **3**. (a) Hexamethyldisilazane (4 equiv), reflux, 1 h. (b) 1-*O*-Acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (0.53 equiv), $SnCl_4$ (0.66 equiv), 1,2-dichloroethane, rt, 4 h. (c) NH_3/MeOH, rt, 44 h. (d) 1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane (1.1 equiv), pyridine/DMF, rt, 2 h. (e) Thiocarbonyldiimidazolide (2.5 equiv), DMF, rt, 14 h. (f) 2,2′-Azobisisobutyronitrile (0.2 equiv), tributyltin hydride (1.5 equiv), toluene, reflux, 1.5 h. (g) 1 M Tetrabutylammonium fluoride, rt, 3 h. (h) 4,4′-Dimethoxytrityl chloride (1.1 equiv), pyridine, rt, 3 h. (i) 2-Cyanoethyl-*N*,*N*-diisopropyl-amino-chloro phosphoramidite (1.5 equiv), *N*,*N*-diisopropylethylamine (1.5 equiv), THF, rt, 30 min. (j) Phosphorus oxytrichloride (1.04 equiv). trimethyl phosphate, 0 °C, 2 h, followed by 0.5 M bis(tri-*n*-butylammonium) pyrophosphate, DMF, tri-*n*-butylamine, 0 °C, 30 min.

The amidite and the triphosphate of **3** were synthesized via the corresponding ribonucleoside (**5**), according to the synthesis of 1-β-D-ribofuranosylpyridin-2-one[16] (Figure 2). The molar absorption coefficient of triphosphate **3** (6580 at λ_{max} = 295 nm) was determined by quantitative analysis of the phosphorus[17] after dephosphorylation with calf intestine alkaline phosphatase. The coupling efficiency of amidite **3** was more than 98% on a DNA synthesizer (PE Applied Biosystems). The sequences of the templates that we synthesized are shown in Figures 3 and 4.

First, we carried out single-nucleotide insertions to test which natural and unnatural dNTPs are favorably inserted opposite the four natural bases and **3** in the templates by a DNA polymerase. A 5′-^{32}P-labeled 15-mer primer (0.5 μM) and each template (at 1 μM) were annealed in a buffer containing 10 mM Tris-HCl, pH 7.5, 7 mM $MgCl_2$, and 0.1 mM DTT. The solution was then incubated at 17 °C for 10 min with 0.4 mM dNTP and 125 nM (1 unit) of KF (exo$^+$) (TAKARA). The insertion products were

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(1) Piccirilli, J. A.; Krauch, T.; Moroney, S. E.; Benner, S. A. *Nature* **1990**, *343*, 33−37.
(2) Horlacher, J.; Hottiger, M.; Podust, V. N.; Hübscher, U.; Benner, S. A. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 6329−6333.
(3) Lutz, M. J.; Held, H. A.; Hottiger, M.; Hübscher, U.; Benner, S. A. *Nucleic Acids Res.* **1996**, *24*, 1308−1313.
(4) Switzer, C.; Moroney, S. E.; Benner, S. A. *J. Am. Chem. Soc.* **1989**, *111*, 8322−8323.
(5) Switzer, C. Y.; Moroney, S. E.; Benner, S. A. *Biochemistry* **1993**, *32*, 10489−10496.
(6) Moran, S.; Ren, R. X.-F.; Rummey, S., IV; Kool, E. T. *J. Am. Chem. Soc.* **1997**, *119*, 2056−2057.
(7) Liu, D.; Moran, S.; Kool, E. T. *Chem. Biol.* **1997**, *4*, 919−926.
(8) Moran, S.; Ren, R. X.-F.; Kool, E. T. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10506−10511.
(9) Morales, J. C.; Kool, E. T. *Nat. Struct. Biol.* **1998**, *5*, 950−954.
(10) Morales, J. C.; Kool, E. T. *J. Am. Chem. Soc.* **1999**, *121*, 2323−2324.
(11) Matray, T. J.; Kool, E. T. *Nature* **1999**, *399*, 704−708.
(12) Kool, E. T. *Biopolymers* **1998**, *48*, 3−17.
(13) Goodman, M. F.; Fygenson, D. K. *Genetics* **1998**, *148*, 1475−1482.
(14) Strazewski, P.; Tamm, C. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 36−57.
(15) Charczuk, R.; Tamm, C.; Suri, B.; Bickle, T. A. *Nucleic Acids Res.* **1986**, *14*, 9530.
(16) Niedballa, U.; Vorbrüggen, H. *Nucleic Acid Chemistry*; John Wiley & Sons: New York, 1978; pp 481−484.
(17) Chen, P. S.; Toribara, T. Y.; Warner, H. *Anal. Chem.* **1956**, *28*, 1756−1758.

10.1021/ja993291o CCC: $19.00
Published on Web 06/09/2000

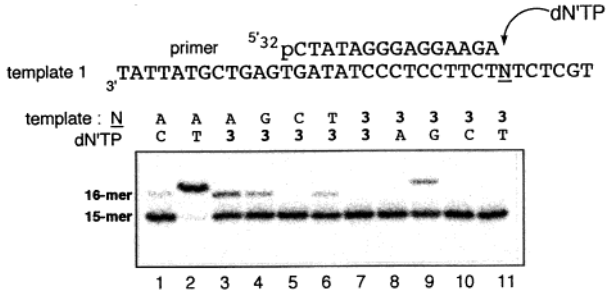

Figure 3. Autoradiograms of denaturing polyacrylamide gel electrophoresis showing single-nucleotide insertions by KF (exo$^+$).

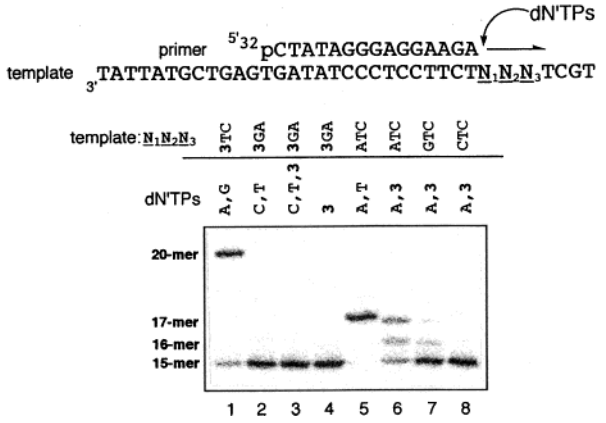

Figure 4. Autoradiograms of denaturing polyacrylamide gel electrophoresis showing primer extensions by KF (exo$^+$).

Table 1. Steady-State Kinetic Parameters for Insertion of Single Nucleotides into a Template−Primer Duplex by KF (exo$^-$)[a]

template base	nucleotide triphosphate	K_M (μM)	V_{max} (% min^{-1})	efficiency (V_{max}/K_M)
A	3	65 (30)	1.1 (0.2)	1.7×10^4
G	3	66 (54)	0.3 (0.1)	4.5×10^3
C	3	465 (186)	0.5 (0.1)	1.1×10^3
T	3	420 (129)	1.9 (0.1)	4.5×10^3
3	A	n.d.[b]	n.d.	
3	G	66 (32)	0.3 (0.1)	4.5×10^3
3	C	n.d.	n.d.	
3	T	n.d.	n.d.	

[a] The procedure is given in Supporting Information. Parenthetic values are standard deviations. [b] No inserted products were detected after incubation for 20 min with 15 mM nucleotide triphosphates.

analyzed by polyacrylamide gel electrophoresis. As expected, only G was successfully inserted opposite **3** in the templates, and the insertion product, a 16-mer, was observed (Figure 3, lanes 7−11). Surprisingly, the insertion of triphosphate **3** showed different specificity opposite the four natural bases in the templates, and it was favorably inserted opposite A, rather than opposite G, in the templates (Figure 3, lanes 3 and 4). Triphosphate **3** was also occasionally inserted opposite T (Figure 3, lane 6). While the hydrophobicity of **3** also expected the self-complementary pairing,[18] no insertion of triphosphate **3** opposite **3** was observed (Figure 3, lane 7).

Quantitative single-nucleotide insertion studies (Table 1) under steady-state conditions using KF (exo$^-$) (Amersham-USB)[9,19] indicate that the selectivity of **3** is similar to that shown in Figure 3. Only dGTP was inserted opposite **3** in the template, with a K_M = 66 μM, and no incorporation of the other native dNTPs (up to 15 mM) was observed. In contrast, triphosphate **3** was preferentially inserted opposite A in the template 3- to 15-fold more efficiently than opposite G, C, and T. While triphosphate **3** was incorporated opposite A and G with the same K_M, the phosphate linkage formation of **3** is more advantageous to **3**-A pairing than to **3**-G pairing, as revealed by comparing the V_{max} values.

Next, we examined primer extensions to see whether DNA synthesis could proceed after base pair formation between **3** and the natural bases in the primer−template duplexes. The experiments were carried out at 17 °C for 30 min in the presence of varied mixtures of dNTPs, various primer−template duplexes, and KF (exo$^+$) (1 unit, 125 nM). Primer extension using the natural triphosphates (0.2 mM each) was not inhibited in the presence of triphosphate **3** (up to 2.4 mM) (data not shown). As shown in Figure 4, lane 1, the primer extension using the template ($N_1N_2N_3$ = **3**TC) efficiently continued in the presence of dGTP and dATP, and terminated before G in the template, resulting in a 20-mer. Since the extension product was not observed in the absence of dGTP (data not shown), we concluded that the extension in Figure 4, lane 1, proceeded after the incorporation of G opposite **3**. By contrast, after the incorporation of triphosphate **3** opposite G, further extension was less effective (Figure 4, lane 7). However, the extension proceeded after the incorporation of **3** opposite A, and the expected 17-mer product was obtained (Figure 4, lane 6). The extensions by the other combinations of base pairing with **3** were blocked (Figure 4, lanes 2−4 and 8). Thus, nucleotide **3** in the templates accepts only dGTP, whereas triphosphate **3** is the preferential substrate opposite A for effective primer extension.

In the primer extension experiments, DNA synthesis efficiently continued only after the formation of G-**3** and **3**-A base pairs in the primer−template duplexes. One of the plausible reasons why nucleotide **3** in the template and triphosphate **3** show different selectivities from each other may be due to the different orientations of the H's of the 4-methyl group of **3**. The rotation of the 4-methyl group of **3** in the template is restricted by stacking with neighboring bases, and the fixed orientation effectively excludes the incorporation of dATP. This is supported by the melting experiments, which showed that the **3**-A pair in a duplex is less stable than the **3**-G pair; the T_m = 35.2 °C of the duplex (5′-CGCAT***3***GTTACC-3′/5′-GGTAAC***A***ATGCG-3′, 5 mM), in 10 mM phosphate buffer, pH 7.0, 100 mM NaCl, and 0.1 mM EDTA, is lower than the T_m = 38.9 °C of the duplex (5′-CGCAT***3***GTTACC-3′/5′-GGTAAC***G***ATGCG-3′, 5 mM). In contrast, the 4-methyl group in the triphosphate easily rotates, and the H of the 2-amino group of A can fit between the H's of this 4-methyl group without strict steric hindrance. Consequently, triphosphate **3** may fit by shape-complementarity with A in the template. To better understand the selectivity of **3**, we may need to perform structural analyses of duplexes containing **3**.

We have demonstrated the selective formation of natural−unnatural base pairs by enzymatic incorporation, which indicates that unnatural bases can be designed on the basis of the H-bonding and the shape-fitting concepts of base pairs.[12−14] Moreover, the unexpected behavior of the 4-methyl group endows **3** with dual specificity of base pairing. While the unnatural base **1** in templates nonspecifically accepts natural bases into the primer strands,[14,15] the 4-methyl derivative (**3**) in the templates serves as a C-analogue, and the triphosphate **3** behaves as a T-analogue rather than a C-analogue. Thereby, the triphosphate **3** could be used as a selective A → G transition mutagen. This specific mutagen would be advantageous for increasing the GC contents of genomes and nucleic acid libraries in in vivo and in vitro mutagenesis, in which the GC contents might be controllable by adjusting the concentration of the triphosphate **3**.

Supporting Information Available: Additional experimental details, gel electrophoretograms, and Hane−Woolf plot (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA993291O

(18) Ogawa, A. K.; Wu, Y.; McMinn D. L.; Liu, J.; Schultz, P. G.; Romesberg, F. E. *J. Am. Chem. Soc.* **2000**, *122*, 3274−3287.
(19) Boosalis, M. S.; Petruska, J.; Goodman, M. F. *J. Biol. Chem.* **1987**, *262*, 14689−14696.