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2-Amino-7-deazaadenine Forms Stable Base Pairs with Cytosine and Thymine

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Abstract—2-Amino-7-deazaadenine (^{AD}A) was incorporated into oligodeoxynucleotides (ODN) and their base-pairing properties with natural nucleobases were investigated. In melting temperature (T_m) experiments, the duplex containing an ^{AD}A/C base pair showed a high stability comparable to that containing ^{AD}A/T base pair. Destabilization of the duplex usually observed for existing degenerate bases was not observed. However, the incorporation efficiency of dCTP was only 1.8% for TTP in single-nucleotide insertion reactions using polymerase.

Synthetic 7-deazapurine, a purine analogue in which the N7 is replaced by a carbon atom, is a promising nucleobase for studying the structure and function of nucleic acids.[1–8] Several groups have incorporated these deazapurine analogues into oligodeoxynucleotides (ODNs), and their binding affinity toward DNA has been studied.[9,10] In recent years, deazapurine has also been used as a tool for probing nucleic acid structures.[11–13] While the thermodynamic parameters for the duplex formation of ODNs containing 7-deazapurine have been thoroughly studied, little is known about the base selectivity and fidelity of the base-pairing of 7-deazapurine. An investigation of the base-pairing properties of 7-deazapurine is important not only for structural studies of nucleic acids, but also for antisense and mutagenesis studies. We now wish to report that 2-amino-7-deazaadenine (**2**), a family of 2-aminoadenine (**3**) which is known to form a tight base pair with thymine by extra hydrogen bonding,[14–16] acts as a superior degenerate base to form a stable base pair with both cytosine and thymine.

Nucleoside **2**[17] was incorporated into ODNs using the conventional phosphoramidite method. The protected phosphoramidite of **2** was prepared as shown in Scheme 1.[18] The thermal stabilities of the duplexes containing **2** and **3** were evaluated by determining the melting temperature (T_m) of the duplexes, 5′-d(TTTGGTT**X**TTT)-3′/5′-(AAA**Y**AACCAAA)-3′ (X = **2** or **3**, Y = T or C), and from the change of A_{260} on heating. The normalized melting profile of the duplexes is shown in Figure 1. The duplex containing a **2**/T base pair showed a T_m value (13.8 °C) close to that of a **3**/T base pair (14.0 °C). The stability observed in the duplex containing a 'mismatched' **3**/C base pair (11.5 °C) remarkably decreased as compared with that for a **3**/T base pair, whereas the T_m of the duplex containing a **2**/C base pair (19.0 °C) was 5.2 °C higher than that containing a **2**/T base pair. The stable base pair with cytosine observed for **2** was striking, and in contrast to the destabilization of the **3**/C base pair.

The thermal stabilities of the duplexes containing **2**/C or **2**/T base pair were compared with those of the duplexes replaced by other base pairs. The T_m values of the duplexes are summarized in Table 1. The duplex containing a **2**/T base pair, which is expected to have extra hydrogen-bonding to thymine, showed a higher T_m than that of a 7-deazaadenine (**1**)/T base pair, as **3**/T duplex was more stable than A/T duplex. When guanine or adenine was incorporated opposite **2**, the duplex was remarkably destabilized. In contrast, the duplex containing a **2**/C base pair showed high duplex stability comparable to the G/C pair. It is worth noting that deazaadenine **2** can form a stable base pair not only with thymine, but also with cytosine.

Base pairs with both thymine and cytosine can be formed by the well-known degenerate bases *O*6-methyl-

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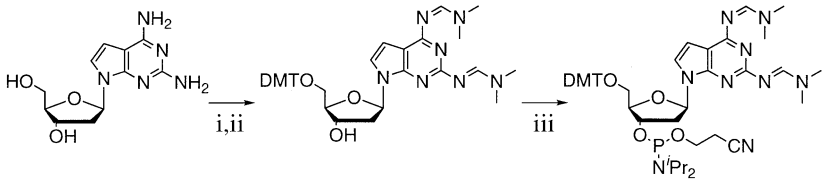

Scheme 1. Synthesis of **2** phosphoramidite. Reagents and conditions: (i) *N*,*N*-dimethylformamide dimethylacetal, DMF, 55 °C, 8 h, 81%; (ii) 4,4′-dimethoxytrityl chloride (DMTCl), DMAP, pyridine, rt, 3 h, 86%; (iii) $(^{i}Pr_2N)_2PO(CH_2)_2CN$, tetrazole, acetonitrile, rt, 4 h, quant.

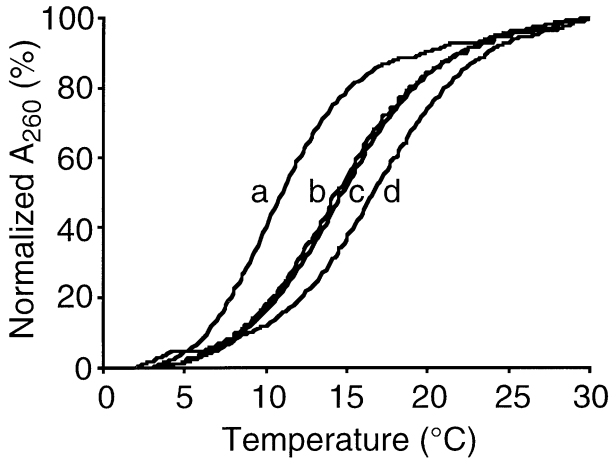

Figure 1. Normalized melting profile of the duplex 5′-d(TTTGGTT**X**TTT)-3′/5′-d(AAA**Y**AACCAAA)-3′ (**X** = **2** or **3**, **Y** = T or C). (a) **3**/C, (b) **2**/T, (c) **3**/T, (d) **2**/C.

guanine (**4**)[19,20] and 2-amino-6-methoxyaminopurine (**5**)[21,22] (Fig. 2). We measured the T_m of the ODNs containing these degenerate bases under the same conditions. Indeed, the degenerate bases **4** and **5** formed base pairs with both thymine and cytosine with similar stabilities (Table 1, entries 11–14). However, these base pairs remarkably destabilized the duplexes when compared to the duplexes containing normal A/T and G/C base pairs. Thus, the T_m experiments demonstrated that **2** is a superior degenerate base which can form a stable base pair with both cytosine and thymine without the duplex destabilization commonly observed for known degenerate bases like **4** and **5**.

High stability of the **2**/C duplex comparable to that of the **2**/T duplex was observed also in the T_m experiments using GC-rich sequences. We measured the T_m of 5′-d(GCGATG**2**GTAGCG)-3′/5′-d(CGCTAC**N**CATCG-C)-3′ (**N** = C and T) in 10 mM PIPES (pH 7.0). The T_m of the duplex containing a **2**/C base pair was 44.4 °C, showing that the **2**/C duplex stability was nearly equal to the **2**/T duplex stability (T_m = 44.7 °C for **2**/T). In contrast, when T_m was measured in high salt concentration, the **2**/C duplex was less stable than that observed for the **2**/T duplex. In 10 mM PIPES in the presence of 100 mM sodium chloride and 10 mM magnesium chloride,[23] the T_ms of the duplexes containing the **2**/C and **2**/T base pairs were 56.1 and 62.0 °C, respectively. Although the T_m of the **2**/C duplex was risen under high salt condition, the stabilization of the **2**/C duplex by high salt was not so great as that observed for the **2**/T duplex.

In order to find out the efficiency of the base-pairing of 7-deazapurines with four natural nucleobases, we examined a single-nucleotide insertion reaction mediated by polymerase using a template containing **2**. The primer–template duplex was incubated with one of four natural deoxynucleotide triphosphates in the presence of a Klenow fragment (*exo*$^-$) at 37 °C, and the incorporation efficiency of these four bases opposite **2** was determined by gel electrophoresis (Table 2).[24] The steady-state kinetic parameters, V_{max} and K_m of the single-nucleotide insertion reaction were derived from a Lineweaver–Burk plot calculated from the intensities of the gel bands.[25] As shown in Table 2, the efficiency of dCTP insertion was considerably low: only 1.8% of that for TTP insertion, although the formation of a stable **2**/C base pair was observed in the T_m experiments. Table 2 shows that the low dCTP insertion efficiency was due to its large K_m value. The K_m of dCTP insertion was 25 times larger than that observed in TTP insertion,

Table 1. The melting temperatures (T_m) of duplexes containing various base pairs[a]

5′-TTTGGTT**X**TTT-3′
3′-AAACCAA**Y**AAA-5′

Entry	**X**/**Y**	T_m (°C)	Entry	**X**/**Y**	T_m (°C)
1	**1**/T	12.2	8	A/T	12.8
2	**2**/T	13.8	9	G/C	19.8
3	**3**/T	14.0	10	A/C	6.8
4	**2**/C	19.0	11	**4**/T	9.3
5	**3**/C	11.5	12	**4**/C	8.5
6	**2**/G	7.0	13	**5**/T	6.7
7	**2**/A	—[b]	14	**5**/C	7.8

[a]Conditions: 2.5 μM duplex, 10 mM sodium cacodylate, pH 7.0.
[b]No sigmoidal melting curve was observed.

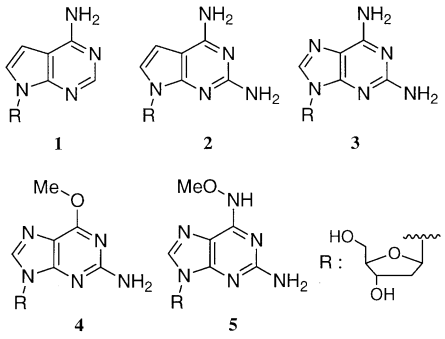

Figure 2. Structures of the synthetic 2′-deoxynucleosides used in this study.

Table 2. The steady-state kinetic parameters for the insertion of a single nucleotide into a template-primer duplex mediated by a Klenow fragment (*exo*$^-$)[a]

5′-TAATACGACTCACTATAGGGAGA(**N**) -3′
3′-ATTATGCTGAGTGATATCCCTCT **2** GTCA-5′

dNTP	V_{max} (pmol U^{-1} min^{-1})	K_m (μM)	Efficiency (V_{max}/K_m)	Relative frequency
TTP	45.3	1.14	39.7	100
dCTP	18.7	25.6	0.730	1.84
dGTP	13.1	62.0	0.211	0.531
dATP	0.994	22.0	0.0452	0.114

[a]Primer–template duplex was incubated with dNTP in the presence of a Klenow fragment (*exo*$^-$) as a polymerase at 37 °C (see ref 23 for details). V_{max} and K_m were calculated from a Lineweaver–Burk plot after three trials.

indicating that **2**/C base-pairing was inefficient in the interior of the enzyme pocket.

In order to confirm the T_m of the duplex elongated by single-nucleotide insertion, the T_m of the duplex consisting of the template and a 12-mer, 5′-d(CTATAGGGAGAPy)-3′, possessing thymine or cytosine at the 3′ end of the truncated primer (opposite **2** on a template strand) was measured. The **2**/C duplex showed a slightly higher T_m (28.0 °C) than the **2**/T duplex (26.2 °C), which was consistent with the T_m results shown in Table 1. Although the **2**/C base pair was as stable as the **2**/T base pair, the insertion of the dCTP opposite **2** was much slower than the TTP insertion. These results suggest that the **2**/C base pair formation in the polymerase reaction is more complicated when compared with the **2**/T base pair formation.

In summary, 7-deazaadenine **2** formed a stable base pair not only with thymine but also with cytosine without any duplex destabilization. While **2** was an effective degenerate base, only TTP was incorporated opposite **2** in a single-nucleotide insertion reaction, and the incorporation of dCTP was inefficient. Owing to its unique base-pairing property, 7-deazaadenine **2** is useful not only as a superior degenerate base that can be used for sequence-alternative hybridization, but also as a potent mutagen for site-directed mutagenesis which can induce the G/C to A/T transition during the DNA replication step.

References and Notes

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18. Selected data for compound **6**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.91 (br, 1 H), 8.67 (br, 1 H), 6.96 (d, 1 H, J = 3.7 Hz), 7.42–6.78 (m, 14 H), 6.51 (d, 1 H, J = 3.8 Hz), 4.55 (m, 1 H), 3.76 (s, 6 H), 3.26 (m, 2 H), 3.14 (d, 6 H, J = 10.5 Hz), 3.09 (d, 6 H, J = 9.3 Hz), 3.05 (m, 1 H), 2.41 (m, 2 H), ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.54, 158.49, 157.52, 157.08, 153.84, 144.66, 135.83, 135.81, 135.71, 135.69, 130.07, 128.17, 127.87, 126.84, 120.64, 113.16, 107.76, 101.52, 86.44, 84.76, 77.21, 72.84, 64.24, 64.08, 55.23, 55.22, 45.77, 41.17, 41.00, 40.57, 35.18, 34.79, MS (FAB, NBA/CH_2Cl_2) m/z (%) 678 [$(M+H)^+$], HRMS (FAB) calcd for $C_{38}H_{43}N_7O_5$ [$(M+H)^+$] 678.3401, found 678.3390.
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24. Primer–template duplex was annealed by mixing in the buffer (10 mM Tris–HCl, pH 7.3, 10 mM $MgCl_2$, 1 mM DTT, and 0.1 mg/mL BSA), heating to 90 °C, and slow cooling to ambient temperature. Primer–template duplex (5 μM) was incubated with dNTP in the presence of Klenow fragment (*exo*$^-$, Ambion) as polymerase at 37 °C. The reaction was stopped at each time by 80% formamide loading buffer (a solution of 80% v/v formamide, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue) and analyzed with PAGE (20% polyacrylamide, 7 M urea). V_{max} and K_m was calculated from Lineweaver–Burk plotting after three trials.
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