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Synthesis of 3-(2-deoxy-β-D-ribofuranosyl)pyridin-2-one and 2-amino-6-(*N*,*N*-dimethylamino)-9-(2-deoxy-β-D-ribofuranosyl)purine derivatives for an unnatural base pair

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Abstract

An unnatural base pair, 2-amino-6-(*N*,*N*-dimethylamino)purine (denoted **x**) and pyridin-2-one (denoted **y**), was designed to prove the structural requirements for base pair formation involving shape complementarity. It was expected that **y** might satisfy the structural requirements for pairing with **x**, in which the bulky 6-dimethylamino group may eliminate base pairing with the natural bases. As chemical or biological substrates for DNA synthesis, the phosphoramidite of **x** and the 2′-deoxy-C3-ribonucleoside triphosphate of **y** (d**y**TP) were synthesized, and the incorporation experiment was demonstrated by using the Klenow fragment of *Escherichia coli* DNA polymerase **I**.

DNA replication is a highly accurate biological event catalyzed by DNA polymerases. To understand the mechanism of the fidelity, enzymatic incorporation experiments using synthetic unnatural nucleotides have been employed.[1–4] Recently, Kool and co-workers have created non-hydrogen bonded base pairs, such as difluorotoluene:benzimidazole[5–7] and pyrene:abasic site.[8] These non-polar bases can be inserted by a 3′-exonuclease-defective Klenow fragment (*exo*$^-$) opposite bases that have shape complementarity.[5,9] This approach demonstrates the importance of steric matching for specific base pair formation in replication.[3,10,11]

On the basis of the steric matching for base pairing, we designed 2-amino-6-(*N*,*N*-dimethylamino)purine (**x**) as a purine analog and pyridin-2-one (**y**) as a pyrimidine analog [Fig. 1(a)]. Through enzymatic incorporation experiments for this base pair, which has different shape complementarity from those of the natural base pairs, the selectivity of the base pairing and the importance of the steric matching were assessed. These bases would be expected to form the **x**:**y** pair favorably and to avoid non-cognate pairs with natural bases, due to the steric hindrance of the

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6-dimethylamino group of **x**. The formations of **x**:T or **x**:C pairs may have disadvantages since the bulky 6-dimethylamino group of **x** clashes with the 4-keto group of T [Fig. 1(b)] and the 4-amino group of C, but not with the 6-hydrogen of **y**. We prepared the 2′-deoxy-C3-ribonucleoside triphosphate of **y** (d**y**TP) and DNA templates containing **x**, and examined the enzymatic incorporation of d**y**TP into DNA using the Klenow fragment.

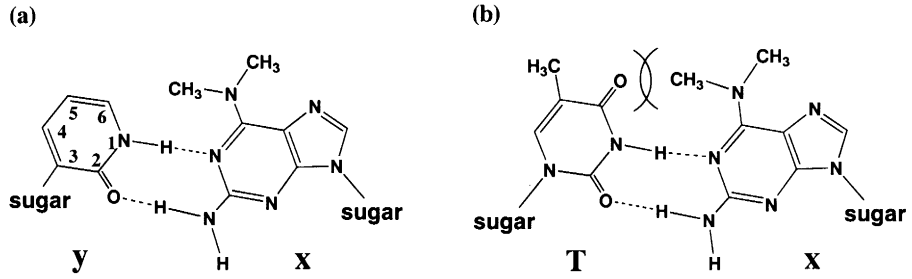


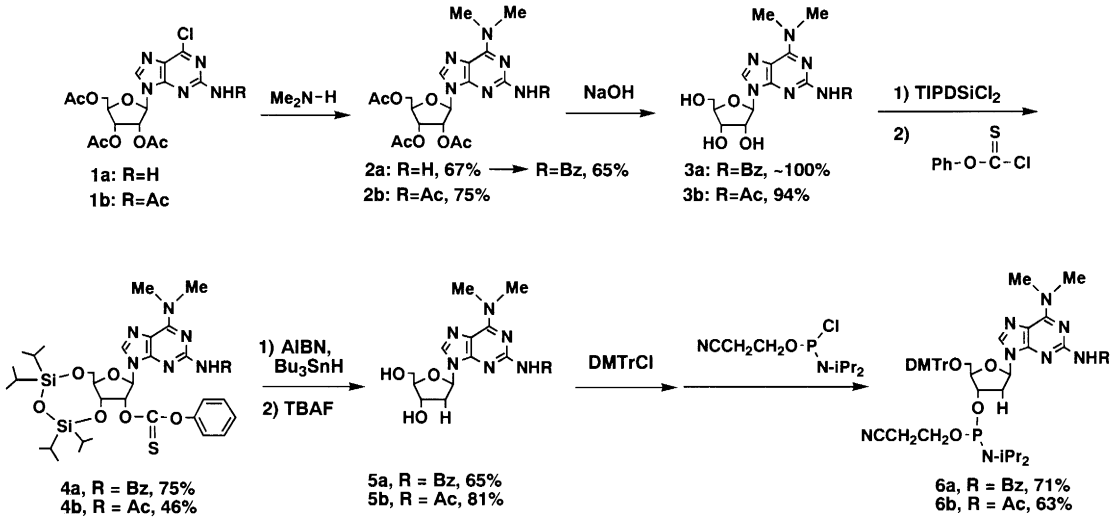
Fig. 1. Cognate and non-cognate base pairs involving unnatural **x** and **y** bases

The triphosphate d**y**TP was synthesized from the corresponding ribonucleosides. Syntheses of both the ribonucleoside[12] and the 2′-deoxyribonucleoside[13,14] of **y** have been reported. To prepare both d**y**TP and the ribonucleoside triphosphate, r**y**TP, for replication and transcription experiments, we first synthesized the ribonucleoside of **y**, and then converted it into the 2′-deoxyribonucleoside. 3-(2-Deoxy-β-D-ribofuranosyl)-pyridin-2-one was obtained in an overall yield of 13% from the ribonucleoside. The deoxynucleoside was converted to d**y**TP[15] with phosphorus oxychloride and pyrophosphate. The molar absorption coefficient of d**y**TP (7.6×10^3 at 298 nm) at pH 7.0 was determined by a quantitative analysis of the phosphorus in the compound. No degradation product of d**y**TP was observed by HPLC analysis after standing at room temperature for seven days in a buffer containing 10 mM tris-HCl, pH 7.5, 7 mM $MgCl_2$, and 0.1 mM DTT. However, heating d**y**TP at 95°C in the solution gradually yielded an unknown decomposition product.

The synthesis of the protected 2′-deoxynucleoside phosphoramidites (**6a** and **6b**) of **x** is illustrated in Scheme 1. Throughout the synthesis, the 2-amino group of the base moiety was protected with a benzoyl or an acetyl group. The substitutions of the 6-chloro groups of **1a** and **1b** with dimethylamino groups was achieved with dimethylamine hydrochloride in 65–75% yields. This approach is more convenient than the previously reported method using trimethylamine.[16] The *N*2-protected ribonucleosides (**3a** and **3b**) were converted to 2′-deoxyribonucleosides (**5a** and **5b**) via the corresponding 3′,5′-*O*-disiloxane derivatives, followed by dimethoxytritylation and phosphitylation to give the phosphoramidite derivatives.[17,18]

Before preparing the templates, the trinucleotide [d(T**x**T)] synthesis was examined by using the **x**-amidites, **6a** and **6b**. The coupling efficiencies of both amidites were similar to those of the commercially available phosphoramidites. After ammonia treatment for removing the protecting groups and releasing the trimer from the CPG-resin, the products were analyzed by reverse-phase HPLC and mass spectrometry. The deprotection with concentrated ammonia at 60°C for 6 h gave two major products, corresponding to d(T**x**T) and the remaining *N*2-acyl-protected trimers. Due to the incomplete removal of the *N*2-acetyl group of **x**, deprotection with ammonia was carried out at 80°C for 10 h. Both the acetyl and benzoyl protecting groups were then removed completely, and d(T**x**T) was obtained without any decomposition of the trimer. Thus, these deprotection conditions were used to prepare the templates containing **x**.

We examined the selectivity of the single-nucleotide insertion of d**y**TP opposite **x** by the Klenow fragment (*exo*$^+$), using a primer-template duplex in which **x** was adjacent to the 3′ end of the primer (Fig. 2). The insertion products were analyzed by gel electrophoresis after an incubation at 17°C for 30 min with each d*N*′TP (where *N*′=A, G, C, T, or **y**), 5′-labeled primer (15-mer), templates containing **x**



Scheme 1. Synthesis of the amidites of **x**

or natural bases at a specific position, and the Klenow fragment. As compared with the natural d*N*′TPs (where *N*′=A, G, C, or T), only d**y**TP was selectively incorporated opposite **x** in the template (N=**x**), and the insertion product was observed as a band corresponding to a 16-mer on the gel (Fig. 2, lanes 7–11). The *C*-nucleotide, d**y**TP, was also incorporated opposite A and G (Fig. 2, lanes 3 and 4); the unnatural base **y** is a shape analog of T and pairs with A, and wobble pairing between **y** and G may lead to the incorporation. However, in the presence of dTTP and dCTP, the insertion of d**y**TP opposite A or G was completely prevented (data not shown). Thus, d**y**TP was selectively incorporated opposite **x** by the Klenow fragment (*exo*$^+$). In the other insertion experiments using the Klenow fragment (*exo*$^-$), the incorporation of dTTP opposite **x** was observed (data not shown). Thus, the 3′-exonuclease activity of the polymerase may be involved in the specificity of the unnatural base pair formation.

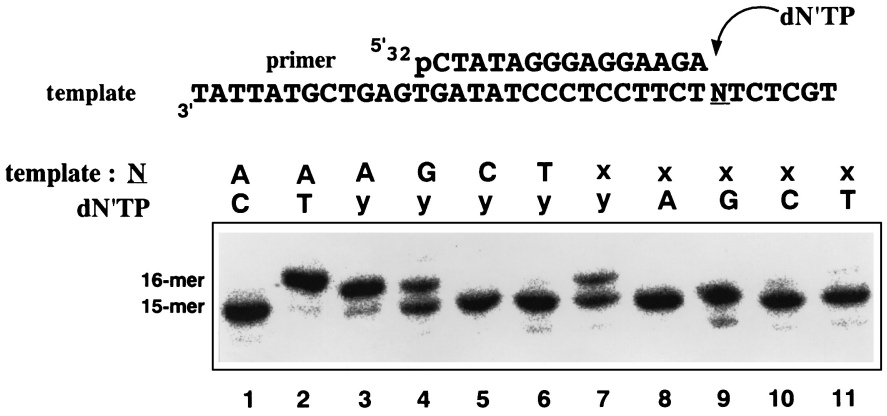


Fig. 2. Single-nucleotide insertion of d**y**TP opposite bases containing **x** in templates. The insertion was carried out at 17°C for 30 min using the Klenow fragment (2 units, 250 nM), 2 μM template, 1 μM 5′-^{32}P-labeled primer, and 0.15 mM dN′TP

We show here that the unnatural **x**:**y** pair fits into the shape complementarity theory and non-cognate base pair formations can be eliminated by introducing steric hindered groups into unnatural bases. To test the selectivity of the **x**:**y** pair formation in replication and transcription by DNA and RNA polymerases, more precise kinetic and thermodynamic analyses are in progress.

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15. ^{1}H NMR (270.16 MHz, D_2O) of dyTP; δ: 7.83 (d, 1H, H4, *J*=4.9 Hz), 7.35 (d, 1H, H6, *J*=4.9 Hz), 6.51 (t, 1H, H5, *J*=4.9 Hz), 5.17 (t, 1H, H1′, *J*=5.0 Hz), 4.56 (br, 1H, H3′), 4.06 (br, 1H, H4′), 3.99 (br, 2H, H5′ and H5′′), 2.19–2.33 (m, 1H, H2′), 1.81–1.98 (m, 1H, H2′′). ^{31}P NMR (109.36 MHz, D_2O) δ: −10.3 (m, 2P, P^1, P^3), −22.7 (m, 1P, P^2); UV (10 mM phosphate buffer pH 7.0): λ_{max}=298 nm (ε=7.6×10^3), 226 nm (ε=7.0×10^3), λ_{min}=247 nm, 211 nm; MS (ESI−): ($M-H^-$) 449.9.
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17. ^{1}H NMR (270.16 MHz, $CDCl_3$) of **6a**; δ: 8.14, 8.13 (s, 1H, H8, diastereomer), 7.72 (s, 1H, BzNH), 7.65–7.70 (m, 2H, Bz-*m*), 7.16–7.47 (m, 12H, Bz-*p*,*o*, DMTr), 6.70–6.75 (m, 4H, DMTr), 6.27–6.41 (m, 1H, H1′), 4.63–4.80 (m, 1H, H3′), 4.20–4.27 (m, 1H, H4′), 3.74 (s, 6H, OCH_3), 3.24–3.72 (m, 10H, H5′, H5′′, NC*H* $(CH_3)_2$, N-CH_3), 2.83–3.00 (m, 1H, H2′), 2.40–2.64 (m, 5H, H2′′, OCH_2CH_2CN), 1.06–1.19 (m, 12H, NCH (CH_3)$_2$); ^{31}P NMR (109.36 MHz, $CDCl_3$) δ: 149.25.
18. ^{1}H NMR (270.16 MHz, $CDCl_3$) of **6b**; δ: 7.81, 7.78 (s, 1H, H8, diastereomer), 7.70 (s, 1H, AcNH), 7.18–7.41 (m, 9H, DMTr), 6.75–6.81 (m, 4H, DMTr), 6.33 (t, 1H, H1′, *J*=6.5 Hz), 4.63–4.70 (m, 1H, H3′), 4.27–4.31 (m, 1H, H4′), 3.78 (s, 6H, OCH_3), 3.30–3.70 (m, 10H, H5′, H5′′, NC*H* $(CH_3)_2$, N-CH_3), 2.72–2.80 (m, 1H, H2′), 2.34–2.63 (m, 5H, H2′′, OCH_2CH_2CN), 1.09–1.20 (m, 12H, NCH (CH_3)$_2$); ^{31}P NMR (109.36 MHz, $CDCl_3$) δ: 149.28, 149.61.