Synthesis of DNA Templates Containing the Fifth Base, 2-Amino-6-(dimethylamino)purine, for Specific Transcription Involving Unnatural Base Pairs

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An unnatural nucleobase, pyridin-2-one (denoted as **y**), can be specifically incorporated into RNA opposite 2-amino-6-(dimethylamino)purine (denoted as **x**) in DNA templates by T7 RNA polymerase. For the easy preparation of DNA templates containing **x**, protecting groups for the 2-amino group of **x** were examined. Although the general N2 protecting groups, such as isobutyryl and acetyl, resisted removal by the usual treatment with concentrated ammonia at 55 °C for 6 h, the N2-phenoxyacetyl group was successfully removed from the protected oligomers. This DNA synthesis would be a useful method for preparation of DNA templates toward enzymatic incorporation of unnatural nucleosides into RNA at desired positions.

Expansion of the genetic alphabet would allow nucleic acids to increase their chemical, physical, and biological abilities.[1,2] We have created a novel base pair, pyridin-2-one (**y**) and 2-amino-6-(dimethylamino)purine (**x**) (Figure 1).[3]

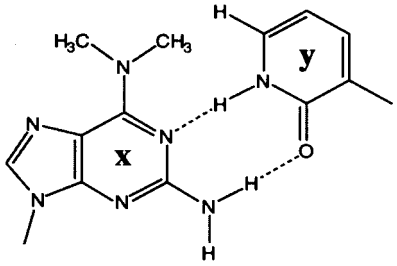

Figure 1. An unnatural base pair, **x** and **y**.

The ribonucleoside triphosphate of **y** can be site-specifically incorporated into RNA opposite **x** in DNA templates.[4] Thus, this base pair would be useful toward a general bioengineering method for the site-specific incorporation of modified nucleosides into RNA. To synthesize the DNA templates containing **x**, we initially employed an isobutyryl, benzoyl or acetyl group for the protection of the exocyclic 2-amino residue of the amidite of **x**. However, even the N2-isobutyryl, which is generally used for the N2 protecting group of guanosine in DNA synthesis, and acetyl groups were too stable for removal from the oligomers by the usual treatment with concentrated ammonia at 55 °C for 6 h (Figure 3a). The complete deprotection of these groups required drastic conditions involving treatment with concentrated ammonia at 80 °C for 10 h. To develop an easy method for the preparation of DNA templates containing several **x**'s with a long chain length (up to 100-mer), we examined base-labile groups, such as phenoxyacetyl,[5–7] chloroacetyl,[8] and dimethylaminomethylene,[9] for the N2 protection of **x**. Among these protecting groups, the N2-chloroacetyl group of **x** was unstable, and the dimethylaminomethylene was not efficiently introduced to the 2-amino group. Here, we show the phenoxyacetyl group as the suitable N2 protecting group of **x** and the convenient synthesis of DNA templates containing **x**.

The phenoxyacetyl group was successfully introduced to the 2-amino group of 2-amino-6-(dimethylamino)-9-(β-D-ribofuranosyl)purine (**1**), in which all of the hydroxy groups were temporarily covered with trimethylsilyl groups, using phenoxyacetyl chloride and hydroxybenzotriazole (Figure 2). The ribonucleoside (**2**) of N2-phenoxyacetylated **x** was converted to the 2'-deoxynucleoside (**4**)[10] by 3',5'-di-*O*-siloxylation, 2'-deoxygenation via the 2'-*O*-imidazolethiocarbonyl derivative (**3**), and de-silylation, in a 52% overall yield (Figure 2, from **2** to **4**). After 5'-*O*-dimethoxytritylation of **4**, the amidite of **x** (**5**) was obtained by phosphitylation. The amidite **5** was characterized by ^{1}H NMR and ^{31}P NMR.[11]

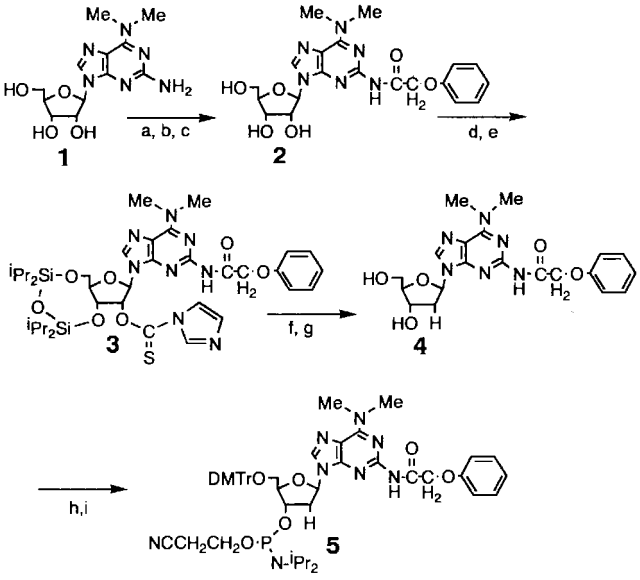

Figure 2. A scheme for the synthesis of amidite **5**. (a) Trimethylchlorosilane, pyridine, rt, 90 min. (b) Phenoxyacetyl-1-*O*-benzotriazolide, rt, 17 h. (c) dil NH_4OH, 0 °C, 15 min, 64% from **1**. (d) 1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane, DMF/pyridine, rt, 1 h, 94%. (e) Thiocarbonyldiimidazolide, DMF, rt, 13 h, 95%. (f) 2,2'-Azobisisobutyronitrile, tributyltin hydride, reflux, 1 h, 98%. (g) Tetrabutylammonium fluoride, THF, rt, 15 min, 59%. (h) 4,4'-Dimethoxytritylchloride, pyridine, rt, 2 h, ~100%. (i) 2-Cyanoethyl diisopropylchlorophosphoramidite, ethyldiisopropylamine, rt, 1 h, 84%.

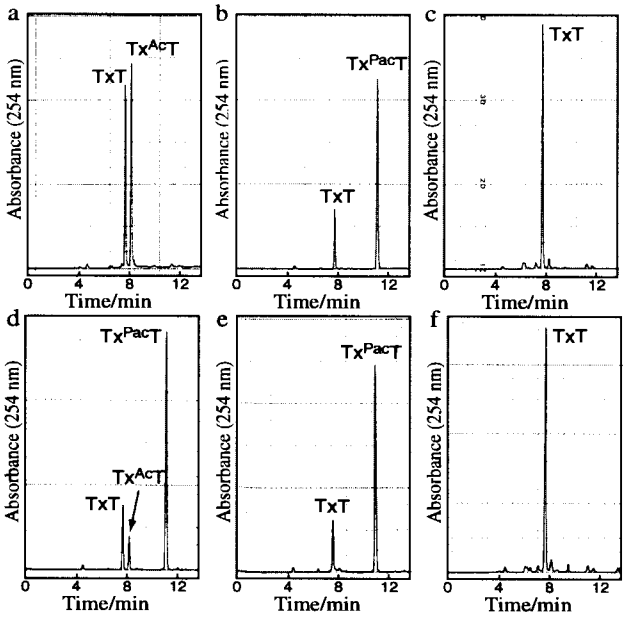

Figure 3. C18-HPLC profiles of TxT trimers after deprotection with NH_4OH at 55 °C for 6 h (a, c, and f) or at room temperature for 1 h (b, d, and e). Elution was performed with a linear gradient of 10 – 100% CH_3CN in 0.1 M TEAA for 15 min.

The synthesis of a trinucleotide (T**x**T) was examined using an automated DNA synthesizer (PE Biosystems, model 392). The coupling efficiency of amidite **5** was similar to that of the commercially available phosphoramidites. After the synthesis, the protected trimer was treated with ammonia for 1 h at room temperature for the removal of 2-cyanoethyl groups and the cleavage from the CPG-support. The solution was analyzed by reverse phase HPLC, and two peaks appeared corresponding to the phenoxyacetyl-protected trimer (T$\mathbf{x}^{\mathbf{Pac}}$T) and T**x**T, which was produced by partial deprotection of T$\mathbf{x}^{\mathbf{Pac}}$T (Figure 3b). While the N2-phenoxyacetyl group of guanosine was easily removed under the same conditions, the N2-protecting group of **x** in the oligomer was rather stable. Thus, the ammonia solution was heated at 55 °C for 6 h. By this treatment, the phenoxyacetyl group was completely removed and T**x**T was obtained (Figure 3c).

It has been suggested that the N2-phenoxyacetyl groups of guanosine in oligomers are partially replaced by acetyl groups during the capping step using acetic anhydride in DNA solid-phase synthesis.[5] We thus examined the replacement of the phenoxyacetyl group of **x** with the acetyl group by the capping reagent. After DNA synthesis, the T$\mathbf{x}^{\mathbf{Pac}}$T-bound CPG was treated with a capping solution (PE Biosystems' reagents: acetic anhydride, 1-methylimidazole, and pyridine in THF) for 30 min at room temperature. After ammonia treatment for 1 h at room temperature, the products were analyzed by HPLC. Even though the N2-phenoxyacetyl group of **x** is much more stable than that of guanine, a peak corresponding to the acetyl-substituted product (T$\mathbf{x}^{\mathbf{Ac}}$T) was observed (Figure 3d). The production of T$\mathbf{x}^{\mathbf{Ac}}$T was also confirmed by mass spectrometry. Thus, in order to avoid the undesirable replacement, the capping reagent was changed to a solution containing 63 mM 4-*t*-butylphenoxyacetic anhydride and 1 M 1-methylimidazole in THF. Using this capping reagent, the undesirable product was eliminated from the products (Figure 3e), and T**x**T was obtained after ammonia treatment at 55 °C for 6 h (Figure 3f). In addition, as the capping reagent, chloroacetic anhydride could be used instead of 4-*t*-butylphenoxyacetic anhydride (data not shown), although the chloroacetyl group was unstable for the N2 protection of **x**.

Oligonucleotides (35-mer and 109-mer) containing one or two **x**'s were synthesized by this method. Further enzymatic reactions using these DNA templates and modified **y** bases are currently in progress.

References and Notes

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10 Compound **4**: ^{1}H NMR (270 MHz, DMSO-d_6) δ 2.26, 2.65 (2H, m, H2',2''), 3.18–3.55 (8H, m, bs, -N(CH_3)$_2$, H5',5''), 3.83 (1H, m, H4'), 4.38 (1H, m, H3'), 4.89 (1H, t, 5'-OH, D_2O exchange, J = 5.1, 5.4 Hz), 5.05 (2H, s, -CO$\underline{CH_2}$O-), 5.27 (1H, d, 3'-OH, D_2O exchange, J = 3.6 Hz), 6.29 (1H, t, H1', J = 6.6, 6.8 Hz), 6.93 (3H, m, phenoxy), 7.28 (2H, m, phenoxy), 8.24 (1H, s, H8), 10.08 (1H, s, -NHCO-, D_2O exchange).
11 Compound **5**: ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.08–1.19 (12H, m, 2$\underline{Me_2}$CH), 2.41–2.75 (4H, m, -O$CH_2\underline{CH_2}$CN, H2',2''), 3.32–3.88 (12H, m, -N(CH_3)$_2$, -O$\underline{CH_2}CH_2$CN, 2$Me_2\underline{CH}$, H5',5''), 3.77 (6H, s, 2CH_3O-), 4.26 (1H, m, H4'), 4.70 (1H, m, H3'), 4.87 (2H, s, -CO$\underline{CH_2}$O-), 6.40 (1H, t, H1', J = 6.3, 6.4 Hz), 6.78, 7.01 (7H, m, phenoxy, DMT-), 7.21–7.41 (11H, m, DMT-), 7.83 (1H, s, s, H8); ^{31}P NMR (109 MHz, $CDCl_3$) δ 149.38, 149.53 (diastereoisomers).