Highly efficient enzymatic synthesis of 3'-deoxyapionucleic acid (apioNA) having the four natural nucleobases

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The synthesis of the 3'-deoxyapionucleoside 3''-triphosphates (apioNTPs) having the four natural nucleobases and their enzymatic incorporation into a DNA-DNA primer-template have been tried. Therminator DNA polymerase was shown to incorporate these apioNTPs effectively giving 43mer DNA-apioNA chimera.

Since nucleic acid aptamers are relatively easily isolated from iterative rounds of selection for binding to various targets with high affinities and selectivities, they are expected to be not only useful therapeutic agents but also biological tools.1 Therapeutic aptamers can act as nucleic acid antibodies against protein targets;2 they can also transport oligonucleotide therapeutic agents into target cells through receptor-mediated endocytosis.3 However, because the unmodified oligonucleotides selected are spontaneously degraded before reaching the target, time-consuming post-selection modifications for stabilizing them against nucleases abundant in biological fluids are usually required.4 Although isolation of fully-modified aptamers during the selection process is desired, it is nevertheless difficult to achieve because of the high substrate specificity of DNA/RNA polymerases required to incorporate modified nucleoside triphosphates, which are necessary to impart nuclease-resistance after incorporation into the oligonucleotides.5 Therefore, the development of a system that incorporates nuclease-resistant chemically modified nucleoside triphosphates having four natural nucleobases still remains a challenge.

Recently, α-L-threo-furanosyl nucleoside 3'-triphosphates (tNTPs) have been incorporated into a DNA-DNA primer-template by DNA polymerases, such as Therminator DNA polymerase, to afford DNA-TNA [α-L-threo-(3'→2') nucleic acid] chimeras up to a 75mer (25mer DNA + 50mer TNA) with >20% yield within 24 h, although the chemical structures of tNTPs are rather different from those of the natural 2'-deoxyribonucleoside 5'-triphosphates (dTTPs).6 Other sugar-modified nucleoside triphosphates such as GNA7, FNA8, LNA/BNA9 and HNA10, were synthesized and their incorporation into DNA by DNA polymerases was also investigated. These results have inspired us to further investigate other sugar-modified nucleoside triphosphates, which could be incorporated into the desired length of oligonucleotides, since oligonucleotides containing these sugar-modified nucleosides would be expected to be nuclease-resistant. Although TNAs are linked together through their 3'- and 2'-hydroxyl groups by a phosphodiester bond and their repeating unit consists of a backbone (5-bonds) one atom shorter than the natural DNA phosphodiester backbone (6-bonds) (Figure 1), tNTPs acted as substrates of DNA polymerases, but not well enough to be able to isolate aptamers. Therefore, we designed two new candidates that could be inserted and elongated much more efficiently by DNA polymerases. These are 2'-deoxy-2'-isonucleoside 5'-triphosphates (iNTPs) and 3'-deoxyapionucleoside 3'-triphosphates (apioNTPs), both of which have a 6-bond phosphodiester backbone similar to DNA although they are regioisomers. We and others have examined the incorporation of iNTPs into DNA by DNA polymerases.11 However, the shape of the iNTPs and local conformational changes of the duplex containing 2'-deoxy-2'-isonucleosides disrupted the elongation reaction to give the full length DNA-INNA product. Therefore, in this paper, we examined the enzymatic insertion and elongation reactions of apioNTPs, which are one-carbon longer homologues of the tNTPs at the 3' position.

The synthesis of the 3'-deoxyapionucleosides having the four natural nucleobases was carried out using the previous methods12 with a slight modification, and their 3' triphosphates (apioNTPs) were prepared (see supporting

Figure 1. Structures of DNA, TNA, INA, apioNA (A) and apioNTP (B).
Addition of Mn$^{2+}$ (1.25 mM) to the enzyme reaction mixture did not show any dramatic effects on the single nucleotide insertion reaction of the apioNTPs. However, we found the optimized primer extension reaction conditions, which were performed using dNTPs or apioNTPs at Mn$^{2+}$ (Figure 3), the full length band was confirmed by MALDI-TOF mass spectroscopy at $m/z$ 8401.8 (Figure S2). Consequently, we found the optimized primer extension reaction conditions, which were 0.8 μM primer-template duplex and 1.7 μM (0.2 U/μL) Thermopol DNA polymerase in the ThermoPol buffer containing 1.25 mM MnCl$_2$ at 44 °C for 1 h.

In order to evaluate the substrate ability of dNTP and apioNTP quantitatively, we determined the kinetic parameters ($K_m$, $V_{max}$; see supporting information) of every triphosphate at various concentrations in the presence or absence of Mn$^{2+}$, and the data are shown in Table 1. In the absence of Mn$^{2+}$, the relative efficiency of incorporation of dTTP into the primer was about twice that of the apioTTP (100 vs 46). The difference is presumably a reflection of their $K_m$ values (0.022 vs 0.058 μM), but not that of their $V_{max}$ values (7.4 vs 9.4 %·min$^{-1}$). Other nucleotides showed a similar effect with thymine nucleotides, namely relatively larger differences in the $K_m$ values (1.5-3.5 times) and smaller differences in the $V_{max}$ values (1.2-1.3 times) between dNTPs and apioNTPs, respectively. These quantitative analyses revealed that Thermopol incorporated the apioNTPs into the DNA/DNA primer/template duplex with 2-5 times lower relative efficiency than the dNTPs. The effect of the addition of Mn$^{2+}$ was next investigated by using single nucleotide insertion reactions. As can be seen from Table 1, the polymerase incorporated apioTTP with almost the same efficiency in the presence or absence of Mn$^{2+}$ (relative efficiency; 48 vs 46), and also with similar $K_m$ (0.086 vs 0.058 μM) and $V_{max}$ (14.0 vs 9.4 %·min$^{-1}$) values. Other nucleotides showed similar results with little difference in $K_m$ (0.4-1.6 times) and $V_{max}$ (0.4-1.5 times) values. Therefore, the addition of Mn$^{2+}$ to the enzyme reaction mixture did not show any dramatic effects on the single nucleotide insertion reaction of the apioNTP.

It is obvious that the elongation reactions of the apioNTPs afford the full length product as essentially one band (lanes 6 and 7). The enzyme concentration was also optimized in Figure S1, the higher concentration of Thermopol DNA polymerase (0.2 U/μL; 1.7 μM) was needed for efficient elongation of apioNA.

**Figure 3.** Primer extension reactions with various reaction temperatures. The reactions were performed with same reaction conditions in general method (see supporting information) except for reaction temperature; 74, 64, 54, 44, and 34 °C (lanes 3-7). Lane 1: primer; Lane 2: control.
Table 1. The kinetic parameters of dNTPs and apioNTPs in the absence
or presence of Mn²⁺ with Therminator DNA polymerase
d

<table>
<thead>
<tr>
<th>NTP (MnCl₂)</th>
<th>[Kₐ] (µM)</th>
<th>Vₘₐₓ</th>
<th>Vₘₐₓ/Kₐ relative efficiency</th>
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<tbody>
<tr>
<td>dTTP</td>
<td>0.022 ± 0.0075</td>
<td>7.4 ± 1.7</td>
<td>3.5 x 10⁸</td>
</tr>
<tr>
<td>apioTTP</td>
<td>0.058 ± 0.026</td>
<td>9.4 ± 1.0</td>
<td>1.6 x 10⁷</td>
</tr>
<tr>
<td>dCTP</td>
<td>0.086 ± 0.020</td>
<td>14.0 ± 1.9</td>
<td>1.7 x 10⁷</td>
</tr>
<tr>
<td>apioCTP</td>
<td>0.040 ± 0.012</td>
<td>5.9 ± 0.74</td>
<td>1.5 x 10⁸</td>
</tr>
<tr>
<td>dATP</td>
<td>0.090 ± 0.020</td>
<td>4.8 ± 1.4</td>
<td>5.4 x 10⁶</td>
</tr>
<tr>
<td>apioATP</td>
<td>0.083 ± 0.003</td>
<td>4.5 ± 1.4</td>
<td>7.1 x 10⁶</td>
</tr>
<tr>
<td>dGTP</td>
<td>0.022 ± 0.0078</td>
<td>7.1 ± 0.82</td>
<td>3.4 x 10⁴</td>
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<tr>
<td>apioGTP</td>
<td>0.078 ± 0.0015</td>
<td>5.5 ± 0.54</td>
<td>7.1 x 10⁷</td>
</tr>
<tr>
<td></td>
<td>0.031 ± 0.007</td>
<td>2.4 ± 0.14</td>
<td>8.0 x 10⁶</td>
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</table>

[a] Assay conditions: Reactions were initiated by adding 1 L of a dNTP or apioNTP solution to 9 L of the reaction mixture containing a 0.8 L of 5'-FAM labeled primer-template complex, 42.5 mM (0.005 U/L) Therminator DNA polymerase in the ThermoPol buffer (see supporting information) in the absence or presence of 1.25 mM MnCl₂ incubating for 3 min at 74 °C, and quenching with the addition of 10 L of loading buffer.

by Therminator were evidently accelerated in the presence of Mn²⁺ as shown in Figure 2. Therefore, we hypothesized that Mn²⁺ influenced the elongation reaction after incorporation of several apionucleotides into the DNA primer. To examine this hypothesis, a chimera oligonucleotide primer (5'-FAM 20mer DNA + 4mer apioNA) was synthesized by the usual phosphoramidite method except for the coupling time of the 15 h - L-threofuranosyl nucleoside 3'-triphosphates (tNTPs) at the 3'-position having the four natural bases (T, C, A and G) and also identified Therminator DNA polymerase as a polymerase that is capable of highly efficient enzymatic apioNA synthesis. Moreover we found that Mn²⁺ and lower temperatures are important for longer elongation of apioNA but are not required for a single nucleotide insertion reaction into a DNA-DNA duplex (Figure 3 and Table 1). A longer elongation polymerase reaction (22mer apioNA elongation) has also been achieved at 34 or 44 °C within 1 h. Therefore, the apioNTPs are some of the most effective sugar-modified triphosphates for enzymatic polymerization by DNA polymerase. Studies of apioNA templated apioNA synthesis by Therminator DNA polymerase are now under investigation.

Notes and references
† Electronic Supplementary Information (ESI) available: Experimental details of synthesis, 1H and 31P NMR of apioNTPs, ODN synthesis, elongation reactions, single nucleotide insertion reactions, and MALDI-TOF mass spectrum of full length 27mer elongated product. See DOI: 10.1039/b000000x/

3 (a) N. S. Que-Gewirth, B. A. Sullenger, Gene Ther. 2007, 14, 283; (b) A. C. Yan, M. Levy, RNA Biol. 2009, 6, 316.