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<th>Highly efficient enzymatic synthesis of 3'-deoxyapionucleic acid (apioNA) having the four natural nucleobases</th>
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<td>Kataoka, Mayumi; Kouda, Yasuo; Sato, Kousuke; Minakawa, Noriaki; Matsuda, Akira</td>
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Supplementary Information

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Contents:

1. General methods (S2-S5).
2. ApioNTP synthesis (S6-S8).
3. Supporting Information Figure 1. A PAGE experiment for the elongation reaction using all four apioNTPs under various concentrations of Therminator DNA polymerase (20mer primer and 27mer template) (S9).
4. Supporting Information Figure 2. MALDI-TOF mass spectrum of the elongated product (20mer primer and 27mer template) (S10).
5. Supporting Information Figure 3. A PAGE experiment for the longer elongation reaction using all four apioNTPs (21mer primer and 43mer template) (S11).
6. $^1$H and $^{31}$P NMR spectra of the new compounds (S12-S19).
7. References (S20).
**General.** $^1$H, $^{13}$C and $^{31}$P NMR spectra were obtained on a JEOL ECX-400P, JEOL ECA-500 or JEOL AL-400 and were reported in parts per million ($\delta$) relative to residual solvent signal for $^1$H NMR spectra, 1,4-dioxane signal (67.2 ppm) as internal standard for $^{13}$C NMR spectra, and 85% phosphoric acid (0.0 ppm) as external standard for $^{31}$P NMR spectra. Coupling constants ($J$) were reported in Hertz (Hz). Abbreviations of multiplicity were as follow: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad. Data were presented as follows: chemical shift (multiplicity, integration, coupling constant). LR- and HR-MS spectra were obtained on a JEOL JMS-HX110, JEOL JMS-700TZ or JEOL JMS-T100LP. UV spectra were measured with Shimadzu UV Visible Spectrophotometer UV-2450. pH was measured with Beckman Coulter F360 pH meter. DNA oligomers were prepared on an Applied Biosystems 3400 DNA Synthesizer. HPLC was performed with Shimadzu LC-10AD-VP or LC-20AB (pump), Shimadzu SPD-M10A-VP or SPD-M20A (UV-visible detector), Shimadzu CTO-10AS-VP or CTO-20A (column oven), CLASS-VP system, LabSolutions (system controller). Sep-pak Plus C18 Cartridge was purchased from Waters. YMC J’sphere ODS-M80 (150 × 4.6 mm) was used as reversed-phase C18 HPLC columns. DNA polymerases were purchased from Takara Shuzo (Klenow (exo$^-$), exTaq), Promega (Tfl, Pfu), New England Biolabs (Vent (exo$^-$), Deep Vent (exo$^-$), Therminator, Therminator II) and Toyobo (KOD Dash). PAGE analysis and purification were performed with 220 × 220 × 0.5 mm and 220 × 220 × 1.5 mm sized gel, respectively. MALDI-TOF mass spectrum was measured with Bruker Daltonics Ultraflex TOF/TOF. Incubation of enzymatic reactions was performed with EYELA MG-1200.

**Optimized methods of the primer extension experiments (20mer primer and 27mer**
The following mixture was used: 0.8 μM duplex consisting of a 5'-FAM labeled primer and a DNA template, Therminator DNA polymerase (0.2 U/μL, 1.7 μM), 200 μM apioNTPs in the ThermoPol buffer [20 mM Tris-HCl (pH 8.8) containing 10 mM KCl, 10 mM (NH₄)₂SO₄ and 2 mM MgSO₄ and 0.1% Triton X-100] containing 1.25 mM MnCl₂ in a final volume of 10 μL. The reaction was performed at 44 °C for 1 h and quenched with the addition of 10 μL loading buffer [1 × TBE buffer (89 mM Tris/89 mM boric acid/2 mM EDTA), 7 M urea, 0.05% xylene cyanol and bromophenol blue]. The reaction mixture (8 μL) was resolved by electrophoresis at 800 V for 5 h using a 20% (19:1) denaturing polyacrylamide gel containing 7 M urea, and the gels were quantified with FLA-2000 (FUJIFILM).

MALDI-TOF mass spectrum of the elongated product (20mer primer and 27mer template). The following mixture was used: 0.8 μM duplex consisting of a primer and a DNA template, Therminator DNA polymerase (0.2 U/μL, 1.7 μM), 200 μM apioNTPs in the ThermoPol buffer containing 1.25 mM MnCl₂ in a final volume of 40 μL. The reaction was performed at 44 °C for 1 h and quenched with the addition of 40 μL of 10 M urea. The mixture was desalted, and counter cations of phosphate groups were exchanged to ammonium salts on ODS column (YMC disposable SPE). The oligonucleotide was then analyzed by MALDI-TOF mass using 3-hydroxypicolinic acid and bis-ammonium citrate as matrix.

Single nucleotide insertion reactions using the steady-state method (20mer primer and 27mer template). Insertion reactions were initiated by adding 1 μL of dNTP or apioNTP solution (0.001-1 μM) to a reaction mixture containing a mixture of the 0.8
µM duplex consisting of a 5'-FAM labeled primer and a DNA template, and Therminator (0.005 U/µL, 42.5 nM) in the ThermoPol buffer [and 1.25 mM MnCl₂ for apioNTP (Mn (+))] in a final volume of 10 µL. The amount of dNTP or apioNTP was adjusted to 25% maximum insertion reaction. The reaction was performed at 74 °C for 3 min and quenched by adding 10 µL of the stop buffer. The reaction mixture (8 µL) was resolved by electrophoresis at 800 V for 5 h using a 20% (19:1) denaturing polyacrylamide gel containing 7 M urea, and the gels were quantified with FLA-2000 (FUJIFILM). Reaction velocities were calculated as the yield of reaction divided by reaction time. Kinetic parameters (Kₘ and Vₘₐₓ) were determined by linear regression analysis of a Hanes-Woolf plot[1] with an average of three independent experiments.

**Synthesis and purification of the oligonucleotides.** Oligonucleotides were synthesized with a DNA Synthesizer (Applied Biosystem Model 3400) by using 3'-deoxyapionucleoside phosphoramidites or commercially available 2'-deoxyribonucleoside phosphoramidite units at 1 µmol scale following the standard procedure described. Each of 3'-deoxyapionucleoside phosphoramidites was used at concentration of 0.1 M in dry MeCN, and the coupling time was extended to 15 min. After completion of the synthesis, the CPG support was treated with concentrated NH₄OH (55 °C, 12 h) and filtered off, and the filtrate was concentrated. The residue was dissolved in 500 µL of 90% formamide and purified by electrophoresis at 400 V for 8 h using a 20% (29 : 1) denaturing polyacrylamide gel (220 × 220 × 1.5 mm). The desired band was cut and extracted in TE buffer at room temperature for overnight. The extract was desalted on Sep-pak C18 column and eluted with aqueous 50% CH₃CN to obtain the desired oligonucleotide.
Single nucletide insertion reactions using the steady-state method (24mer primer and 27mer template). Insertion reactions were initiated by adding 1 µL of apioATP solution (0.001-1 µM) to a reaction mixture containing a mixture of the 0.8 µM duplex consisting of a 5'-FAM labeled primer and a DNA template, and Therminator (0.2 U/µL, 1.7 µM) in the ThermoPol buffer [and 1.25 mM MnCl₂ for apioATP (Mn (+))] in a final volume of 10 µL. The amount of apioATP was adjusted to 25% maximum insertion reaction. The reaction was performed at 74 °C for 3 min and quenched by adding 10 µL of the stop buffer. The reaction mixture (8 µL) was resolved by electrophoresis at 800 V for 4 h using a 20% (29:1) denaturing polyacrylamide gel containing 7 M urea, and the gels were quantified with FLA-2000 (FUJIFILM). Reaction velocities were calculated as the yield of reaction divided by reaction time. Kinetic parameters ($K_m$ and $V_{max}$) were determined by linear regression analysis of a Hanes-Woolf plot$^{[1]}$ with an average of three independent experiments.
The synthesis of 3'-deoxyapionucleoside 3''-triphosphates (apioNTPs)

1-{(2R,3R,4R)-3-Hydroxy-4-[(triphosphoryl)methyl]tetrahydrofuran-2-yl}thymine sodium salt (apioTTP): A solution of 2'-O-acetyl-3'-deoxy-D-apiothymidine[^2] (69 mg, 0.24 mmol) in pyridine/1,4-dioxane (1/3, 960 µL) was treated with 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (73 mg, 0.36 mmol) in 1,4-dioxane (480 µL) at room temperature for 10 min. The reaction mixture was treated with 0.5 M solution of bis(tri-n-butylammonium)pyrophosphate in dry DMF (960 µL, 0.48 mmol) and tri-n-butylamine (320 µL, 1.3 mmol) at room temperature for 10 min. The reaction mixture was treated with 1% iodine in pyridine/water (98/2) (ca. 3 mL) for 5 min, and which was treated with 5% aqueous solution of NaHSO₃ (ca. 2 mL) for additional 30 min. The reaction mixture was concentrated in vacuo, and the residue was treated with saturated aqueous ammonia solution (20 mL) at room temperature overnight. The reaction mixture was concentrated in vacuo, and the residue was dissolved in H₂O (300 mL), and the solution applied to a DEAE Sephadex column, which was eluted with a linear gradient of 750 mL each of H₂O and 1.0 M TEAB (pH 8.0). Fractions containing desired product were concentrated in vacuo and coevaporated with H₂O/EtOH (1/1). The residue was dissolved in H₂O (10 mL), and the solution was applied to a column of DIAION PK 212 (H⁺ form), which was eluted with H₂O. The eluate was applied to a DIAION WK 40 (Na⁺ form), which was eluted with H₂O. Fractions containing apioTTP were concentrated in vacuo to give apioTTP (51%) as a white solid. ¹H NMR (D₂O, 500 MHz) δ 7.55 (1H, s, H-6), 5.84 (1H, d, H-1', J₁',₂' = 6.4 Hz), 4.45 (1H, dd, H-2', J₂',₁' = 6.4, J₂',₃' = 6.8 Hz), 4.30 (1H, dd, H-4'a J₆₇ = 8.6, J₄₇₃₇ = 8.6 Hz), 4.15 (1H, dd,
H-4'b $J_{\text{gem}} = 8.6$, $J_{4'b,3'} = 9.7$ Hz), 4.13 (2H, dd, H-3''), $J_{\text{gem}} = 9.7$, $J_{3'',3'} = 7.4$ Hz), 2.74 (1H, m, H-3'), 1.89 (3H, s, Me). $^3$P NMR (D$_2$O, 202 MHz) δ $-10.45$, $-10.67$, $-22.79$. ESIMS-LR $m/z$ 481 [M-H]; ESIMS-HR calcd for C$_{10}$H$_{16}$N$_2$O$_{14}$P$_3$ 480.9814, found 480.9802.

1-{(2R,3R,4R)-3-Hydroxy-4-[(triphosphoryl)methyl]tetrahydrofuran-2-yl}cytosine sodium salt (apioCTP, 54%, as a white solid) was obtained from 2'-O-acetyl-N'-benzoyl-3'-deoxy-$\alpha$-apiocytidine$^{[2]}$ (129 mg, 0.34 mmol) as described for the synthesis of apioTTP.$^1$H NMR (D$_2$O, 500 MHz) δ 7 73 (1H, d, H-6, $J_{6,5} = 7.4$ Hz), 6.05 (1H, d, H-5, $J_{5,6} = 7.4$ Hz), 5.85 (1H, d, H-1', $J_{1',2'} = 5.7$ Hz), 4.41 (1H, dd, H-2', $J_{2',1'} = 5.7$, $J_{2',3'} = 6.3$ Hz), 4.29 (1H, dd, H-4'a $J_{\text{gem}} = 8.6$, $J_{4'a,3'} = 8.0$ Hz), 4.12 (1H, dd, H-4'b $J_{\text{gem}} = 8.6$, $J_{4'b,3'} = 8.0$ Hz), 4.06 (2H, dd, H-3'', $J_{\text{gem}} = 5.7$, $J_{3'',3'} = 5.2$ Hz), 2.70 (1H, m, H-3'). $^3$P NMR (D$_2$O, 202 MHz) δ $-6.33$, $-10.03$, $-21.91$. ESIMS-LR $m/z$ 466 [M-H]; ESIMS-HR calcd for C$_9$H$_{15}$N$_3$O$_{13}$P$_3$ 465.9818, found 465.9817.

9-{(2R,3R,4R)-3-Hydroxy-4-[(triphosphoryl)methyl]tetrahydrofuran-2-yl}adenine sodium salt (apioATP, 55%, as a white solid) was obtained from 2'-O-acetyl-N$^6$-benzoyl-3'-deoxy-$\alpha$-apioadenosine$^{[2]}$ (78 mg, 0.20 mmol) as described for the synthesis of apioTTP.$^1$H NMR (D$_2$O, 500 MHz) δ 35 8 (1H, s, H-2), 8.11 (1H, s, H-8), 5.94 (1H, d, H-1', $J_{1',2'} = 5.4$ Hz), 4.84 (1H, d, H-2', $J_{2',1'} = 5.4$, $J_{2',3'} = 6.9$ Hz), 4.13 (2H, dd, H-3''), $J_{\text{gem}} = 9.7$, $J_{3'',3'} = 7.4$ Hz), 2.74 (1H, m, H-3'), 1.89 (3H, s, Me). $^3$P NMR (D$_2$O, 202 MHz) δ $-10.45$, $-10.67$, $-22.79$. ESIMS-LR $m/z$ 481 [M-H]; ESIMS-HR calcd for C$_{10}$H$_{16}$N$_2$O$_{14}$P$_3$ 480.9814, found 480.9802.

S7
Hz), 4.36 (1H, dd, H-4'a \( \lambda_{gem} = 9.2, \lambda_{4'a,3'} = 8.7 \) Hz), 4.19 (1H, dd, H-4'b \( \lambda_{gem} = 9.2, \lambda_{4'b,3'} = 8.7 \) Hz), 4.18 (2H, dd, H-3'', \( \lambda_{gem} = 8.6, \lambda_{3'',3'} = 7.4 \) Hz), 2.86 (1H, m, H-3'). 3 P NMR (D₂O, 202 MHz) \( \delta \) –8.43, –10.17, –21.68. ESIMS-LR \( m/z \) 490 [M-H]; ESIMS-HR calcd for C₁₀H₁₃N₅O₁₂P₃ 489.9930, found 489.9913.

9-\{(2R,3R,4R)-3-Hydroxy-4-[(triphosphoryl)methyl]teterahydropuran-2-yl\}guanine sodium salt (apioGTP, 44%, as a white solid) was obtained from 2'-O-acetyl-N²-benzoyl-3'-deoxy-D-apioguanosine[^2] (37 mg, 0.09 mmol) as described for the synthesis of apioTTP. ¹H NMR (D₂O, 500 MHz) \( \delta \) 8 (1H, s, H-8), 5.78 (1H, d, H-1', \( \lambda_{1',2'} = 5.7 \) Hz), 4.91 (1H, dd, H-2', \( \lambda_{2',1'} = 5.7, \lambda_{2',3'} = 7.4 \) Hz), 4.31 (1H, dd, H-4'a \( \lambda_{gem} = 8.6, \lambda_{4'a,3'} = 8.6 \) Hz), 4.21 (2H, dd, H-3'', \( \lambda_{gem} = 8.6, \lambda_{3'',3'} = 8.6 \) Hz), 4.19 (1H, dd, H-4'b \( \lambda_{gem} = 8.6, \lambda_{4'b,3'} = 8.6 \) Hz), 2.86 (1H, m, H-3'). ³ P NMR (D₂O, 202 MHz) \( \delta \) –5.28, –10.00, –20.97. ESIMS-LR \( m/z \) 506 [M-H]; ESIMS-HR calcd for C₁₀H₁₃N₅O₁₃P₃ 505.9879, found 505.9874.
A PAGE experiment for the elongation reaction using all four apiONTPs under various concentrations of Therminator DNA polymerase (20mer primer and 27mer template).

**Figure S1.** A PAGE experiment for the elongation reaction in the presence of 1.25 mM MnCl₂ using all four apiONTPs at 44 °C for 1 h under various concentrations of Therminator DNA polymerase. Lane 1; primer, lanes 2-7; the elongation product by using apiONTPs under various concentrations of Therminator DNA polymerase. Sequence of the primer-template complex and apiON inline elongation product were shown in Figure 2A.
MALDI-TOF mass spectrum of elongated product (20mer primer and 27mer template).

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**Figure S2.** MALDI-TOF mass spectrum of the elongation product by using apioNTPs. The Spectrum was obtained from elongation reaction followed by ODS column purification.
A PAGE experiment for the longer elongation reaction using all four apioNTPs (21mer primer and 43mer template).

A) Sequence of the primer (21mer)-template (43mer) complex and the apioNA elongation product. Elongated apioNA is shown in bold letters.

5'-32P-d(TAATACGACTCATATAGGGCC)-3'
3' d(ATTATGCTGAGTGATATCCGGGGCCGTCGACCACGCAAAATG)-5'

5'-32P-d(TAATACGACTCATATAGGGCCGACTGTCGTAC)-3'
3' d(ATTATGCTGAGTGATATCCGGGGCCGTCGACCACGCAAAATG)-5'

B) Primer extension experiments: 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), respectively. Lane 1; primer, lane 2; control.

Figure S3. A) Sequence of the primer (21mer)-template (43mer) complex and the apioNA elongation product. Elongated apioNA is shown in bold letters. B) Primer extension experiments: 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), respectively. Lane 1; primer, lane 2; control.
$^1$H and $^{31}$P NMR spectra of apioNTPs.
References
