Electronic Supplementary Information

Synthesis of DNA fragments containing 2’-deoxy-4’-selenonucleoside units using DNA polymerases: comparison of dNTPs with O, S and Se at the 4’-position in replication

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General methods

Physical data were measured as follows: $^1$H, $^{13}$C, and $^{31}$P NMR spectra were recorded at 400 MHz (Bruker AV 400N) instruments in CDCl$_3$, DMSO-$d_6$ or D$_2$O as the solvent with tetramethylsilane or phosphoric acid as an internal standard. Chemical shifts are reported in parts per million (δ), and signals are expressed as s (singlet), d (doublet), t (triplet), m (multiplet), or br (broad). Mass spectra were measured on Waters LCT Premier 2695 (ESI). TLC was done on Merck Kiesel gel F254 precoated plates. Silica gel used for column chromatography was KANTO Chemical silica gel 60N (neutral).

Natural dNTPs were purchased from GE Healthcare, Japan. The following DNA polymerases were purchased: KF exo– DNA polymerase from Promega; Deep Vent exo–, Deep Vent; Vent exo–, Terminator, 9°N$_m$ DNA polymerases from New England Biolabs; KOD Dash DNA polymerase from TOYOBO. ODNs used in this study were purchased from FASMAC, Japan.
1-(3-O-Acetyl-2-deoxy-5-O-dimethoxytrityl-4-seleno-β-D-ribofuranosyl)thymine (S1).

To a solution of 4'-selenothymidine (SeT) \(^5\) (260 mg, 0.85 mmol) in dry pyridine (5 mL) was added DMTrCl (344 mg, 1.0 mmol), and the mixture was stirred for 4.5 h at room temperature. Then \(\text{Ac}_2\text{O}\) (240 \(\mu\)L, 2.5 mmol) was added to a solution, and the mixture was stirred for 5.5 h at room temperature. The reaction was quenched by addition of ice. The reaction mixture was partitioned between EtOAc and \(\text{H}_2\text{O}\), and the organic layer was washed with saturated aqueous NaHCO\(_3\) and brine, dried (Na\(_2\)SO\(_4\)) and concentrated in vacuo. The residue was purified by a silica gel column, eluted with hexane/AcOEt (2:1-1:5), to give S1 (440 mg, 80% as a white solid): \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 8.09 (1 H, br s, exchangeable with D\(_2\)O, NH), 7.45 (2 H, m, Ph), 7.29–7.37 (8 H, m, Ph and H-6), 6.84 (4 H, m, Ph), 6.62 (1 H, dd, \(J = 6.6, 9.1\) Hz, H-1’), 5.50 (1 H, m, H-3’), 3.87 (1 H, m, H-4’), 3.79 (6 H, s, OMe \(\times 2\) ), 3.53 (1 H, dd, \(J = 5.4, 10\) Hz, H-5’a), 3.34 (1 H, dd, \(J = 6.6, 10\) Hz, H-5’b), 2.48 (1 H, ddd, \(J = 3.1, 6.6, 14\) Hz, H-2’a), 2.18 (1 H, m, H-2’b), 2.08 (3 H, s, OAc), 1.67 (3 H, d, \(J = 1.0\) Hz, CH\(_3\)); \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\) 170.21, 162.99, 158.74, 150.26, 144.23, 136.67, 135.51, 135.39, 130.18, 130.15, 128.29, 127.98, 127.15, 113.24, 112.06, 87.01, 78.00, 65.47, 55.25, 54.27, 50.25, 40.85, 21.17, 12.41; ESI-LRMS \(m/z\) 673 (M+Na); ESI-HRMS calcd for \(\text{C}_{33}\text{H}_{55}\text{N}_2\text{O}_7\text{Se}\) (M+H) 651.1610, found 651.1609.

1-(2-Deoxy-5-O-dimethoxytrityl-4-seleno-β-D-ribofuranosyl)thymine (S2).

To solution of S1 (140 mg, 0.22 mmol) in CHCl\(_3\) was added trichloroacetic acid (TCA) (60 \(\mu\)L) and the mixture was stirred for 2 h at room temperature. The reaction was quenched by addition of saturated aqueous NaHCO\(_3\). The reaction mixture was partitioned between CHCl\(_3\) and \(\text{H}_2\text{O}\), and the organic layer was washed with saturated aqueous NaHCO\(_3\) and brine, dried (Na\(_2\)SO\(_4\)) and concentrated in vacuo. The residue was purified by a silica gel column, eluted with hexane/CHCl\(_3\) (3:1) to give S2 (137 mg, 75% as a white solid): \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 8.09 (1 H, br s, exchangeable with D\(_2\)O, NH), 7.63 (2 H, m, Ph), 7.22 (8 H, m, Ph and H-6), 6.83 (4 H, m, Ph), 6.64 (1 H, dd, \(J = 6.6, 9.1\) Hz, H-1’), 5.53 (1 H, m, H-3’), 3.92 (1 H, m, H-4’), 3.25 (6 H, s, OMe \(\times 2\) ), 3.69 (1 H, dd, \(J = 5.4, 10\) Hz, H-5’a), 3.34 (1 H, dd, \(J = 6.6, 10\) Hz, H-5’b), 2.48 (1 H, ddd, \(J = 3.1, 6.6, 14\) Hz, H-2’a), 2.18 (1 H, m, H-2’b), 2.06 (3 H, s, OAc), 1.66 (3 H, d, \(J = 1.0\) Hz, CH\(_3\)); \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\) 170.16, 162.99, 158.76, 150.27, 144.23, 136.58, 135.51, 135.39, 130.18, 128.30, 127.99, 127.15, 113.24, 112.07, 87.02, 78.00, 65.47, 55.25, 54.27, 50.25, 40.82, 21.17, 12.41; ESI-LRMS \(m/z\) 673 (M+Na); ESI-HRMS calcd for \(\text{C}_{33}\text{H}_{55}\text{N}_2\text{O}_7\text{Se}\) (M+H) 651.1610, found 651.1609.
and concentrated in vacuo. The residue was purified by a silica gel column, eluted with 0–6% MeOH in CHCl₃, to give S2 (53 mg, 68% as a white solid): ¹H NMR (CDCl₃) δ 8.06 (1 H, br s, exchangable with D₂O, NH), 7.66 (1 H, d, J = 1.3 Hz, H-6), 6.62 (1 H, dd, J = 6.5, 9.3 Hz, H-1’), 5.46 (1 H, m, H-3’), 4.00 (1 H, dd, J = 6.8, 14 Hz, H-5’a), 3.86 (2 H, m, H-4’ and H-5’b), 2.56 (1 H, ddd, J = 3.8, 6.5, 14 Hz, H-2’a), 2.47 (1 H, ddd, J = 4.0, 9.3, 14 Hz, H-2’b), 2.11 (3 H, s, OAc), 1.95 (3 H, d, J = 1.3 Hz, CH₃); ¹³C NMR (CDCl₃) δ 170.57, 163.39, 150.47, 137.03, 112.08, 78.46, 64.17, 54.98, 52.77, 41.38, 21.20, 12.82; ESI-LRMS m/z 371 (M+Na); ESI-HRMS calcd for C₁₂H₁₇N₂O₂Se (M+H) 349.0303, found 349.0305.

4’-Selenothymidine 5’-triphosphate (SeTTP).

To a solution of S2 (50 mg, 0.14 mmol) in dry pyridine (150 µL) and 1,4-dioxane (450 µL) was added an 1 M solution of 2-chloro-4H-1,2,3-dioxaphosphorin-4-one in 1,4-dioxane (0.17 mmol, 170 µL). After 15 min, a 0.5 M solution of bis(tri-n-butylammonium)pyrophosphate in dry DMF (0.23 mmol, 460 µL) and tri-n-butylamine (0.63 mmol, 150 µL) were added, and the reaction mixture was stirred for 10 min. A solution of 1% iodine in pyridine/water (98/2, v/v) (ca. 5 mL) was then added. After 5 min excess iodine was decomposed by adding 5% aqueous solution of Na₂S₂O₃ (ca. 2 mL), and the reaction mixture was stirred for 5 min. The reaction mixture was concentrated in vacuo, and ammonium hydroxide (28%, 5 mL) was added to the residue. After 15 h, the solution was concentrated in vacuo, and the residue was dissolved in water (300 mL). The solution was applied to a DEAE Sephadex column, which was eluted with a linear gradient of 600 mL each of water and 1.0 M TEAB buffer. Fractions containing SeTTP were concentrated in vacuo, and the residue was coevaporated with EtOH. The residue was dissolved in water (5 mL), which was applied to a column of DIAION PK 212 (H⁺ form) then DIAION WK 20 (Na⁺ form), and the fractions containing SeTTP were concentrated in vacuo to give SeTTP as a Na salt (61 mg, 69% as a white solid): ¹H NMR (D₂O) δ 7.86 (1 H, s, H-6), 6.46 (1 H, dd, J = 6.6, 9.4 Hz, H-1’), 4.71 (1 H, m, H-3’), 4.05–4.24 (2 H, m, H-4’ and H-5’a), 3.90 (1 H, m, H-5’b), 2.48 (1 H, ddd, J = 2.8, 6.6, 14 Hz, H-2’a), 2.39 (1 H, ddd, J = 4.1, 9.4, 14 Hz, H-2’b), 1.88 (3 H, s, CH₃); ³¹P NMR (D₂O) δ −7.07, −11.24, −21.93; ESI-LRMS m/z 610 (M–H); ESI-HRMS calcd for C₁₆H₁₅N₂Na₃O₁₃P₃Se (M–H) 610.8489, found 610.8501.
**Primer extension reaction using various DNA polymerases**

Annealed 5’-FITC labeled primer (0.4 μM) and template (0.5 μM) mixture, SeTTP, TTP or TTP (50 μM), and DNA polymerases (0.04–0.05 units/μL) in a reaction buffer (20 μL) was incubated at 37 or 74 °C. After 60 min, 4 μL aliquots of the reaction mixture was sampled and added into the loading solution (4 μL) containing 10 M urea, 20% w/v sucrose, 0.1% SDS, 0.05% BPB in 1 × TBE. The samples were analyzed by 20% denaturing polyacrylamide gel electrophorese (PAGE) containing 8 M urea, which was visualized and analyzed with a bio-imaging analyzer (Molecular Imager FXpro system WOM).

**Primer extension reactions in the presence of SeTTP, STTP or TTP with dATP, dGTP and dCTP using random sequence was used as a template (Fig. S1)**

![Fig. S1 Denaturing polyacrylamide gel image of the products. The duplex of 5’-FITC labeled primer (0.4 μM) and template (0.5 μM), corresponding triphosphates (each 50 μM), and each DNA polymerase (0.04–0.05 units/μL) in a reaction buffer (20 μL) was incubated at 37 or 74 °C.](image-url)
Steady-state kinetics for the single-nucleotide insertion using KF exo–

A primer (20-mer) labeled with FITC at the 5’-end was annealed with a template (30-mer) in 50 mM Tris-HCl (pH 7.2) buffer containing 10 mM MgSO₄ and 0.1 mM DTT. The primer-template duplex solution (0.4 µM) was mixed with each dYTP solution (10–1000 µM). The mixture was incubated for more than 2 min at 37 °C, and then the reactions were initiated by adding an enzyme solution (0.01–0.1 unit/µL) to each duplex–dYTP mixture (10 µL) at 37 °C. The amount of enzyme, the reaction time (2–10 min), and the gradient concentration of dYTP were adjusted to give reaction extents of 20% or less. The reactions were quenched with 10 µL of a loading solution (10 M urea, 20% w/v sucrose, 0.1% SDS, 0.05% BPB in 1 × TBE). The diluted products were resolved by electrophoresis on a 20% polyacrylamide gel containing 8 M urea, and the gels were visualized with Molecular Imager FXpro system WOM (BioRad) equipped with Quantity One software. Relative velocities ($v_0$) were calculated as the extents of the reaction divided by the reaction time and were normalized to the duplex and enzyme concentration (0.4 µM, 0.02 unit/µL) for the various concentrations used. The kinetic parameters ($K_m$ and $V_{max}$) were obtained from Hanes–Woolf plots of [dYTP]/$v_0$ against [dYTP].

Schematic image of conformation changes of the sugar ring in thymidine analogue upon substituting O4’ with selenium or sulfur (Fig. S2)

Fig. S2 Bond lengths and angles were referred from *J. Am. Chem. Soc.*, 1992, 114, 9936–9943 (ref. 10) for 4’-O and 4’-S thymidine, and *Org. Lett.*, 2010, 12, 2242–2245 (ref. 5) for 4’-Se thymidine.
**PCR amplification**

The PCR was performed in 20 µL of corresponding buffer containing DNA polymerase (0.100–0.125 units/µL), 87 mer DNA template (5’-gggactagctacggtcctctattattgtgtaagtcgtaagtcacgggcaatctccagacctctataaatatttac-3’; 5.0 nM), 0.2 mM each of SeTTP, STTP or TTP with dATP, dGTP and dCTP, and 0.5 µM of primers (Forward; 5’-gacggaatataagctggtgg-3’, Reverse; 5’-taatacgacatataggaactagctacggtc-3’). The reaction mixtures were gently vortexed and then amplified using a thermal cycler. PCR was performed under the condition [94 °C, 15 sec; (94 °C, 30 sec; 50 °C, 30 sec; 72 °C, 30 sec, 10 min or 20 min) × 15 cycles; 72 °C, 15 min]. PCR products were analyzed by 6.4% PAGE and were quantified by measuring the fluorescence intensities of the corresponding bands by a Molecular Imager FX Pro (Bio–Rad). After a second PCR with dNTPs, the products were purified with High Pure PCR Product Purification Kit (Roche) and were sequenced with each of PCR primer by an ABI PRISM® 3100 Genetic Analyzer.

**Real-time RT-PCR amplification**

The PCR reaction (20 µL) was performed in KOD Dash reaction buffer with 2 µM each of TTP, SeTTP or STTP with dATP, dGTP and dCTP, 10 nM each primers, 100,000-fold diluted SYBR Green I, 50-fold diluted ROX as a reference dye, 0.1 unit/µL of KOD Dash DNA polymerase, and DNA template at different concentrations (final concentrations: 20 pM, 40 pM, 60 pM, 80 pM and 100 pM) on a StepOnePlus Real-Time PCR System (Applied Biosystems). RT-PCR was performed under the condition [50 °C, 2 min; 95 °C, 10 min; (95 °C, 15 sec, 60 °C, 60 sec) × 40 cycles]. Data collection was performed with the detection filters for SYBR and ROX, and the data were analyzed with the StepOne Software v2.1 (Applied Biosystems).
Sequence analysis of the PCR products from all dSNTPs using forward (A) and reverse (B) primer. (C) Summary of the sequence analysis (Fig. S3)

As shown here, the SeTTP was incorporated during PCR cycle without losing the sequence information of the template.

FIG. S3

3′-d(gggactagctacgagtgctccctgtatacttactcgtaagtcgtcgaactaccacggccacttcagaccaccagcttatattccgtc)-5′

5′-d(ggtggtcgaatataaggcag)-3′

5′-d(taatacgactcactatagggactacgtgactc)-3′

3′-d(gggactagctacgagtgctccctgtatacttactcgtaagtcgtcgaactaccacggccacttcagaccaccagcttatattccgtc)-3′

5′-d(ggtggtcgaatataaggcag)-3′

5′-d(taatacgactcactatagggactacgtgactc)-3′

PCR with SeTTP

PCR with dNTPs

Primer extension (Sequencing)
compound S2
4'-selenotymidine 5'-triphosphate (SeTTP)