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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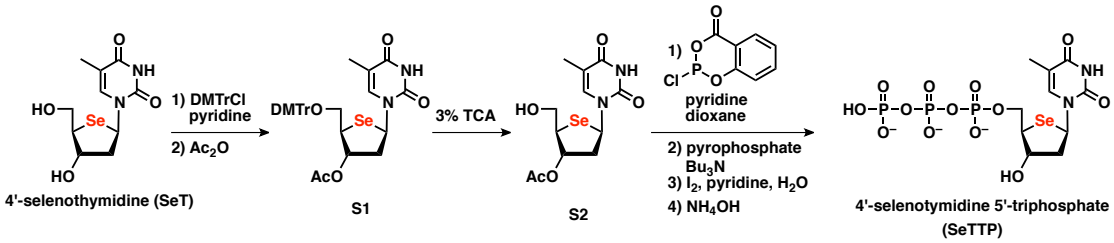
General methods

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_2O 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.

Synthesis of SeTTP (Scheme S1)



Scheme S1

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 Ac_2O (240 μL, 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 H_2O, and the organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried (Na_2SO_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 ($CDCl_3$) δ 8.09 (1 H, br s, exchangable with D_2O, 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 × 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 ($CDCl_3$) δ 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 $C_{33}H_{35}N_2O_7Se$ (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 $CHCl_2$ was added trichloroacetic acid (TCA) (60 μL) 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 H_2O, and the organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried (Na_2SO_4)

and concentrated *in vacuo*. The residue was purified by a silica gel column, eluted with 0–6% MeOH in $CHCl_3$, to give S2 (53 mg, 68% as a white solid): ^{1}H NMR ($CDCl_3$) δ 8.06 (1 H, br s, exchangable with D_2O, 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, CH_3); ^{13}C NMR ($CDCl_3$) 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 $C_{12}H_{17}N_2O_5Se$ (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-4*H*-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 $Na_2S_2O_3$ (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): ^{1}H NMR (D_2O) 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, CH_3); ^{31}P NMR (D_2O) δ –7.07, –11.24, –21.93; ESI-LRMS *m/z* 610 (M–H); ESI-HRMS calcd for $C_{10}H_{13}N_2Na_3O_{13}P_3Se$ (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)

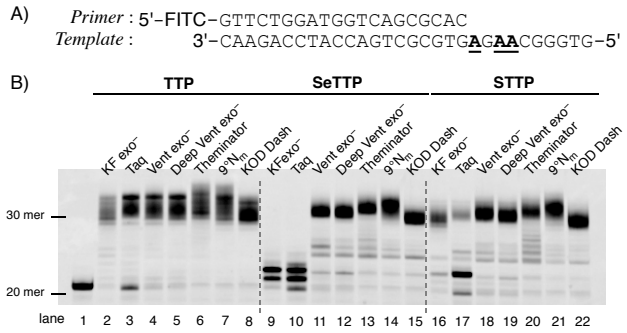
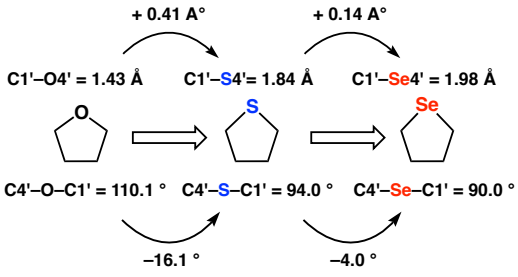


Fig. S1 Denaturing polyacrylamide gel image of the products. The duplex of 5'-FITC labeled primer (0.4 μM) and template (0.5 μM), corresponding triphosphates (each 50 μM), and each DNA polymerase (0.04–0.05 units/μL) in a reaction buffer (20 μL) was incubated at 37 or 74 °C.

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 $MgSO_4$ 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 electrophorese on a 20% polyacrylamide gel containing 8 M urea, and the gels were visualized with Molecular Imager FXpro system WOM (BioRad) equiped 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)



	4'-O[Ref.10]	4'-S[Ref.10]	4'-Se[Ref.5]
	Bond lengths (Å) and angles (deg)		
C1'-C2'	1.52	1.53	1.53
C2'-C3'	1.52	1.51	1.50
C3'-C4'	1.53	1.55	1.54
C1'-O[a]	1.43	1.84	1.98
C4'-O[a]	1.46	1.83	1.96
C1'-C2'-C3'	102.7	106.2	108.0
C2'-C3'-C4'	102.1	106.6	108.4
C3'-C4'-O[a]	104.4	105.4	105.5
C4'-O-C1'[a]	110.1	94.0	90.0
O-C1'-C2'[a]	106.5	105.9	104.8

[a]O represents S4' or Se4'

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'-gggactagctacgagtgctccctgtatacttactcgtaagtcgtcgaactaccacggccacttcagaccaccagcttatattccgtc-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'-taatacgactcactatagggactagctacgagtgctc-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 a 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.

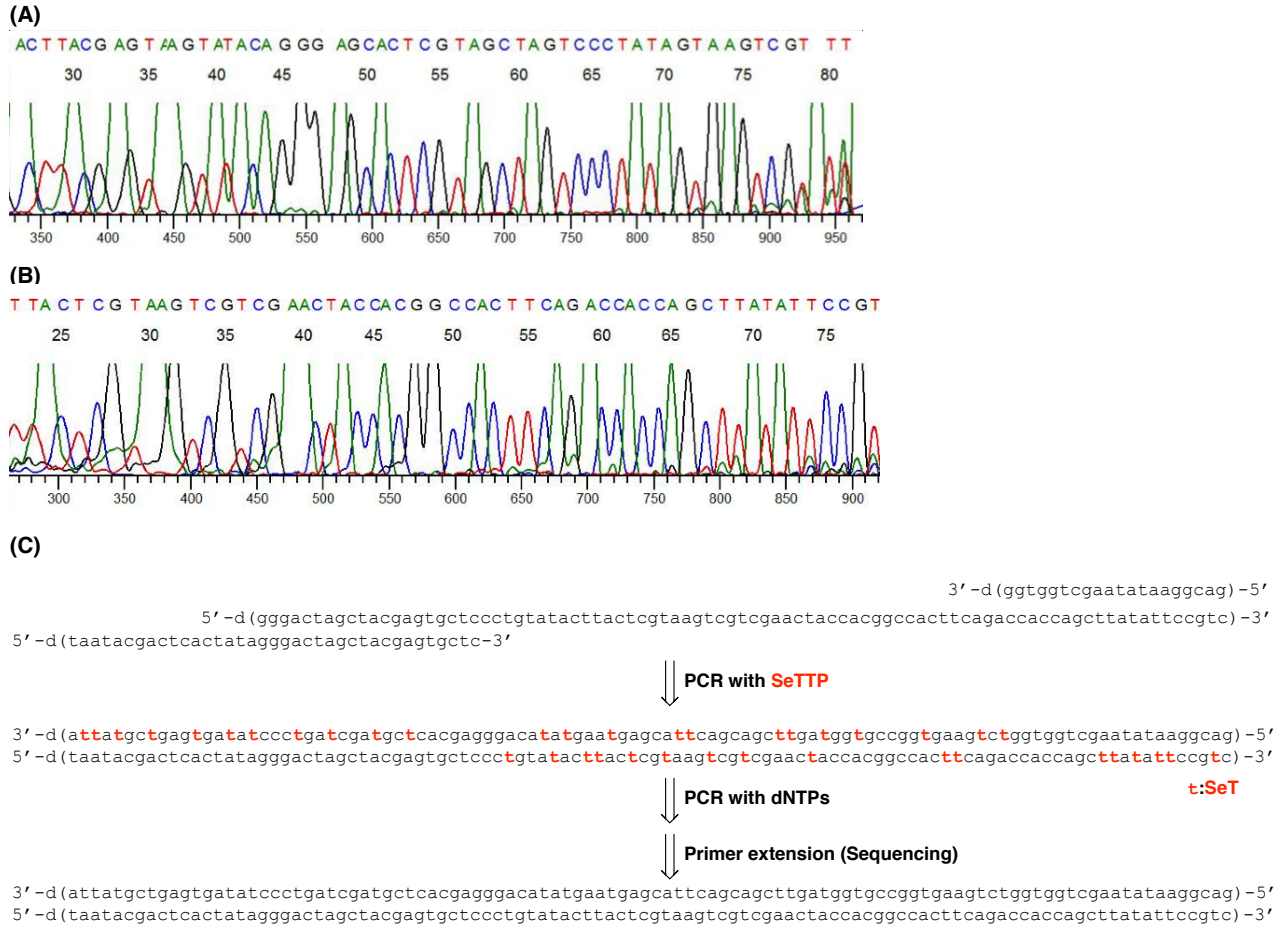
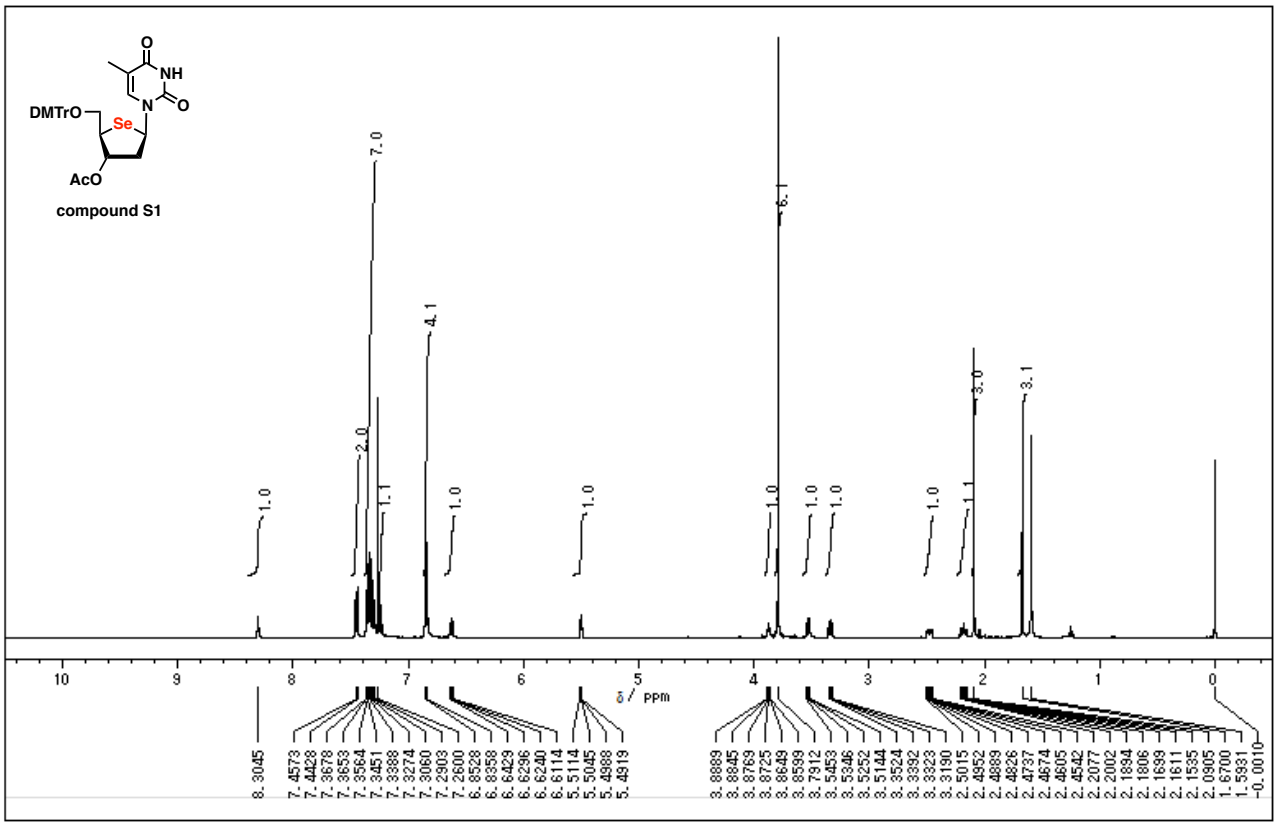
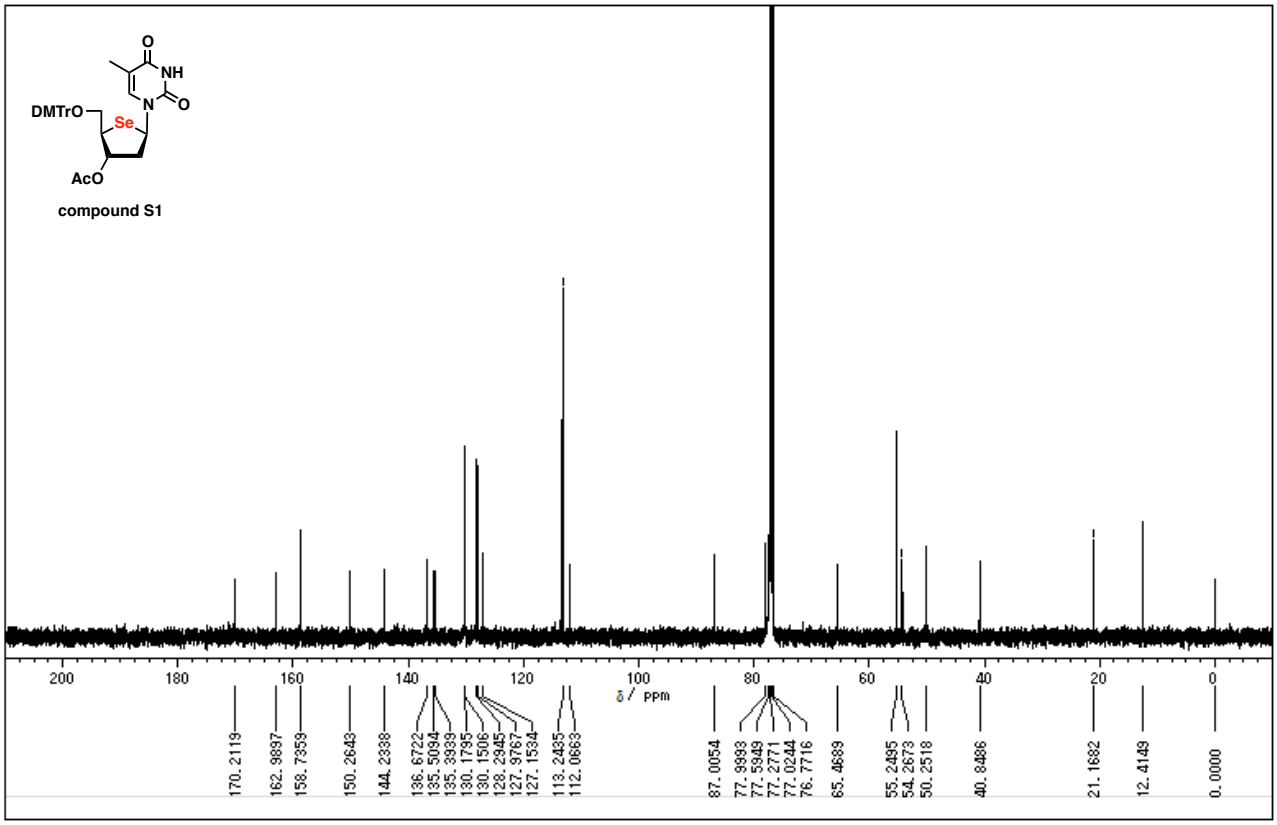
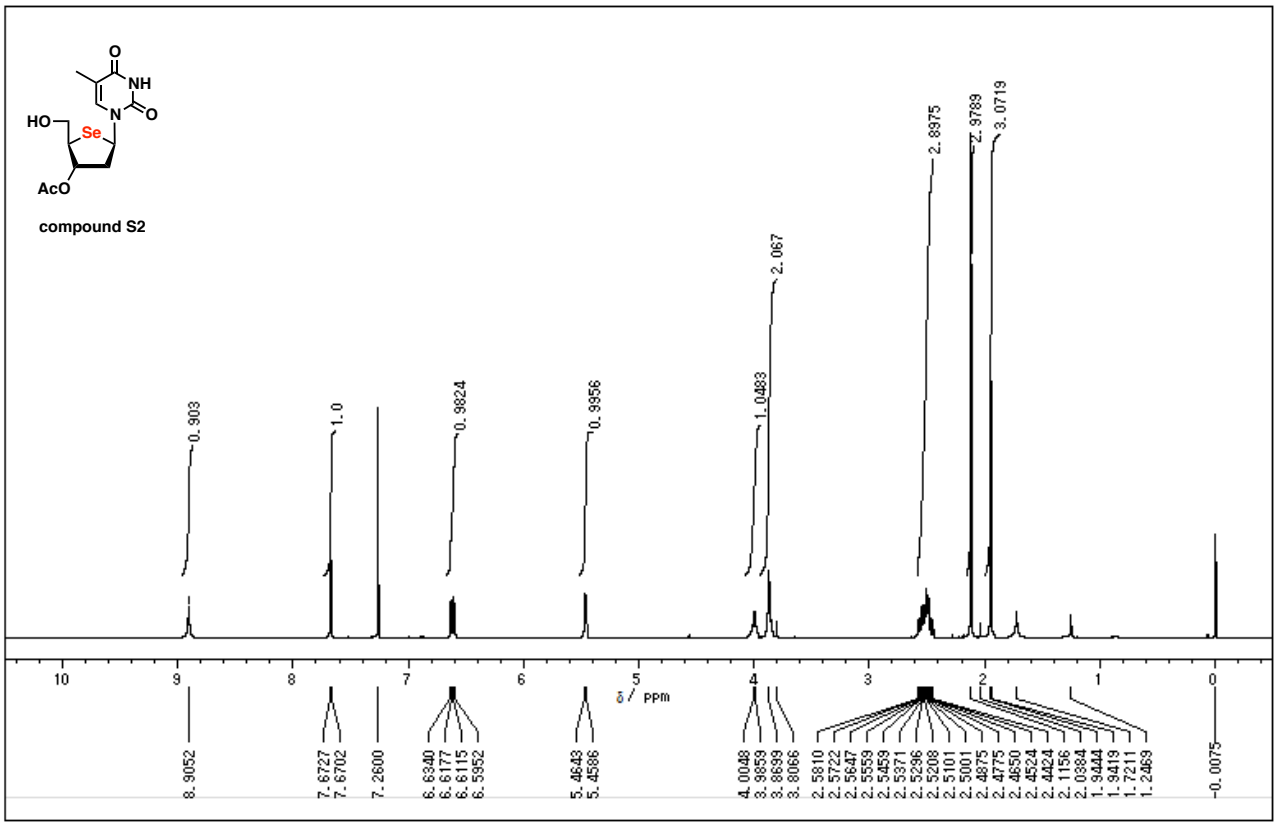


Fig. S3


DMTrO
Se
AcO
NH
O
O
N
compound S1
δ / ppm


DMTrO
Se
AcO
NH
O
O
N
compound S1
δ / ppm


compound S2
0.903
1.0
0.9824
0.9956
1.0483
2.067
2.8375
2.9789
3.0719
δ / ppm
8.9052
7.6727
7.6702
7.2600
6.6340
6.6177
6.6115
6.5952
5.4643
5.4586
4.0048
3.9859
3.8699
3.8066
2.5810
2.5722
2.5647
2.5559
2.5459
2.5371
2.5296
2.5208
2.5101
2.5001
2.4875
2.4775
2.4650
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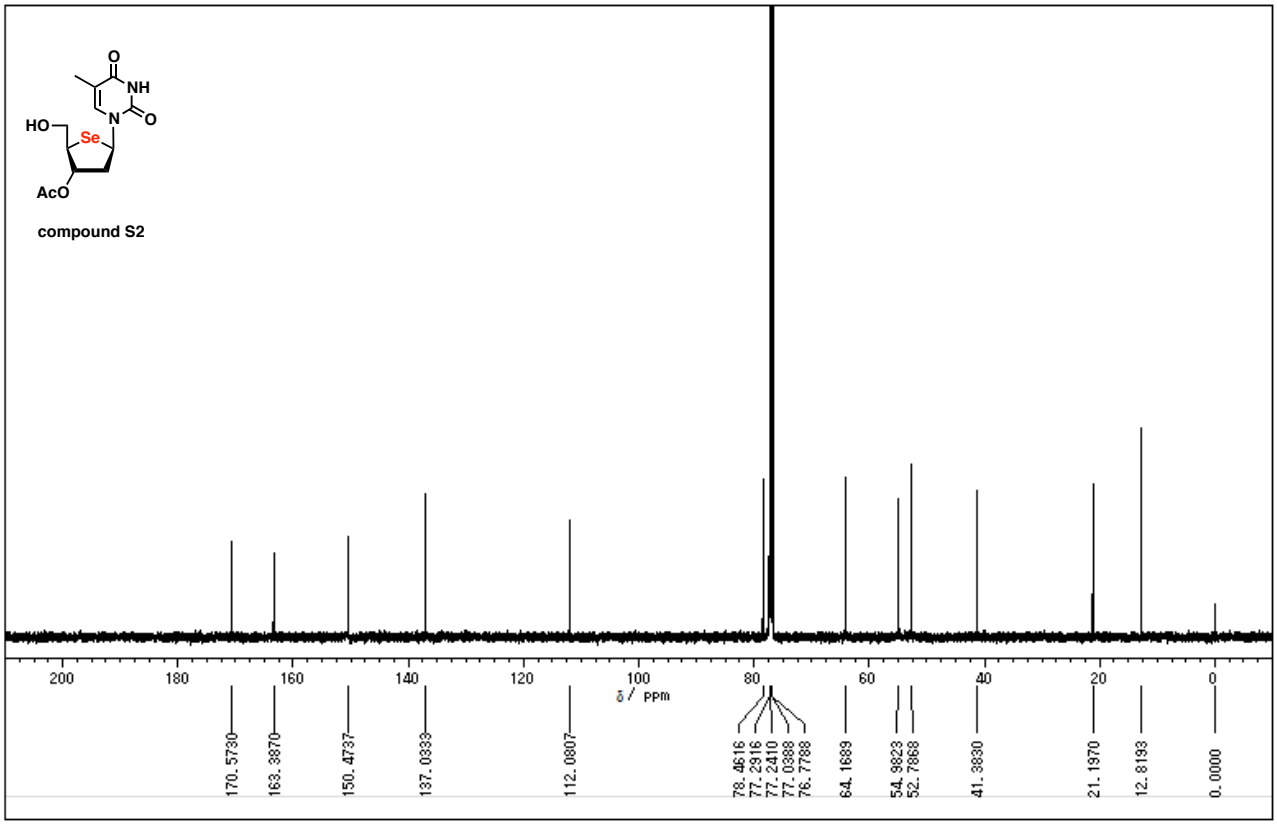
1.9419
1.7211
1.2469
-0.0075


compound S2
δ / ppm
170.5730
163.3870
150.4737
137.0333
112.0807
78.4616
77.2916
77.2410
77.0388
76.7788
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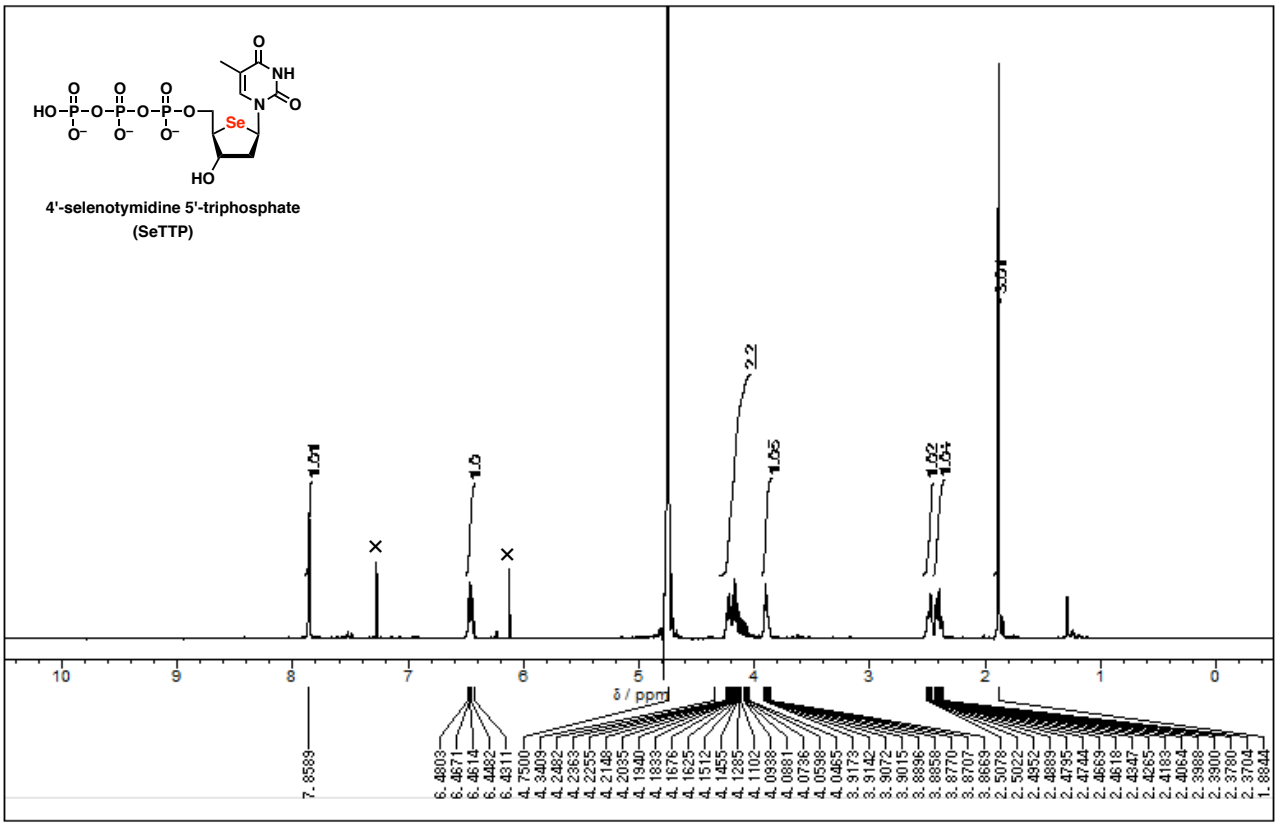
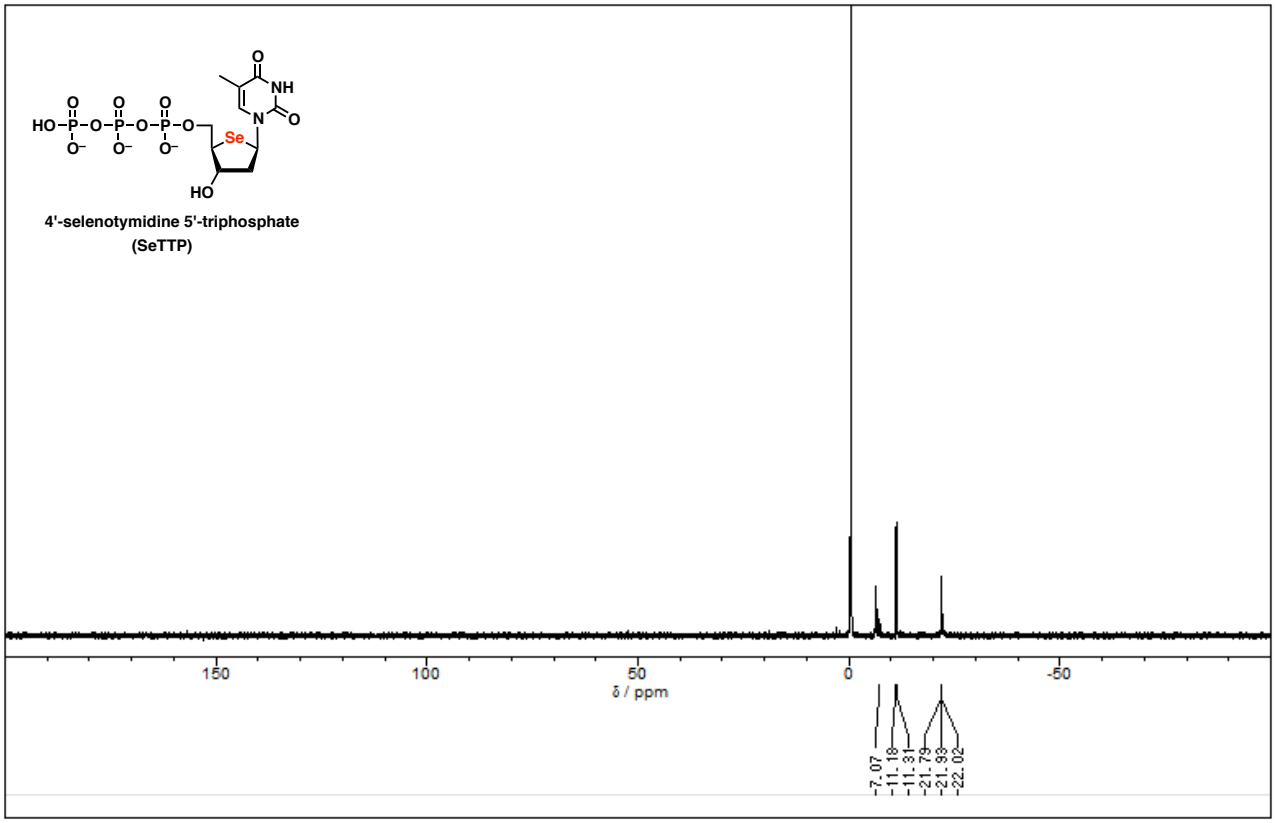

4'-selenotymidine 5'-triphosphate
(SeTTP)
δ / ppm


4'-selenotymidine 5'-triphosphate
(SeTTP)
δ / ppm