Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information

Amplification of Fluorescent DNA through Enzymatic Incorporation of a Highly Emissive Deoxyguanosine Analogue

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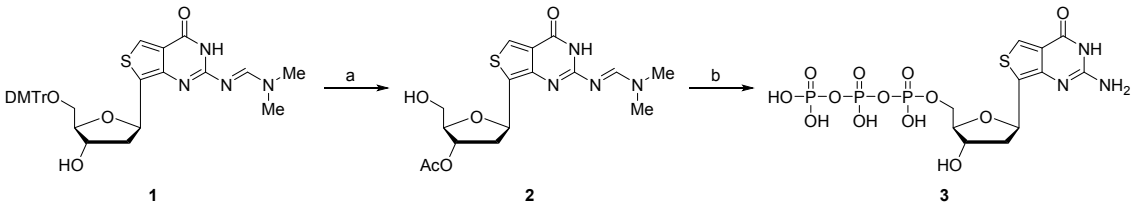
Materials

Dichloroacetic acid and tri-*n*-butylamine were received from Wako Chemicals and used without further purification. bis(tributylammonium)pyrophosphate was purchased from Sigma-Aldrich Chemicals Co. (Milwaukee, WI). 2-chloro-1,3,2-benzodioxaphosphorin-4-one and iodine were received from TCI. Acetic anhydride was purchased from Nacalai Tesque . All other chemicals and solvents were purchased from Sigma-Aldrich Chemicals Co., Wako Pure Chemical Ind. Ltd., TCI, Nacalai Tesque, or Kanto Chemical Co. Inc. *O*$^{5'}$-Dimethoxytrityl-*N*^{2}-DMF-2-aminothieno[3,4-*d*]pyrimidine G mimic deoxynucleoside was prepared by following the literature procedures [1]. Water was deionized (specific resistance of ≥ 18.0 MΩ cm at 25 ^{o}C) by a Milli-Q system (Millipore Corp.).

Methods

NMR spectra were obtained on a JEOL JNM ECA-600 spectrometer operating at 600 MHz for ^{1}H NMR and in $CDCl_3$ unless otherwise noted. Flash column chromatography was performed employing Silica Gel 60 (70–230 mesh, Merck Chemicals). Silica-gel preparative thin-layer chromatography (PTLC) was performed using plates from Silica gel 70 PF_{254} (Wako Pure Chemical Ind. Ltd.).

Synthesis of thdGTP



Reagents and conditions: (a) (i) acetic anhydride, pyridine; (ii) dichloro acetic acid, DCM, 0 ºC, 51%; (b) 2-chloro 4H-1,3,2-benzodioxaphosphorin 4-one, dioxane, pyridine, tri-*n*-butylamine, bis(tri-*n*-butylammonium)pyrophosphate, DMF, then I_2/pyridine/H_2O, 39%

***O*^{3}'-acetyl-*N*^{2}-DMF-2-aminothieno[3,4-*d*]pyrimidine G mimic deoxynucleoside (2)**

O^{5}'-Dimethoxytrityl-*N*^{2}-DMF-2-aminothieno[3,4-*d*]pyrimidine G mimic deoxynucleoside (192 mg, 0.3 mmol) was coevaporated to dryness with pyridine three times. To the residue in pyridine (3.0 mL), acetic anhydride (113 μL, 1.2 mmol) was added. The reaction mixture was stirred at room temperature for 12 h and was separated by ethyl acetate and 5% aqueous $NaHCO_3$. The organic layer was washed with saturated NaCl, dried with Na_2SO_4, and evaporated *in vacuo*. The residue in CH_2Cl_2 (30 mL) was added dichloroacetic acid (300 μL) at 0 ºC. The reaction mixture was stirred at 0 ºC for 15 min. To the reaction mixture was added 5% aqueous $NaHCO_3$, and the organic layer was washed with 5% $NaHCO_3$ and saturated NaCl. After drying over $NaSO_4$, the solvent was concentrated. The residue was loaded on preparative TLC. CH_2Cl_2/MeOH = 10/1 was used to PTLC and eluted with EtOAc and MeOH, to afford 56.4 mg of *O*^{3}'-acetyl-*N*^{2}-DMF-2-aminothieno[3,4-*d*]pyrimidine G mimic deoxynucleoside (**2**) in 51% yield. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.50 (s, 1H), 8.30 (br, 1H), 8.06 (s, 1H), 5.45 (d, J = 5.46 Hz, 1H), 5.41 (dd, J = 11.55, 5.43 Hz,1H), 5.02 (br, 1H), 4.17 (s, 1H), 3.86 (d, J = 2.67 Hz, 1H), 3.82 (m, 1H), 3.17 (s, 3H), 3.06 (s, 3H),

2.95 (ddd, J = 15.47, 9.53, 6.12 Hz, 1H), 2.28 (dd, J = 13.60, 5.41 Hz, 1H), 2.12 (s, 1H); ^{13}C NMR (150 MHz, $CDCl_3$): δ 170.75, 159.01, 158.15, 154.71, 146.34, 126.48, 126.13, 124.44, 86.15, 78.30, 76.13, 63.45, 41.10, 39.76, 34.88, 21.18; ESI-HRMS calculated for $C_{16}H_{19}N_4O_5S$ $[M-H]^-$ 379.1082, found 379.1098.

$O^{5'}$-triphosphate -2-aminothieno[3,4-*d*]pyrimidine G mimic deoxynucleoside (3)

$O^{3'}$-acetyl-N^2-DMF-2-aminothieno[3,4-*d*]pyrimidine G mimic deoxynucleoside (**2**) (30 mg, 82 μmol) was coevaporated with pyridine to dryness. The residue was dissolved in pyridine (80 μL) and dioxane (160 μL). 2-chloro-1,3,2-benzodioxaphosphorin-4-one (18.3 mg, 90 μmol) was added. After 10 min, tri-*n*-butylamine (82 μL) and 0.5 M bis(tributylammonium)pyrophosphate in DMF (246 μL, 123 μmol) were added to the reaction mixture, which was stirred at room temperature for 10 min. A solution of 1% iodine in pyridine/water (98/2, v/v, 1.64 mL) was added. After 15 min, 5% aqueous solution of $NaHSO_3$ (123 μL), followed by water (6 mL), was added to the reaction mixture, which was stirred at room temperature for 30 min, and then 28% NH_4OH (16 mL) was added. Ammonolysis was performed at room temperature for 4 h. After the reaction mixture was concentrated *in vacuo*, the product was dissolved in small amount of water and purified by C18-HPLC (eluted by a gradient of 0% to 50% CH_3CN in 50 mM TEAA) to give $O^{5'}$-triphosphate -2-aminothieno[3,4-*d*]pyrimidine G mimic deoxynucleoside (**3**) (16.7 mg, 39%). ^{1}H NMR (600 MHz, D_2O): δ 8.19 (s, 1H), 5.59 (dd, J = 10.16, 6.12 Hz, 1H), 4.68 (m, 1H), 4.23 (m, 2H), 4.19 (m, 1H), 2.38-2.33 (m, 2H); ^{31}P NMR (240 MHz, D_2O): δ -22.50 (t, J = 19.75 Hz, 1P), -10.57 (d, J = 19.75 Hz, 1P), 9.80 (d, J = 19.75 Hz, 1P); ESI-HRMS calculated for $C_{11}H_{15}N_3O_{13}P_3S^-$ $[M-H]^-$ 521.9544, found 521.9994.

Photophysical data for thdGTP monomer

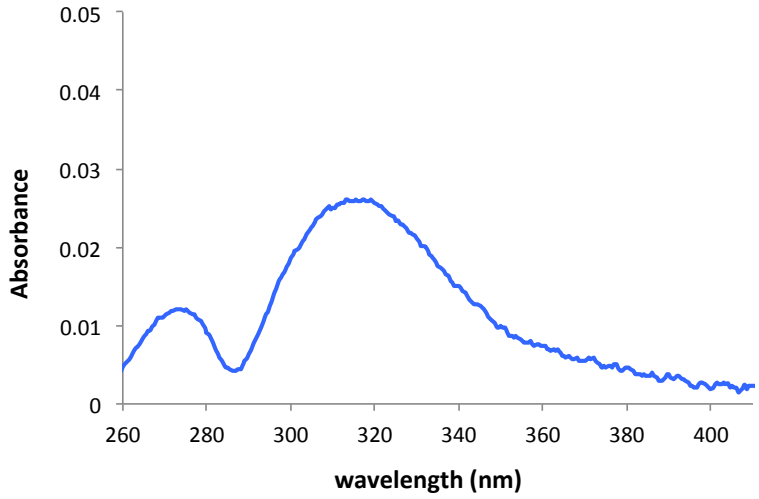


Figure S1. JASCO V-650 UV/VIS spectrophotometer was used to record absorption spectra with a 0.5 nm resolution. The cuvette temperature was kept at 25 °C by JASCO PAC-743R. Sample was prepared with 10 μM in H_2O.

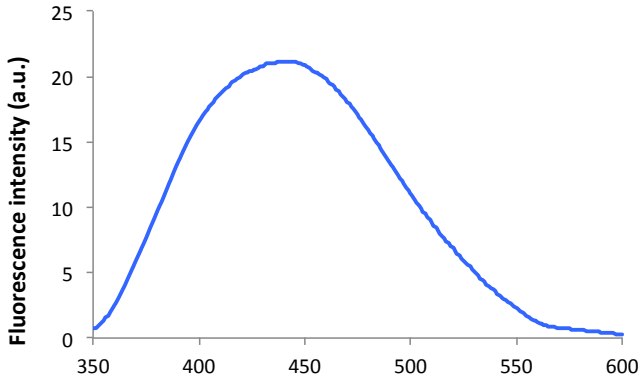


Figure S2. Fluorescence measurements were conducted using a JASCO FP-6300 spectrofluorometer. The sample temperature was controlled with a JASCO EHC-573 at 20 °C. Measurements were performed using fluorescence cells with a 0.5-cm path length. Sample was prepared with 10 μM in H_2O. Excitation wavelength was 325 nm.

Primer extension

The FAM-labeled primer (10-mer) was annealed with the 17-mer templates in 2x Klenow Fragment Buffer (20 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$), by heating at 95 °C and slow cooling to rt. The duplex solution (10 μM, 5 μl) was mixed with 2 μl of three dNTPs solution (200 μM dCTP, dTTP, and dATP) and 2 μl of dGTP or thdGTP solution (200 μM), and the reaction was started by adding 1 μl of a solution containing the exonuclease-proficient Klenow fragment (2 U, Wako). The reaction mixture was incubated at 37°C for 30 min and was terminated by adding 10 μl of a dye solution (0.05% BPB in formamide) and heating at 75 °C for 3 min. The products were fractionated on a 15% (v/v) polyacrylamide gel containing 7 M urea (100 V, 10 mA, 60 min), and were analyzed with a bio-imaging analyzer (FLA-3000, Fujifilm). Similar experiments using 10-mer primer without FAM-label was also conducted. After the fractionation by polyacrylamide gel electrophoresis, the photo was taken under UV irradiation, followed by CYBR Gold dyeing.

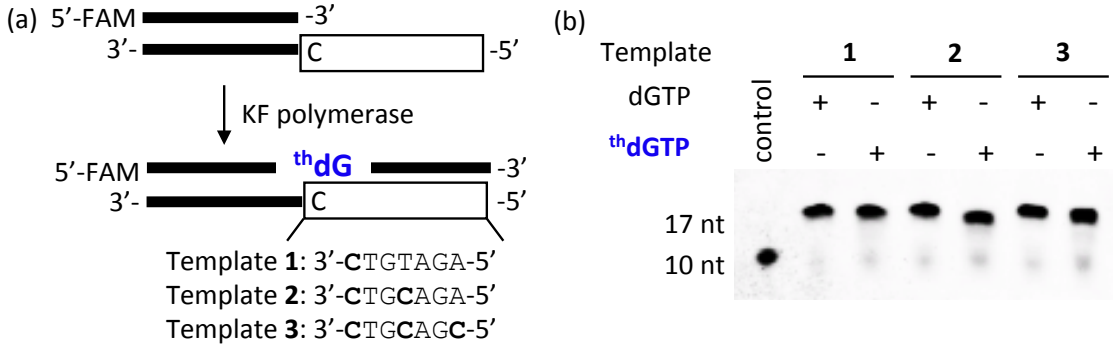


Figure S3. (a) Primer extension experiments with 17-mer templates **1**–**3**, in the presence of either natural dGTP or **thdGTP** and three dNTPs (dATP, dTTP, and dCTP). (b) Analyses by denaturing gel electrophoresis of primer-extended products with each

template after 30 min incubation at 37 °C. Lane 'control' corresponds to incubation with a mixture of primer, and dNTPs without the Klenow fragment.

PCR amplification

PCR amplification (50 µl) of the 56-mer DNA fragments (5'-TGGAGAGTATAGTGAGTGGTATTATGTACGTATTTCACACACCAAACACCTATCAT-3') was performed by using 1 µM each reverse primer (24-mer; 5'-ATGATAGGTGTTTGGTGTGTGAAA-3') and forward primer (26-mer; 5'-TGGAGAGTATAGTGAGTGGTATTATG-3') and each DNA pol at the following concentration (KOD –Plus-, 1.0 U; One Taq, 5.0 U; Deep Vent (exo-), 2.0 U; Deep Vent, 2.0 U), in the 1x reaction buffer accompanying each DNA pol, supplemented with 5 µl of 2 mM dNTPs (dCTP, dTTP, dATP, and thdGTP). PCR conditions were 2 m at 98 ºC, 15 s at 95 ºC, 30 s at 53 ºC, and 10 s at 68 ºC for 40 cycles. The PCR products were fractionated by native 15% (v/v) PAGE (200 V, 20 mA, 60 min).

PCR amplification with the mixture of thdGTP and dGTP

PCR amplification (50 μl) of 338-mer templates with the mixture of thdGTP and dGTP contained 1 μM of the primer set, 90 ng of pET28a plasmid and 1.0 U of KOD –Plus- polymerase, in the 1x reaction buffer accompanying each DNA pol, supplemented with 5 μl of 2 mM three dNTPs (dCTP, dTTP, and dATP) and total 2 mM of dGTP and thdGTP mixture (1:0, 1:1, 1:3, ..., 1:9, 0:1). PCR conditions were 2 m at 98 ºC, 15 s at 95 ºC, 30 s at 53 ºC, and 30 s or 1 m at 68 ºC for 40 cycles. After an aliquot (3 μl) of PCR products were fractionated by 2% agarose gel electrophoresis, the residue was purified with GenElute PCR Clean-Up Kit (Sigma-Aldrich) to remove dNTPs. A portion of each purified DNA solution was diluted to 1.3 μM (100 μl), and fluorescent measurement was conducted. The residual solution was incubated at 70 °C for 2 h with nuclease P_1 (Wako) and at 37 °C for 2.5 h with Antarctic Phosphatase (New England Biolabs). All of the reaction mixture was used for HPLC analysis.PCR amplification (50 μl) of 298, 480, and 761-mer templates with the mixture of dNTPs and 5 equiv of thdGTP contained 1 μM of the primer set, 90 ng of pUC18 or 150 ng of pGEM plasmids and 1.0 U of KOD –Plus- polymerase, supplemented with 5 μl of 2 mM dNTPs mixed solution (dCTP, dTTP, dATP, and dGTP) and 25 μl of 2 mM thdGTP. PCR conditions were 2 m at 98 ºC, 15 s at 95 ºC, 30 s at 53 ºC, and 30 s or 1 m at 68 ºC for 40 cycles. After an aliquot (5 μl) of PCR products were fractionated by 2% agarose gel electrophoresis, the residue was purified with GenElute PCR Clean-Up Kit (Sigma-Aldrich) to remove dNTPs. A portion of each purified DNA solution was diluted to 1.3 μM (100 μl), and fluorescent measurement was conducted. The residual solution was incubated at 70 °C for 1 h with nuclease P_1 (Wako) and at 37 °C for 1 h with Antarctic Phosphatase (New England Biolabs). All of the reaction mixture was used for HPLC analysis.

template	primers	
cDNA of Zα domain from ADAR (338-mer)[a]	Forward primer	5′-TTAATGAATTCCTGAGTATCTACCAAGATCAGGAACAAAG-3′
	Reverse primer	5′-CCGCTGAGCAATAACTAGCAT-3′
pUC18 F413-710 (298-mer)	Forward primer	5′-GCAGGTCGACTCTAGAGGAT-3′
	Reverse primer	5′-GAGTCAGTGAGCGAGGAAG-3′
pGEM F601-1080 (480-mer)	Forward primer	5′-ATCGACGCTCAAGTCAGAGG-3′
	Reverse primer	5′-GTTTGCCGGATCAAGAGCTACC-3′
pUC18 F905-1665 (761-mer)	Forward primer	5′-ATCGACGCTCAAGTCAGAGG-3′
	Reverse primer	5′-GATCGCTGAGATAGGTGCCTC-3′

Table S1. Templates and primers used for PCR amplification.

[a] pET28a (338-mer)

5'-

TTAATGAATTCCTGAGTATCTACCAAGATCAGGAACAAAGGATCTTAAAGT
TCCTGGAAGAGCTTGGGGAAGGGAAGGCCACCACAGCACATGATCTGTCTG
GGAAACTTGGGACTCCGAAGAAAGAAATCAATCGAGTTTTATACTCCCTGG
CAAAGAAGGGCAAGCTACAGAAAGAGGCAGGAACACCCCCTTTGTGGAAA
ATCGCGGTCTCCACTCAGTGATGAAGCTTGCGGCCGCACTCGAGCACCACC
ACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGT
TGGCTGCTGCCACCGCTGAGCAATAACTAGCATTTAATGAATTCCTGAGTAT
CTACCAAGATCAGGAACAAAGTTAATGAATTCCTGAGTATCTACCAAGATC
AGGAACAAAG-3'

Reference

1 S.Park, H. Otomo, L. Zheng, H. Sugiyama, *Chem. Comm.,* **2014**, *50*, 1573–1575.