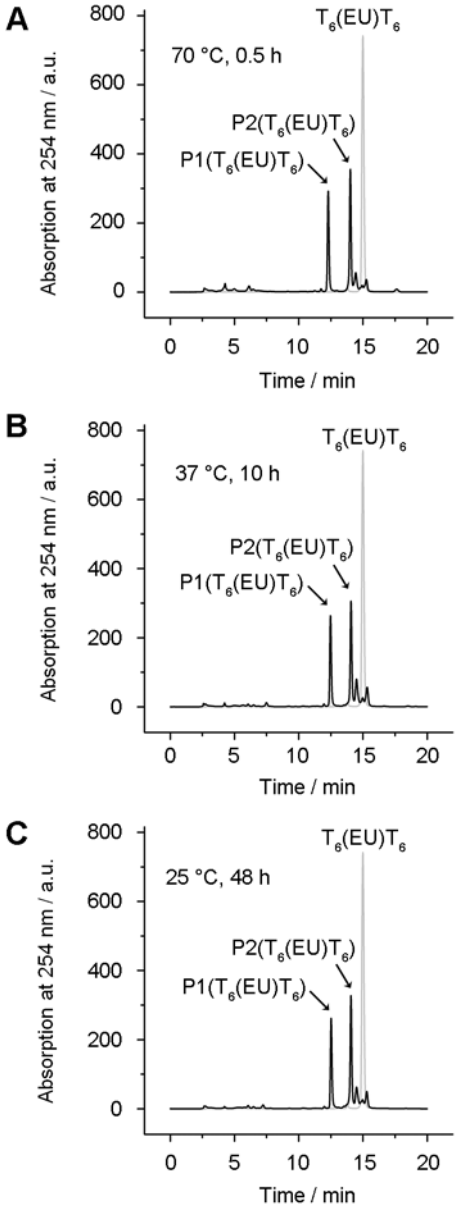
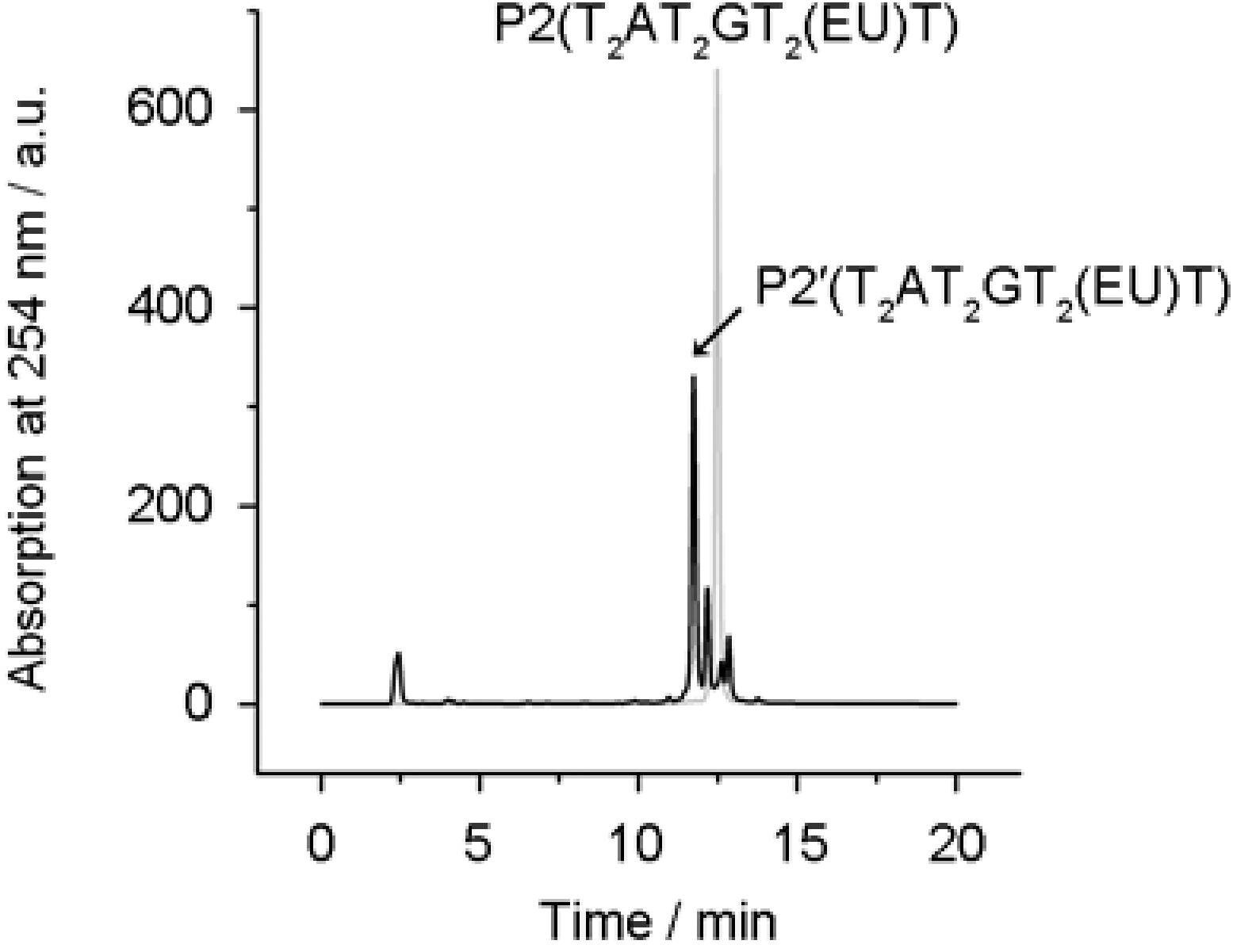
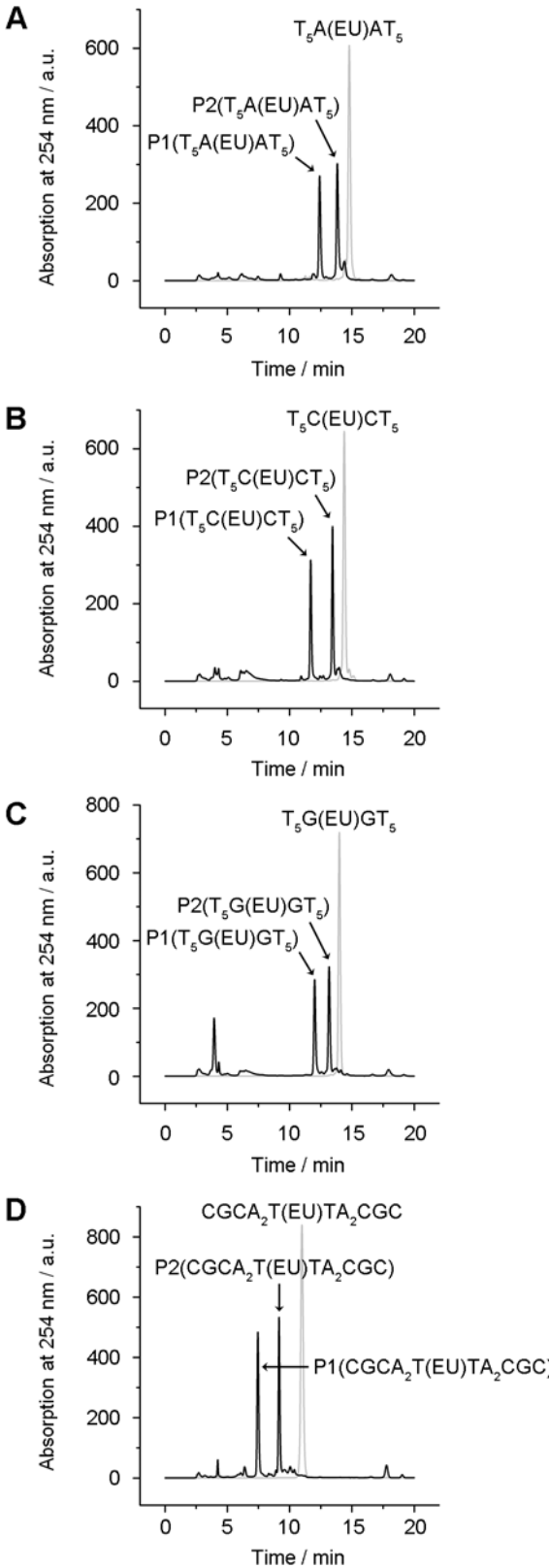
Data S1

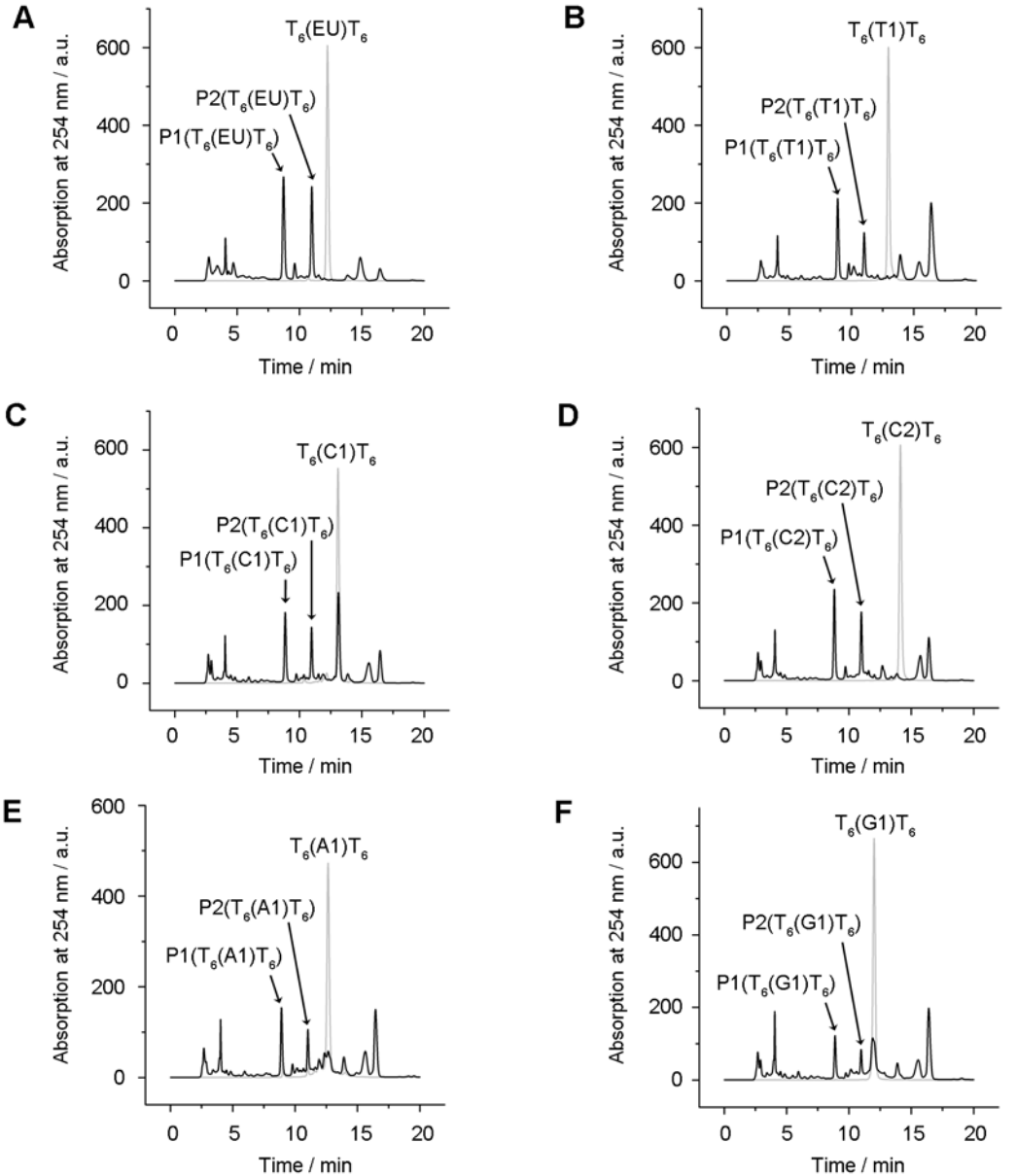
MALDI TOF mass data of cleavage products

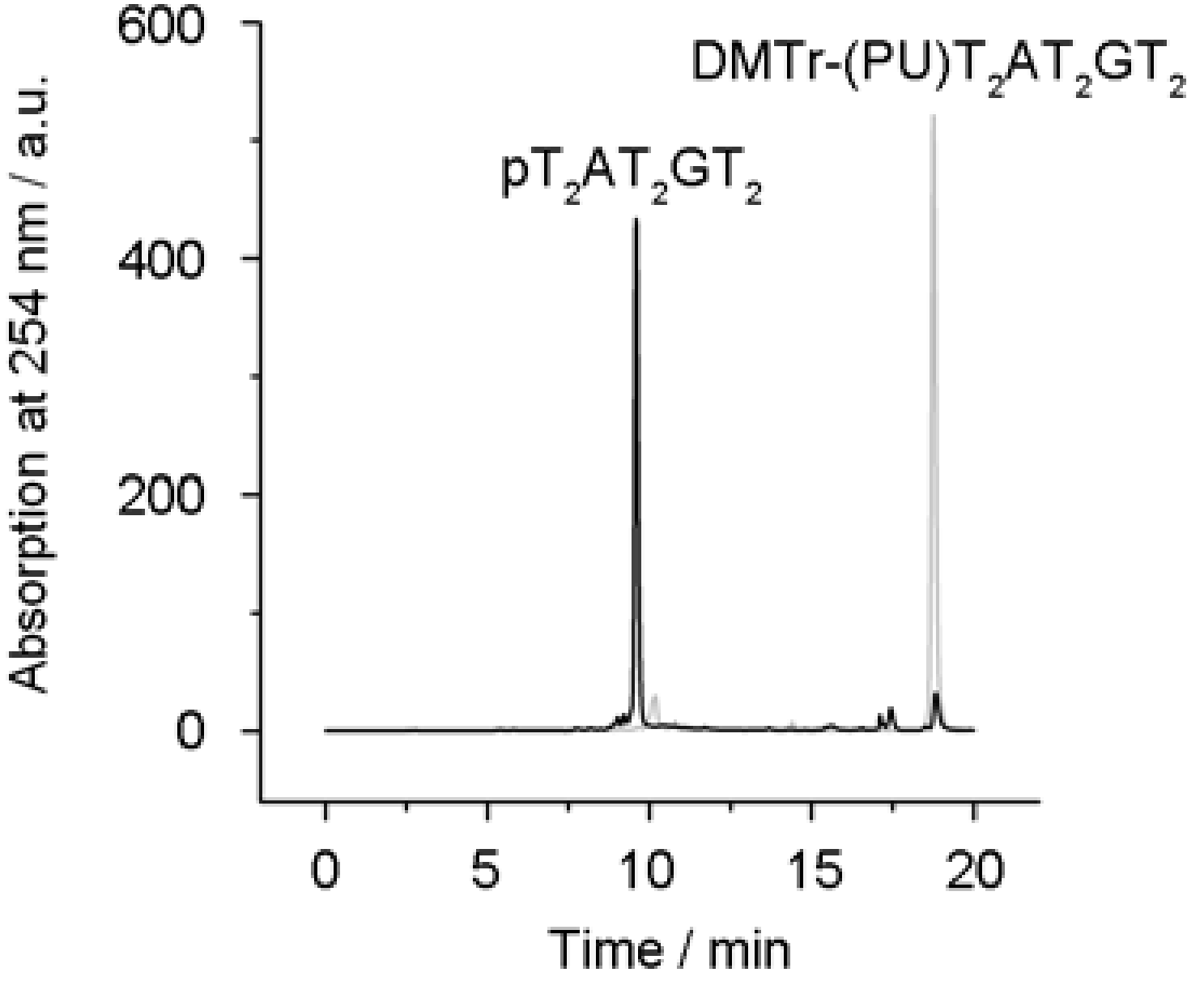
Products from $T_6(EU)T_6$; pT_6 (P1), calcd $C_{60}H_{79}N_{12}O_{43}P_6$ 1842.2 $[M-H]^-$; found 1841.8; T_6pR (P2), calcd $C_{66}H_{90}N_{13}O_{45}P_6$ 1971.3 $[M-H]^-$; found 1971.3; Product from $(EU)T_2AT_2GT_2$; $pT_2AT_2GT_2$ (P1), calcd for $C_{80}H_{103}N_{22}O_{54}P_8$ 2484.6 $[M-H]^-$; found 2484.6; Product from $T_2AT_2GT_2(EU)T$; $T_2AT_2GT_2pR$ (P2), calcd for $C_{86}H_{114}N_{23}O_{56}P_8$ 2613.7 $[M-H]^-$; found 2613.8; Products from $T_5A(EU)AT_5$; pAT_5 (P1), calcd $C_{60}H_{78}N_{15}O_{41}P_6$ 1851.2 $[M-H]^-$; found 1850.9; T_5ApR (P2), calcd $C_{66}H_{89}N_{16}O_{43}P_6$ 1980.3 $[M-H]^-$; found 1980.2; Products from $T_5C(EU)CT_5$; pCT_5 (P1), calcd $C_{59}H_{78}N_{13}O_{42}P_6$ 1827.2 $[M-H]^-$; found 1827.0; T_5CpR (P2), calcd $C_{65}H_{89}N_{14}O_{44}P_6$ 1956.3 $[M-H]^-$; found 1956.3; Products from $T_5G(EU)GT_5$; pGT_5 (P1), calcd $C_{60}H_{78}N_{15}O_{42}P_6$ 1867.2 $[M-H]^-$; found 1867.2; T_5GpR (P2), calcd $C_{66}H_{89}N_{16}O_{44}P_6$ 1996.3 $[M-H]^-$; found 1996.2; Products from $CGCA_2T(EU)TA_2CGC$; pTA_2CGC (P1), calcd $C_{58}H_{74}N_{23}O_{36}P_6$ 1855.2 $[M-H]^-$; found 1855.0; $CGCA_2TpR$ (P2), calcd $C_{64}H_{85}N_{24}O_{38}P_6$ 1984.3 $[M-H]^-$; found 1984.3. R ($C_6H_{11}NO_2$) was calculated on the supposition that the cleavage reaction was accompanied by elimination of EU and addition of methylamine and OH^-.

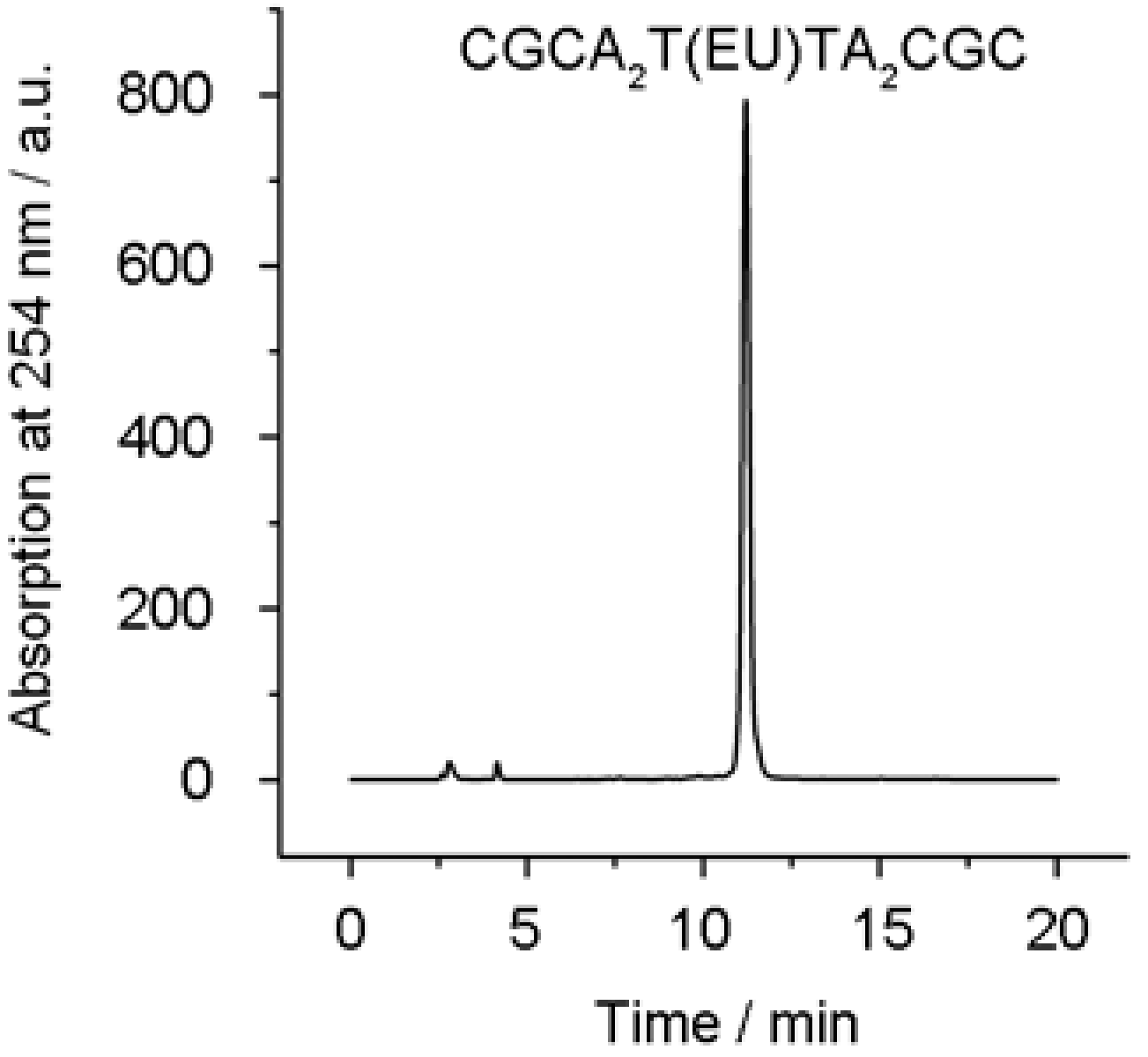

A
B
C
T6(EU)T6
70 °C, 0.5 h
37 °C, 10 h
25 °C, 48 h
P2(T6(EU)T6)
P1(T6(EU)T6)
Absorption at 254 nm / a.u.
Time / min
0
200
400
600
800
0
5
10
15
20


P2(T2AT2GT2(EU)T)
P2′(T2AT2GT2(EU)T)
Absorption at 254 nm / a.u.
600
400
200
0
0
5
10
15
20
Time / min


A
T5A(EU)AT5
P2(T5A(EU)AT5)
P1(T5A(EU)AT5)
Absorption at 254 nm / a.u.
Time / min
B
T5C(EU)CT5
P2(T5C(EU)CT5)
P1(T5C(EU)CT5)
Absorption at 254 nm / a.u.
Time / min
C
T5G(EU)GT5
P2(T5G(EU)GT5)
P1(T5G(EU)GT5)
Absorption at 254 nm / a.u.
Time / min
D
CGCA2T(EU)TA2CGC
P2(CGCA2T(EU)TA2CGC)
P1(CGCA2T(EU)TA2CGC)
Absorption at 254 nm / a.u.
Time / min


A
B
C
D
E
F
Absorption at 254 nm / a.u.
Time / min
T6(EU)T6
P2(T6(EU)T6)
P1(T6(EU)T6)
T6(T1)T6
P2(T6(T1)T6)
P1(T6(T1)T6)
T6(C1)T6
P2(T6(C1)T6)
P1(T6(C1)T6)
T6(C2)T6
P2(T6(C2)T6)
P1(T6(C2)T6)
T6(A1)T6
P2(T6(A1)T6)
P1(T6(A1)T6)
T6(G1)T6
P2(T6(G1)T6)
P1(T6(G1)T6)


600
400
200
0
Absorption at 254 nm / a.u.
DMTr-(PU)T2AT2GT2
pT2AT2GT2
0
5
10
15
20
Time / min


CGCA2T(EU)TA2CGC
Absorption at 254 nm / a.u.
800
600
400
200
0
0
5
10
15
20
Time / min

Method S1

Preparation of DNA oligonucleotides containing 5-ethynyluracil (EU)

DNA oligonucleotides containing EU were synthesized on the DNA synthesizer by using the standard phosphoramidite method. The phosphoramidite to incorporate EU into DNA oligonucleotides was synthesized according to the literature [12,13]. Phosphoramidites of natural DNA nucleosides (^{Bz}dA, ^{Ac}dC, ^{dmf}dG, and T) were purchased from Glen research. The coupling time of EU phosphoramidite was extended to 30 minutes to ensure high coupling efficiency. The last DMTr group was not deprotected on the DNA synthesizer (DMTr-ON). After automated DNA synthesis, the CPG-support was put into a screw-cap tube and 1 mL of 50 mM K_2CO_3/MeOH was added into the tube. The suspension was left at 25°C for 16 hours or 65°C for 4 hours. A small amount of H_2O was added to the suspension and MeOH in the suspension was removed by speed-vac. H_2O was added to the residue to reach a total volume of ≈500 µL and 28% NH_3aq (500 µL) was added to the suspension. The suspension was left at 25°C for 16 hours. NH_3 in the suspension was removed by speed-vac. The suspension was passed through a 0.45 µm filter. The DMTr-ON DNA oligonucleotides were purified by reversed-phase HPLC with a linear gradient over 20 minutes from 5 to 50% CH_3CN in 50 mM ammonium formate (AF). After speed-vac and freeze-drying to remove the solvent and AF, the DMTr group on the DNA oligonucleotides was removed in 80% AcOH aqueous solution (100 µL) at 25°C for 30 minutes. H_2O was added to the solution to reach a total volume of ≈1 mL. The DNA oligonucleotides were purified by reversed-phase HPLC with a linear gradient over 20 minutes from 5 to 20% CH_3CN in 50 mM AF. The purified DNA oligonucleotides were desalted and identified by MALDI TOF mass spectrometry except for long primers (≥ 30 mer): T_6(EU)T_6, calcd for $C_{131}H_{167}N_{26}O_{89}P_{12}$ 3901.5 $[M-H]^-$; found 3901.8; (EU)$T_2AT_2GT_2$, calcd for $C_{91}H_{113}N_{24}O_{58}P_8$ 2718.8 $[M-H]^-$; found 2718.9; $T_2AT_2GT_2$(EU)T, calcd for $C_{101}H_{126}N_{26}O_{65}P_9$ 3023.0 $[M-H]^-$; found 3023.4; T_5A(EU)AT_5, calcd for $C_{131}H_{165}N_{32}O_{85}P_{12}$ 3919.6 $[M-H]^-$; found 3920.3; T_5C(EU)CT_5, calcd for $C_{129}H_{165}N_{28}O_{87}P_{12}$ 3871.5 $[M-H]^-$; found 3872.0; T_5G(EU)GT_5, calcd for $C_{131}H_{165}N_{32}O_{87}P_{12}$ 3951.6 $[M-H]^-$; found 3952.5; CGCA_2T(EU)TA_2CGC, calcd for $C_{127}H_{157}N_{48}O_{75}P_{12}$ 3927.6 $[M-H]^-$; found 3928.1; AG_2TGCT_3A(EU)GACTCT-GC$_2$G, calcd for $C_{206}H_{257}N_{74}O_{128}P_{20}$ 6437.1 $[M-H]^-$; found 6437.8.

Methods S2

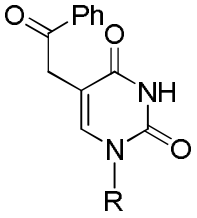
PCR amplification by using primers containing 5-ethynyluracil (EU)

KOD Fx neo (TOYOBO) was used for PCR. Before PCR, $CGCA_2T(EU)TA_2CGC$ in ×1 buffer for KOD Fx neo was heated with a temperature program, 94°C, 2 min → [98°C, 10 sec → 60°C, 30 sec → 68°C, 90 sec] ×30 → 4°C. After heating, the sample was directly analyzed by reversed-phase HPLC with a linear gradient over 20 minutes from 5 to 20% CH_3CN in 50 mM AF (Fig. S5). PCR solution (50 μL) contained primers (0.4 μM, each), dNTPs (400 μM, each), buffer (×1), KOD Fx neo (1 U), and template DNA, EcoRI-digested pBluescript-sk(–) (200 ng) or linear λ phage DNA (1 ng). The linear λ phage DNA (N3011) was purchased from New England Biolabs. DNA fragments for the construction of the 3.7 kbp plasmid were amplified using a temperature program, 94°C, 2 min → [98°C, 10 sec → 60°C, 30 sec → 68°C, 90 sec] ×30 → 4°C. The PCR-amplified DNA fragments were analyzed on a 1% agarose gel containing ethidium bromide in TAE buffer by electrophoresis.

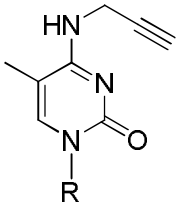
Methods S3

DNA base analogues for DNA cleavage

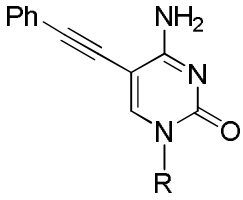
We found that the DNA base analogues described below can cause the same DNA cleavage as that induced by EU. Their synthetic schemes are shown following their respective synthetic procedures.



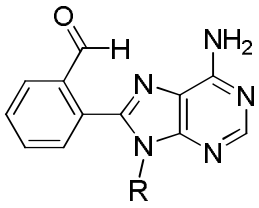
T1



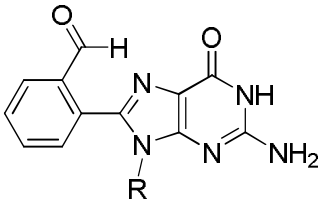
C1



C2



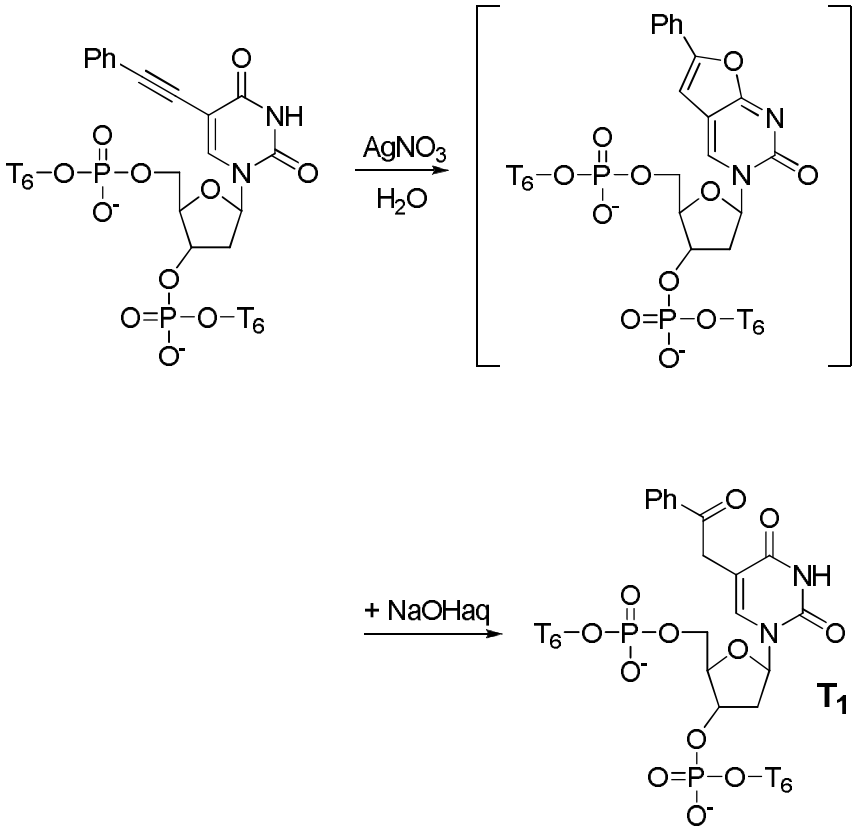
A1



G1

Preparation of $T_6(T1)T_6$

$T_6(T1)T_6$ was postsynthetically prepared from the DNA oligonucleotide containing 5-ethynyluracil (PU) [17]. $AgNO_3$aq (100 mM, 200 µL) was added to $T_6(PU)T_6$ aqueous solution (1 mM strand concentration, 200 µL) [18]. The solution was mixed and left at 25°C for 16 hours. After addition of NaOHaq (10 M, 20 µL) [19], the solution was mixed and left at 25°C for 2 hours. H_2O (600 µL) containing acetic acid (20 µL) was added to the solution. The solution was filtered with 0.45 nm filter. The product was purified by reversed-phase HPLC with a linear gradient over 20 minutes from 8 to 20% CH_3CN/50 mM AF. The retention time of the product was 12.5 minutes. After desalination, the product was identified by MALDI TOF mass spectrometry: $T_6(T1)T_6$, calcd for $C_{137}H_{173}N_{26}O_{90}P_{12}$ 3995.6 $[M-H]^-$; found 3995.6.



6-*N*-(3-propynyl)-5-methyl-2'-deoxycytidine (1)

Thymidine (1.21 g, 5.00 mmol) was suspended in CH_3CN (20 mL). Triethylamine (5.58 mL, 40.0 mmol) and acetic anhydride (1.89 mL, 20.0 mmol) were added to the suspension. After stirring at 25°C for 21 hours, methanol (10 mL) was added to the solution and stirred for 2 hours. The solvent was removed by evaporation. AcOEt (100 mmL) and saturated NaClaq (100 mL) were added to the residue and the product was extracted to the organic layer. The organic layer was washed with saturated NaClaq (100 mL) twice, dried over magnesium sulfate, and filtered. The solvent was removed by evaporation and the residue was dried under reduced pressure. 1,2,4-triazole (3.45 g, 50 mmol), CH_3CN (30 mL), and triethylamine (7.67 mL, 55.0 mmol) were added to the residue. $POCl_3$ (932 µL, 10.0 mmol) was slowly added to the solution under constant stirring. After stirring for 1 hour, the solvent was removed by evaporation. AcOEt (100 mmL) and saturated NaClaq (100 mL) were added to the residue and the product was extracted to the organic layer. The organic layer was washed with saturated NaClaq (100 mL) twice, dried over magnesium sulfate, and filtered. The solvent was removed by evaporation and the residue was dried under reduced pressure

[20]. 1,4-dioxane (30 mL) was added to the residue and the solution was stirred. Propargylamine (961 μL, 15.0 mmol) was added to the solution. After stirring at 25°C for 2 hours, methanol (30 mL) and 28% NH_3aq (30 mL) were added to the solution. The solution was stirred at 25°C for 24 hours. The solvent was removed by evaporation and the product **1** was purified by silica gel column chromatography (10–15% MeOH/CH_2Cl_2). The product **1** was obtained as a white powder (873 mg, 3.13 mmol, 63%): ^{1}H NMR (DMSO-d6) *δ* 7.65 (d, *J* = 0.9, 1H), 7.57 (t, *J* = 5.7, 1H), 5.19 (d, *J* = 4.6, 1H), 5.01 (t, *J* = 5.3, 1H), 4.23-4.18 (m, 1H), 4.14-4.01 (m, 2H),3.76-3.74 (m, 1H),3.62-3.50 (m, 2H), 3.04 (t, *J* = 2.5 , 1H),2.12-2.05 (m, 1H),1.99-1.92 (m, 1H),1.84 (s, 3H); ^{13}C NMR (DMSO-d6) *δ* 162.4, 154.9, 138.0, 101.7, 87.2, 84.8, 81.5, 72.6, 70.4, 61.4, 40.3, 29.4, 13.0; HRMS (ESI) calcd for $C_{13}H_{17}N_3O_4Na$ ([M+Na]$^+$) 302.1116, found 302.1117.

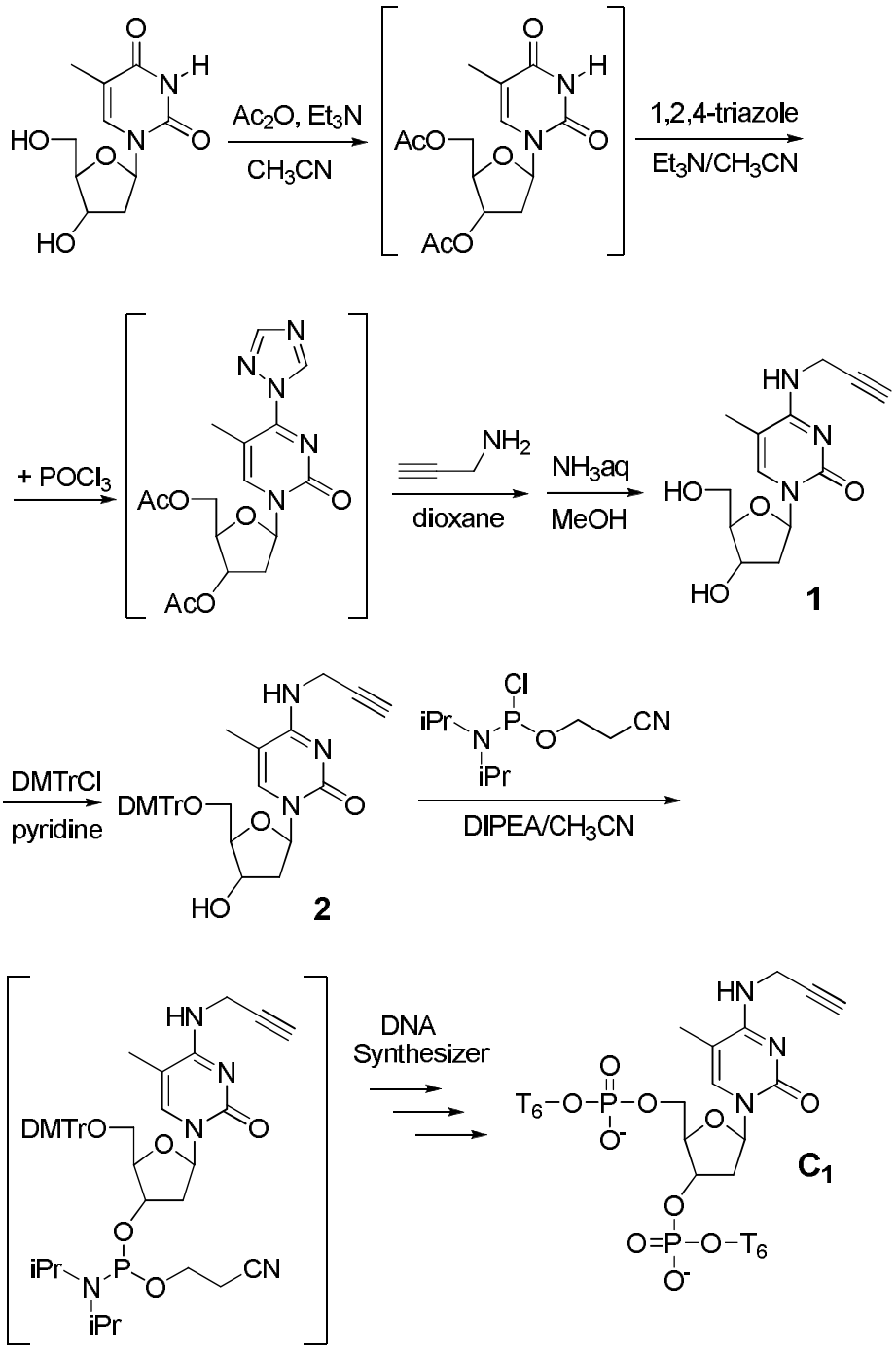
5'-*O*-dimetoxytrityl-6-*N*-(3-propynyl)-5-methyl-2'-deoxycytidine (2)

Pyridine (10 mL) was added to **1** (312 mg, 1.12 mmol) and DMTrCl (454 mg, 1.34 mmol). The solution was stirred at 25°C for 1 hour. After addition of methanol (10 mL), the solvent was removed by evaporation. The residual pyridine was removed by coevaporation with CH_2Cl_2 and hexane. The product **2** was purified by silica gel column chromatography (2–5% MeOH, 2% Et_3N/CH_2Cl_2). The product **2** was obtained as a white powder (631 mg, 0.924 mmol, 83%): ^{1}H NMR (DMSO-d6) *δ* 7.63 (t, *J* = 5.7, 1H), 7.51 (s, 1H), 7.39-7.35 (m, 2H),7.32-7.19 (m, 7H), 6.90-6.86 (m, 4H),6.21 (t, *J* = 6.6, 1H), 5.34 (d, *J* = 4.1, 1H), 4.31-4.25 (m, 1H), 4.14-4.02 (m, 2H),3.90-3.86 (m, 1H),3.72 (s, 6H), 3.22-3.16 (m, 2H),3.09-3.00 (m, 6H),2.21-2.06 (m, 2H),1.50 (s, 3H), 1.23-1.15 (m, 9H); ^{13}C NMR (DMSO-d6) *δ* 162.3, 158.1, 157.9, 154.8, 144.7, 137.4, 135.5, 135.3, 130.0, 127.9, 127.7, 126.8, 113.2, 113.0, 101.9, 85.8, 85.4, 84.6, 81.4, 72.6, 70.5, 63.6, 55.0, 45.3, 29.4, 8.4; HRMS (ESI): calcd for $C_{34}H_{35}N_3O_6Na$ ([M+Na]$^+$) 604.2525, found 604.2424.

Preparation of T_6(C1)T_6

2 (291 mg, 0.426 mmol) was dissolved in CH_3CN (5 mL). Disopropylethylamine (261 μL, 1.50 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (223 μL, 1.00 mmol) was added to the solution. The solution was stirred at 25°C for 30 minutes. AcOEt (25 mL) and saturated NaClaq (25 mL) were added to the suspension. The product was extracted to the organic layer. The organic layer was washed with saturated NaClaq (25 mL) twice, dried over magnesium sulfate,

and filtered. The solvent was removed by evaporation and the residue was dried by coevaporation with acetonitrile and further dried under reduced pressure. After dissolving the residue to CH_3CN (10 mL), the phosphoramidite solution was used directly for automated DNA synthesis. $T_6(C1)T_6$ was synthesized by standard phosphoramidite method on the DNA synthesizer. The protected oligonucleotide was cleaved by 28% NH_3aq from the solid support and deprotected at 25°C for 16 hours in 28% NH_3aq. After removal of ammonia by speed-vac, the aqueous solution was filtered through 0.45 μm filter. $T_6(C1)T_6$ was purified by reversed-phase HPLC with a linear gradient over 20 minutes from 0 to 50% CH_3CN/50 mM AF. The retention time of the product was 10.6 minutes. After the desalination, the product was identified by MALDI TOF mass spectrometry: $T_6(C1)T_6$, calcd for $C_{133}H_{172}N_{27}O_{88}P_{12}$ 3928.6 $[M-H]^-$; found 3928.8.



4-*N*-[*N*,*N*-di(*n*-butyl)aminomethylene]-5-phenylethynyl-2'-deoxycytidine (3)

5-phenylethynyl-2'-deoxycytidine (708 mg, 2.16 mmol) [21] was dissolved in methanol (10 mL). *N*,*N*-di(*n*-butyl)formamide dimethyl acetal (2.0 mL, 8.6 mmol) [22] was added to the solution. The solution was stirred at 25°C for 2 hours. The solvent was removed by evaporation. The product **3** was purified by silica gel column chromatography (0–10% MeOH/CH_2Cl_2). The product **3** was obtained as

a pale brown oil (700 mg, 1.50 mmol, 69%): ^{1}H NMR (DMSO-d6) δ 8.64 (s, 1H), 8.44 (s, 1H), 7.43-7.34 (m, 5H), 6.12 (t, *J* = 6.2, 1H), 5.23 (d, *J* = 4.1, 1H), 5.13 (t, *J* = 4.8, 1H), 4.26-4.21 (m, 1H), 3.84-3.81 (m, 1H), 3.70-3.44 (m, 6H), 2.26-2.20 (m, 1H), 2.08-2.01 (m, 1H), 1.66-1.52 (m, 4H), 1.32-1.22 (m, 4H), 0.90 (t, *J* = 7.3, 3H), 0.82 (t, *J* = 7.3, 3H); ^{13}C NMR (DMSO-d6) δ 169.7, 157.4, 153.4, 145.3, 130.7, 128.6, 128.1, 123.2, 97.5, 91.6, 87.6, 85.8, 84.5, 69.7, 60.7, 54.9, 51.4, 45.4, 41.0, 30.4, 28.5, 19.6, 19.1, 13.5; HRMS (ESI) calcd for $C_{26}H_{35}N_4O_4$ ($[M+H]^+$) 467.2658, found 467.2653.

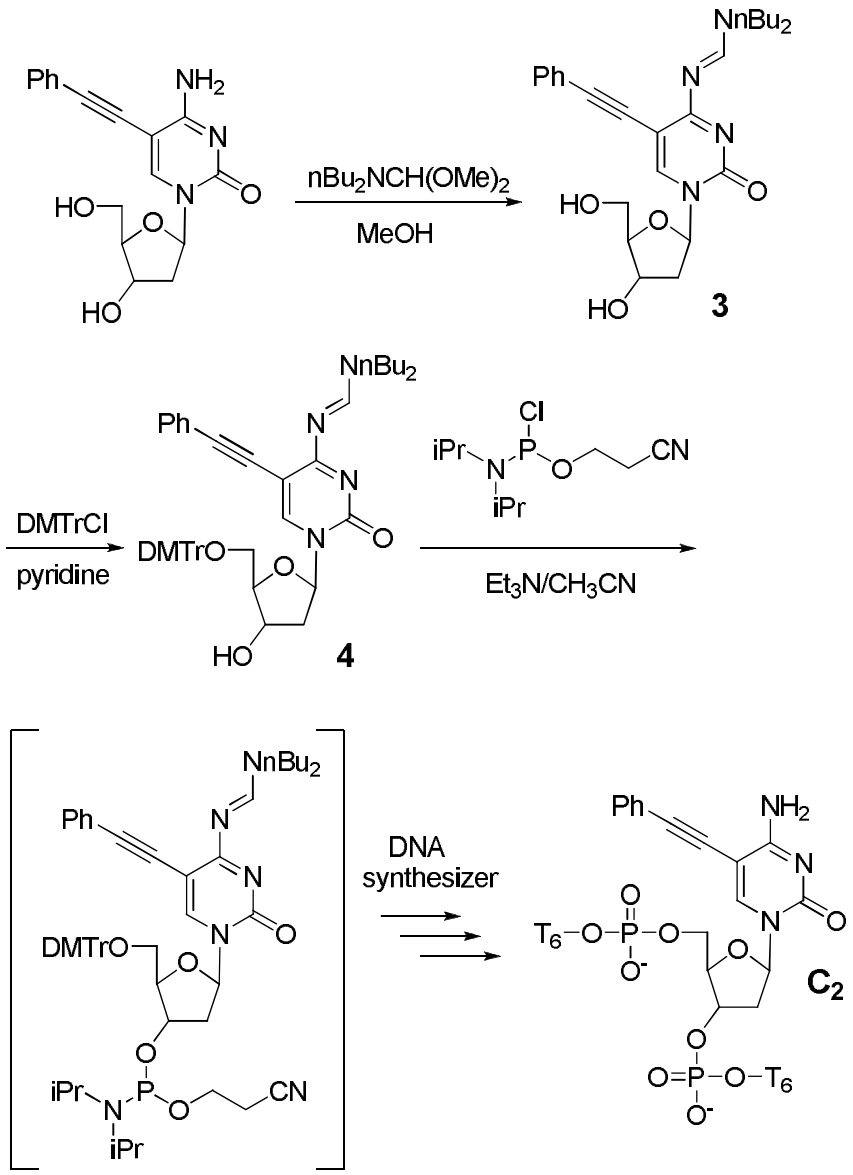
5'-*O*-dimethoxytrityl-4-*N*-[*N*,*N*-di(*n*-butyl)aminomethylene]-5-phenylethynyl -2'-deoxycytidine (4)

Pyridine (10 mL) was added to **3** (700 mg, 1.50 mmol) and DMTrCl (763 mg, 2.25 mmol). The solution was stirred at 25°C for 1 hour. After addition of methanol (10 mL), the solvent was removed by evaporation. The residual pyridine was removed by coevaporation with CH_2Cl_2 and hexane. The product **4** was purified by silica gel column chromatography (0–5% MeOH, 2% Et_3N/CH_2Cl_2). The product **4** was obtained as white powder (924 mg, 1.20 mmol, 80%): ^{1}H NMR (DMSO-d6) δ 8.65 (s, 1H), 8.17 (s, 1H), 7.44-6.81 (m, 18H), 6.14 (t, *J* = 6.6, 1H), 5.32 (d, *J* = 4.6, 1H), 4.31-4.26 (m, 1H), 4.02-3.97 (m, 1H), 3.644 (s, 3H), 3.637 (s, 3H), 3.57-3.49 (m, 4H), 3.23-3.16 (m, 2H), 2.36-2.30 (m, 1H), 2.17-2.10 (m, 1H), 1.66-1.53 (m, 4H), 1.32-1.22 (m, 4H), 0.91 (t, *J* = 7.3, 3H), 0.82 (t, *J* = 7.3, 3H); ^{13}C NMR(DMSO-d6) δ 169.7, 158.03, 158.01, 157.5, 153.4, 144.7, 144.3, 135.6, 135.4, 130.6, 129.7, 129.6, 128.3, 127.93, 127.86, 127.6, 126.6, 123.0, 113.2, 97.9, 91.7, 86.2, 85.9, 83.9, 70.6, 63.5, 54.9, 54.5, 45.4, 41.2, 30.4, 28.5, 19.6, 19.1, 13.5; HRMS (ESI): calcd for $C_{47}H_{53}N_4O_6$ ($[M+H]^+$) 769.3965, found 769.3985.

Preparation of $T_6(C2)T_6$

4 (109 mg, 0.142 mmol) was dissolved in CH_3CN (5 mL). Triethylamine (157 µL, 1.13 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (126 µL, 0.565 mmol) was added to the solution. The solution was stirred at 25°C for 30 minutes. AcOEt (25 mL) and saturated NaClaq (25 mL) were added to the suspension. The product was extracted to the organic layer. The organic layer was washed with saturated NaClaq (25 mL) twice, dried over magnesium sulfate, and filtered. The solvent was removed by evaporation and the residue was dried by coevaporation with acetonitrile and further dried under reduced pressure.

After dissolving the residue to CH_3CN (4.7 mL), the phosphoramidite solution was used directly for automated DNA synthesis. $T_6(C2)T_6$ was synthesized by the standard phosphoramidite method on the DNA synthesizer. The last DMTr group was not deprotected on the DNA synthesizer. The protected oligonucleotide was cleaved by 28% NH_3aq from the solid support and deprotected at 25°C for 16 hours in 28% NH_3aq. After removal of ammonia by speed-vac, the aqueous solution was filtered through a 0.45 μm filter. 5'-DMTr-$T_6(C2)T_6$ was purified by reversed-phase HPLC with a linear gradient over 20 minutes from 5 to 50% CH_3CN/50 mM AF. The retention time of the 5'-DMTr-$T_6(C2)T_6$ was 15.2 minutes. After speed-vac and freeze-drying to remove the solvent and AF, the DMTr group was removed at 25°C for 30 minutes in 80% AcOHaq (100 μL). The solution was diluted with H_2O to be ≈1 mL and passed through a 0.45 m filter. $T_6(C2)T_6$ was purified by reversed-phase HPLC with a linear gradient over 20 minutes from 8 to 20% CH_3CN/50 mM AF. The retention time of the product was 13.6 minutes. After the desalination, the product was identified by MALDI TOF mass spectrometry: $T_6(C2)T_6$, calcd for $C_{137}H_{172}N_{27}O_{88}P_{12}$ 3976.6 $[M-H]^-$; found 3977.1.



8-(2-formylphenyl)-2'-deoxyadenosine (5)

8-bromo-2'-deoxyadenosine (2.20 g, 6.66 mmol) [23], 2-formylbenzene boronic acid (2.00 g, 13.3 mmol), palladium (II) acetate (150 mg, 0.666 mmol), triphenylphosphine (525 mg, 2.00 mmol), and potassium carbonate (1.84 g, 13.3 mmol) were stirred in acetonitrile (40 mL) and H_2O (20 mL) at 80°C for 4 hours [24,25]. The solvent was removed by evaporation. Methanol (200 mL) was added to the residue. The resulting solution was filtered. The solvent was removed by evaporation. The product **5** was purified by silica gel column chromatography (10% MeOH/CH_2Cl_2). The product **5** was obtained as white powder (1.81 g, 5.09 mmol, 76%): ^{1}H NMR (DMSO-d6) δ 9.93 (s, 1H), 8.18 (s,

1H), 8.09-8.07 (m, 1H), 7.90-7.80 (m, 2H), 7.74-7.72 (m, 1H), 7.46 (br, 2H), 5.90 (dd, *J* = 8.5, 6.2, 1H), 5.49 (dd, *J* = 8.3, 4.1, 1H), 5.17 (d, *J* = 4.1, 1H), 4.39-4.35 (m, 1H), 3.82-3.79 (m, 1H), 3.65-3.59 (m, 1H), 3.50-3.44 (m, 1H), 3.19 (ddd, *J* = 12.8, 8.7, 6.0, 1H), 2.06 (ddd, *J* = 13.0, 6.2, 1.8, 1H); ^{13}C NMR (DMSO-d6) *δ* 191.7, 156.2, 152.3, 149.6, 147.4, 135.4, 133.8, 131.5, 131.4, 130.8, 129.5, 119.3, 88.3, 85.7, 71.3, 62.2, 37.4; HRMS (ESI) calcd for $C_{17}H_{18}N_5O_4$ ($[M+H]^+$) 356.1359, found 356.1364.

6-*N*-[*N*,*N*-di(*n*-butyl)aminomethylene]-8-(2-formylphenyl)-2'-deoxyadenosine (6)

5 (1.55 g, 4.36 mmol) was dissolved in methanol (20 mL). *N*,*N*-di(*n*-butyl)-formamide dimethyl acetal (8.14 mL, 34.8 mmol) was added to the solution [22]. The solution was stirred at 25°C for 2 hours. The solvent was removed by evaporation. The product **6** was purified by silica gel column chromatography (2–5% MeOH/CH_2Cl_2). The product **6** was obtained as a pale brown oil (1.40 g, 2.83 mmol, 65%): ^{1}H NMR (DMSO-d6) *δ* 9.92 (s, 1H), 8.91 (s, 1H), 8.45 (s, 1H), 8.09 (d, *J* = 7.8, 1H), 7.91-7.82 (m, 2H), 7.74 (d, *J* = 7.3, 1H), 5.94 (dd, *J* = 7.8, 6.8, 1H), 5.28 (dd, *J* = 7.6, 4.4, 1H), 5.18 (d, *J* = 4.1, 1H), 4.40-4.36 (m, 1H), 3.81-3.77 (m, 1H), 3.64-3.41 (m, 6H), 3.25-3.18(m, 1H), 2.09-2.04 (m, 1H), 1.62-1.52 (m, 4H), 1.33-1.22 (m, 4H), 0.94-0.83 (m, 6H); ^{13}C NMR(DMSO-d6) *δ* 191.8, 159.5, 157.9, 151.7, 151.6, 149.3, 135.5, 133.8, 131.5, 131.3, 130.9, 129.7, 125.8, 88.2, 85.5, 71.2, 62.1, 50.9, 44.4, 37.2, 30.4, 28.7, 19.6, 19.1,13.7, 13.5; HRMS (ESI) calcd for $C_{26}H_{35}N_6O_4$ ($[M+H]^+$) 495.2720, found 495.2718.

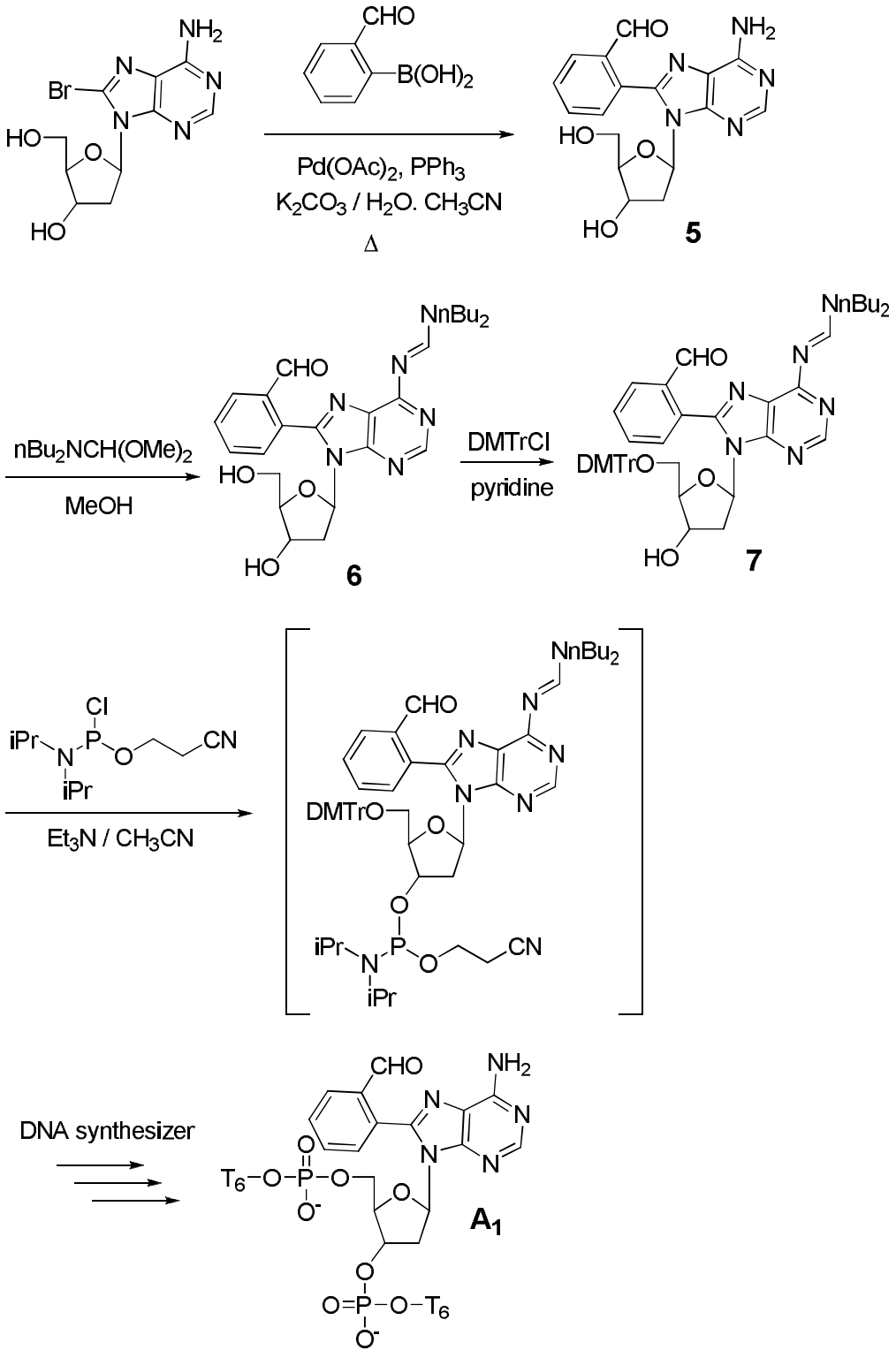
5'-*O*-dimethoxytrityl-6-*N*-[*N*,*N*-di(*n*-butyl)aminomethylene]-8-(2-formylphenyl)-2'-deoxyadenosine (7)

Pyridine (10 mL) was added to **6** (1.31 g, 2.64 mmol) and DMTrCl (991 mg, 2.92 mmol). The solution was stirred at 25°C for 1 hour. After addition of methanol (10 mL), the solvent was removed by evaporation. The residual pyridine was removed by coevaporation with CH_2Cl_2 and hexane. The product **7** was purified by silica gel column chromatography (2% MeOH, 2% Et_3N/CH_2Cl_2). The product **7** was obtained as a white powder (1.24 g, 1.56 mmol, 59%): ^{1}H NMR (DMSO-d6) *δ* 9.89 (s, 1H), 8.88 (s, 1H), 8.25 (s, 1H), 8.06-8.04 (m, 1H), 7.88-7.78 (m, 3H), 7.33-7.31 (m, 2H), 7.21-7.15 (m, 7H), 6.81-6.73 (m, 4H), 5.98 (t, *J* = 6.9, 1H), 5.25 (d, *J* = 4.6, 1H), 4.57-4.53 (m, 1H), 3.94-3.90 (m, 1H), 3.71 (s, 3H), 3.69 (s, 3H), 3.59-3.53 (m, 2H), 3.44-3.32 (m, 3H), 3.20-3.12 (m, 2H),

2.11 (ddd, *J* = 13.0, 7.3, 4.3, 1H), 1.62-1.52 (m, 4H), 1.34-1.23 (m, 4H), 0.90-0.87 (m, 6H); ^{13}C NMR(DMSO-d6) *δ* 191.6, 159.2, 157.9, 157.9, 157.7, 151.8, 149.5, 145.0, 135.7, 135.60, 135.55, 133.6, 131.7, 131.4, 130.7, 129.7, 129.5, 129.1, 127.6, 126.5, 125.6, 113.0, 112.9, 85.8, 85.2, 84.8, 71.0, 63.6, 54.94, 54.90, 50.9, 44.4, 36.3, 30.4, 28.7, 19.6, 19.1, 13.7, 13.5; HRMS (ESI): calcd for $C_{47}H_{53}N_6O_6$ ($[M+H]^+$) 797.4027, found 797.4046.

Preparation of $T_6(A1)T_6$

7 (159 mg, 0.200 mmol) was dissolved in CH_3CN (5 mL). Triethylamine (223 µL, 1.60 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (178 µL, 0.800 mmol) were added to the solution. The solution was stirred at 25°C for 30 minutes. AcOEt (25 mL) and saturated NaClaq (25 mL) were added to the suspension. The product was extracted to the organic layer. The organic layer was washed with saturated NaClaq (25 mL) twice, dried over magnesium sulfate, and filtered. The solvent was removed by evaporation and the residue was dried by coevaporation with acetonitrile and further dried under reduced pressure. After dissolving the residue in CH_3CN (10 mL), the phosphoramidite solution was used directly for automated DNA synthesis. $T_6(A1)T_6$ was synthesized by the standard phosphoramidite method on the DNA synthesizer. The last DMTr group was not deprotected on the DNA synthesizer. The protected oligonucleotide was cleaved from the solid support using 28% NH_3aq and deprotected at 25°C for 16 hours in 28% NH_3aq. After removal of ammonia by speed-vac, the aqueous solution was filtered through a 0.45 µm filter. 5'-DMTr-$T_6(C2)T_6$ was purified by reversed-phase HPLC with a linear gradient over 20 minutes from 0 to 50% CH_3CN/50 mM AF. The retention time of the 5'-DMTr-$T_6(A1)T_6$ was 16.5 minutes. After speed-vac and freeze-drying to remove the solvent and AF, the DMTr group was removed at 25°C for 30 minutes in 80% AcOHaq (100 µL). The solution was diluted with H_2O to 1 mL and filtered through a 0.45 m filter. $T_6(C2)T_6$ was purified by reversed-phase HPLC with a linear gradient over 20 minutes from 5 to 20% CH_3CN/50 mM AF. The retention time of the product was 14.9 minutes. After the desalination, the product was identified by MALDI TOF mass spectrometry: $T_6(A1)T_6$, calcd for $C_{137}H_{172}N_{29}O_{88}P_{12}$ 4004.7 $[M-H]^-$; found 4005.2.



8-(2-formylphenyl)-2'-deoxyguanosine (8)

8-bromo-2'-deoxyguanosine (3.46 g, 10.0 mmol) [26], 2-formylbenzene boronic acid (3.00 g, 20.0 mmol), palladium (II) acetate (224 mg, 1.00 mmol),

triphenylphosphine (787 mg, 3.00 mmol), and potassium carbonate (2.76 g, 20 mmol) were stirred in acetonitrile (80 mL) and H_2O (40 mL) at 80°C for 4 hours [24,25]. The solvent was removed by evaporation. Methanol (200 mL) was added to the residue. The resultant solution was filtered. The solvent was removed by evaporation. The product **8** was purified by silica gel column chromatography (10–20% MeOH/CH_2Cl_2). The product **8** was obtained as a pale yellow powder (2.97 g, 8.00 mmol, 80%): ^{1}H NMR (DMSO-d6) δ 10.80 (br, 1H), 9.85 (s, 1H), 7.98 (dd, *J* = 7.8, 1.4, 1H),7.84-7.80 (m, 1H), 7.75-7.67 (m, 2H), 6.47 (br, 2H), 5.92 (dd, *J* = 8.2, 6.4, 1H), 5.10 (d, *J* = 4.1 Hz, 1H), 4.89-4.86 (m, 1H), 4.21-4.18 (m, 1H), 3.72-3.69 (m, 1H), 3.49-3.38 (m, 2H), 2.98 (ddd, *J* = 13.2, 8.2, 5.9, 1H), 4.20 (ddd, *J* = 13.3, 6.4, 2.3, 1H); ^{13}C NMR(DMSO-d6) δ 191.7, 156.5, 153.3, 151.8, 143.4, 135.5, 133.5, 132.5, 131.4, 130.1, 128.4, 117.5, 87.8, 84.6, 71.0, 61.9, 36.8; HRMS (ESI) calcd for $C_{17}H_{17}N_5O_4Na$ ([M+Na]$^+$) 394.1127, found 394.1115.

2-*N*-[*N*,*N*-di(*n*-butyl)aminomethylene]-8-(2-formylphenyl)-2'-deoxyguanosine (9)

8 (2.32 g, 6.25 mmol) was dissolved in methanol (20 mL). *N*,*N*-di(*n*-butyl)-formamide dimethyl acetal (11.7 mL, 50.0 mmol) was added to the solution [22]. The solution was stirred 25°C for 2 hours. The solvent was removed by evaporation. The product **9** was purified by silica gel column chromatography (5–10% MeOH/CH_2Cl_2). The product **9** was obtained as a pale yellow powder (2.10 g, 4.11 mmol, 66%): ^{1}H NMR (DMSO-d6) δ 11.51 (s, 1H), 9.89 (s, 1H), 8.53 (s, 1H), 8.02-8.00 (m, 1H), 7.87-7.83 (m, 1H), 7.77-7.73 (m, 1H), 7.69-7.67 (m, 1H), 5.92 (dd, *J* = 7.8, 6.9, 1H), 5.16 (d, J = 4.2, 1H), 4.85-4.82 (m, 1H), 4.33-4.29 (m, 1H), 3.76-3.73 (m, 1H), 3.58-3.35 (m, 6H), 3.14 (ddd, *J* = 13.7, 7.3, 6.4, 1H), 2.05 (ddd, *J* = 13.3, 6.4, 2.8, 1H), 1.63-1.54 (m, 4H), 1.35-1.23 (m, 4H), 0.94-0.89 (m, 6H); ^{13}C NMR(DMSO-d6) δ 191.7, 157.8, 157.4, 157.3, 150.5, 144.6, 135.5, 133.6, 132.1, 131.3, 130.2, 128.7, 120.5, 87.8, 85.0, 71.0, 62.0, 51.2, 44.9, 37.3, 30.3, 28.5, 19.6, 19.1, 13.7, 13.5; HRMS (ESI) calcd for $C_{26}H_{34}N_6O_5Na$ ([M+Na]$^+$) 533.2488, found 533.2481.

5'-*O*-dimethoxytrityl-2-*N*-[*N*,*N*-di(*n*-butyl)aminomethylene]-8-(2-formylphenyl)-2'-deoxyguanosine (10)

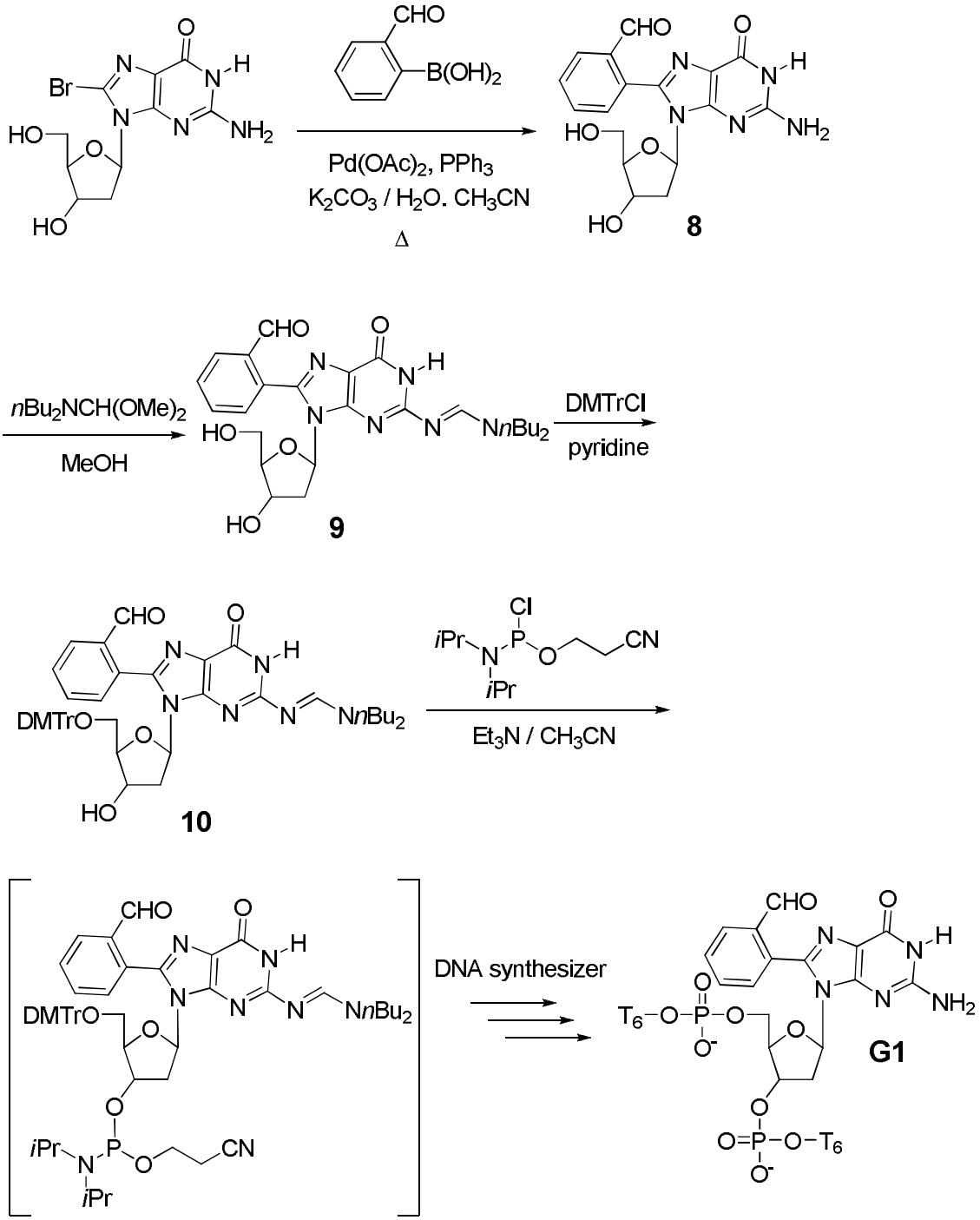
Pyridine (16 mL) was added to **9** (2.10 g, 4.11 mmol) and DMTrCl (1.53 g, 4.52 mmol). The solution was stirred at 25°C for 1 hour. After addition of methanol (10 mL), the solvent was removed by evaporation. The residual pyridine was

removed by coevaporation with CH_2Cl_2 and hexane. The product **10** was purified by silica gel column chromatography (80–98% AcOEt, 2% Et_3N/*n*-hexane). The product **10** was obtained as a pale yellow powder (2.20 g, 2.71 mmol, 66%): ^{1}H NMR (DMSO-d6) δ 11.52 (s, 1H), 9.88 (s, 1H), 8.38 (s, 1H), 7.98 (d, J = 7.8 Hz, 1H), 7.80-7.79 (m, 2H), 7.74-7.70 (m, 1H), 7.31-7.29 (m, 2H), 7.20-7.15 (m, 7H), 6.79-6.72 (m, 4H), 6.00 (dd, J = 8.0, 4.8 Hz, 1H), 5.19 (d, J = 5.1 Hz, 1H), 4.54-4.48 (m, 1H), 3.84-3.80 (m, 1H), 3.70 (s, 3H), 3.69 (s, 3H), 3.47-3.43 (m, 2H), 3.31-3.26 (m, 1H), 3.21-3.13 (m, 3H), 3.07-3.04 (m, 1H), 2.17 (ddd, J = 13.7, 8.0, 5.8 Hz, 1H), 1.59-1.51 (m, 2H), 1.45-1.36 (m, 2H), 1.33-1.24 (m, 2H), 1.15-1.09 (m, 2H), 0.93-0.89 (m, 3H), 0.79-0.75 (m, 3H); ^{13}C NMR(DMSO-d6) δ 191.6, 158.0, 157.9, 157.5, 157.3, 156.9, 150.3, 144.9, 144.8, 135.7, 135.6, 133.4, 132.5, 131.2, 130.1, 129.6, 129.4, 128.3, 127.6, 126.5, 120.4, 112.9, 85.4, 85.2, 84.0, 70.6, 63.6, 55.0, 54.9, 51.2, 45.0, 37.6, 30.2, 28.5, 19.6, 19.0, 13.7, 13.5; HRMS (ESI): calcd for $C_{47}H_{53}N_6O_7$ ($[M+H]^+$) 813.3976, found 813.3976.

Preparation of T_6(G1)T_6

10 (163 mg, 0.200 mmol) was dissolved in CH_3CN (5 mL). Triethylamine (223 µL, 1.60 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (178 µL, 0.800 mmol) were added to the solution. The solution was stirred at 25°C for 30 minutes. AcOEt (25 mL) and saturated NaClaq (25 mL) were added to the suspension. The product was extracted to the organic layer. The organic layer was washed with saturated NaClaq (25 mL) twice, dried over magnesium sulfate, and filtered. The solvent was removed by evaporation and the residue was dried by coevaporation with acetonitrile and further dried under reduced pressure. After dissolving the residue in CH_3CN (10 mL), the phosphoramidite solution was used directly for automated DNA synthesis. T_6(G1)T_6 was synthesized by standard phosphoramidite method on the DNA synthesizer. The last DMTr group was not deprotected on the DNA synthesizer. The protected oligonucleotide was cleaved by 28% NH_3aq from the solid support and deprotected at 25°C for 16 hours in 28% NH_3aq. After removal of ammonia by speed-vac, the aqueous solution was filtered through a 0.45 µm filter. 5'-DMTr-T_6(G1)T_6 was purified by reversed-phase HPLC with a linear gradient over 20 minutes from 5 to 50% CH_3CN/50 mM AF. The retention time of the 5'-DMTr-T_6(G1)T_6 was 15.2 minutes. After speed-vac and freeze-drying to remove the solvent and AF, the DMTr group was removed at 25°C for 30 minutes in 80% AcOHaq (100 µL). The solution was diluted with H_2O to 1 mL and filtered through 0.45 m filter. T_6(C2)T_6

was purified by reversed-phase HPLC with a linear gradient over 20 minutes from 5 to 20% CH_3CN/50 mM AF. The retention time of the product was 14.6 minutes. After desalination, the product was identified by MALDI TOF mass spectrometry: $T_6(G1)T_6$, calcd for $C_{137}H_{172}N_{29}O_{89}P_{12}$ 4020.7 $[M-H]^-$; found 4022.0.



DNA cleavage reaction using designed DNA base analogues

DNA cleavage was carried out using an aqueous solution of DNA oligonucleotides (100 μM) in a screw-cap tube. The same volume of 40% methylamine was added to the sample and the resulting solution was heated at 70°C in a water bath. After removal of methylamine by speed-vac, the sample was directly analyzed by reversed-phase HPLC with a linear gradient over 20 min from 8% to 20% CH_3CN in 50 mM AF. The cleavage products, P1 and P2 were purified, desalted by the HPLC and identified by MALDI TOF mass spectrometry: P1 (pT_6), calcd for $C_{60}H_{79}N_{12}O_{43}P_6$ 1843.2 $[M-H]^-$; found 1841.6 ($P1(T_6(T1)T_6)$), 1841.8 ($P1(T_6(C1)T_6)$), 1842.0 ($P1(T_6(C2)T_6)$), 1842.0 ($P1(T_6(A1)T_6)$), and 1842.2 ($P1(T_6(G1)T_6)$); P2 (T_6pR), calcd for $C_{66}H_{90}N_{13}O_{45}P_6$ 1971.3 $[M-H]^-$; found 1971.0 ($P2(T_6(T1)T_6)$), 1971.4 ($P2(T_6(C1)T_6)$), 1971.1 ($P2(T_6(C2)T_6)$), 1971.2 ($P2(T_6(A1)T_6)$), and 1971.0 ($P2(T_6(G1)T_6)$).

Methods S4

5'-phosphorylation of the DNA oligonucleotide using 5-phenylethynyluracil (PU)

Here, we used 5-phenylethynyluracil (PU) instead of 5-ethynyluracil because of the more facile synthesis of the corresponding phosphoramidite [17]. DMTr-(PU)$T_2AT_2GT_2$ was synthesized on the automated DNA synthesizer by the DMTr ON method. DMTr-(PU)$T_2AT_2GT_2$ was deprotected in 28% NH_3aq at 25°C for 16 hours. After removal of ammonia by speed-vac, the solution was passed through a 0.45 μm filter and the solution was analyzed by reversed-phase HPLC with a linear gradient over 20 min from 0% to 50% CH_3CN in 50 mM AF. The major product was purified, desalinated by reversed-phase HPLC, and identified by MALDI TOF mass spectrometry: DMTr-(PU)$T_2AT_2GT_2$, calcd for $C_{118}H_{136}N_{24}O_{60}P_8$ 3099.3 $[M-H]^-$; found 3097.7. The degradation of the DMTr-containing moiety of DMTr-(PU)$T_2AT_2GT_2$ was carried out by heating in 50% ethylenediamine aqueous solution at 70°C for 8 hours. The solution was neutralized by AcOH. The solution was passed through a 0.45 μm filter and the solution was analyzed by reversed-phase HPLC. The major product was purified, desalinated by reversed-phase HPLC, and identified by MALDI TOF mass spectrometry: p$T_2AT_2GT_2$, calcd for $C_{80}H_{103}N_{22}O_{54}P_8$ 2484.6 $[M-H]^-$; found 2484.6.