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# SYNTHESIS OF ALKYNYL *C*-NUCLEOTIDE TRIPHOSPHATES TOWARD ENZYMATIC ELONGATION OF ARTIFICIAL DNA

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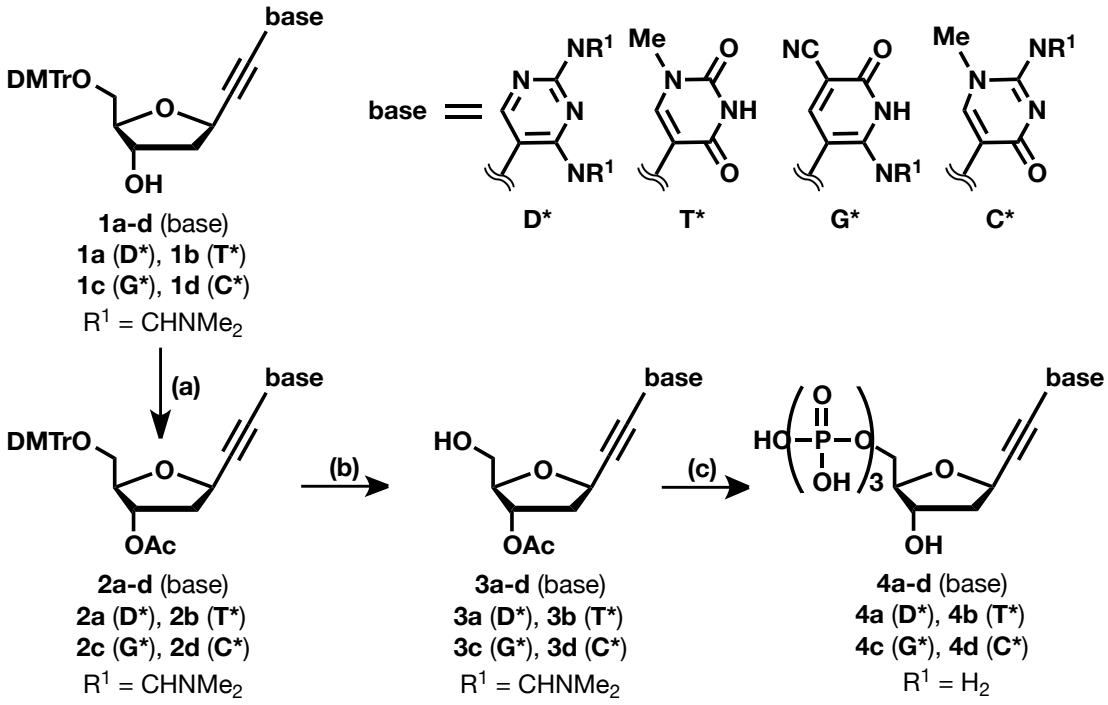
**Abstract** – Four types of alkynyl *C*-nucleotide triphosphates were synthesized, in which heterocyclic compounds as a non-natural nucleobase were tethered to deoxyribose through acetylene bonds. The non-natural nucleobases are D*, T*, G*, and C*, corresponding to natural A, T, G, and C, respectively, from a hydrogen-bonding pattern of view. All the non-natural triphosphates were found to be a good substrate for terminal deoxynucleotidyl transferase (TdT), a natural nucleic acid-relevant enzyme.

In addition to the two canonical base pairs in DNA, development of non-natural base pairs has been pursued for expanding genetic alphabets in recent years. Some of modified DNAs possessing such non-natural bases proved to work in replication system.[1] Therefore, creation of non-natural nucleobases has drawn our attention in terms of purely scientific interests as well as of biotechnological applications.[2] We have developed artificial DNAs made exclusively of non-natural alkynyl *C*-nucleotides with four types of heterocyclic compound as a non-natural nucleobase (D*, T*, G*, and C* in Scheme 1, $R^1 = H_2$, corresponding to natural A, T, G, and C, respectively).[3,4] The artificial DNA-like oligomers formed right-handed duplexes and triplexes in a sequence specific manner with an antiparallel orientation. The artificial duplexes exhibited thermal stabilities fairly close to those of natural double-stranded DNA having the same sequence context. Our artificial DNA is one of the well-fabricated imitations of natural DNA in terms of the above points, which encouraged us to apply the artificial DNA to some nucleic acid-relevant enzymes. Herein, we report the synthesis of alkynyl *C*-nucleotide triphosphates possessing four types of non-natural nucleobase and the adaptability of them to a natural enzyme, terminal deoxynucleotidyl transferase (TdT).

First, the 3'-hydroxy group of alkynyl *C*-nucleosides **1a**–**d**[3] was protected by acetyl (Ac) group to give **2a**–**d** (Scheme 1). Deprotection of 4,4'-dimethoxytrityl (DMTr) group of **2a**–**d** was then performed under

an acidic condition to furnish **3a**–**d**. A conventional phosphorylation reported by Ludwig et al.[5] was applied to the non-natural *C*-nucleosides **3a**–**d**, and the subsequent removal of all of the protecting groups with $NH_4OH$ afforded alkynyl *C*-nucleotide triphosphates **4a**–**d**, successfully. The triphosphates were purified by reverse-phase HPLC, and identified by electrospray ionization (ESI) high-resolution mass (HRMS), $^1$H NMR, and $^{31}$P NMR measurements. For example, a reverse-phase HPLC profile of D*-nucleotide triphosphate **4a** after purification was shown in Figure 1a, in which a single peak appeared at the retention time around 18 min. In the ESI-HRMS spectrum of **4a** under a negative ion mode, a monovalent peak was observed at $m/z$ = 488.9977 for $[M\text{-}H^+]^-$ (Figure 1b). In addition, $^{31}$P NMR spectrum of **4a** by using of 85% $H_3PO_4$ as an external reference showed that a triplet-like double doublet was observed around –23 ppm assignable as a $\beta$-positioned phosphorus in a triphosphate with two P-O-P couplings, whereas two doublets around –10 ppm were for $\alpha$- and $\gamma$-positions (Figure 1c).




Scheme 1. (a) $Ac_2O$, DMAP, $Et_3N$, pyridine; (b) $CCl_3CO_2H$, pyridine, $CH_2Cl_2$, (59% for **3a**, 69% for **3b**, 90% for **3c**, 52% for **3d**, in 2 steps); (c) 1) 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one, pyridine, MeCN, 2) bis(tri-*n*-buthylammonium)pyrophosphate, tributylamine, DMF, 3) iodine (1%) in pyridine–$H_2O$, 4) $NH_4OH$, $H_2O$, (3% for **4a**, 28% for **4b**, 2% for **4c**, 4% for **4d**).

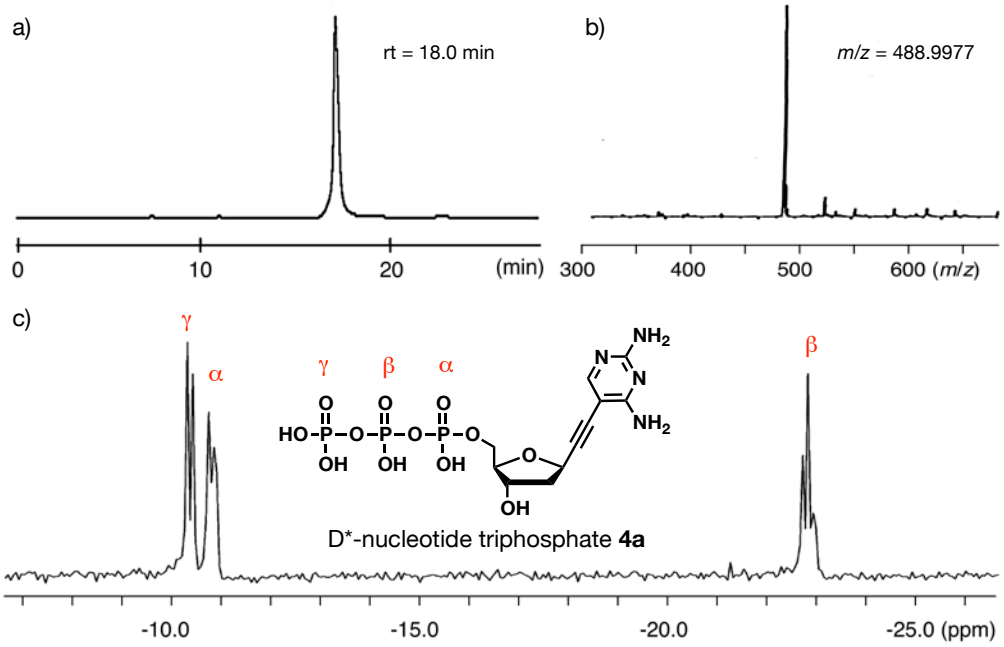



Figure 1. (a) A reverse-phase HPLC profile of D*-nucleotide triphosphate **4a** after purification ($5C_{18}$-AR-II column: 100 mM triethylammonium acetate (pH 7.0)/MeCN, 0 → 40%, 90 min). (b) ESI-HRMS spectrum of **4a** under a negative ion mode (calcd for $[M-H]^-$, $C_{11}H_{16}N_4O_{12}P_3$: 488.9983). (c) $^{31}P$ NMR (160 MHz) spectrum of **4a** in $D_2O$ at room temperature.

Next, we set about applying these four types of alkynyl *C*-nucleotide triphosphate **4a**–**d** to natural nucleic acids-relevant enzymes. Terminal deoxynucleotidyl transferase, TdT,[6] catalyzes the addition of deoxynucleotides to the 3'-OH terminus of single- or double-stranded DNA without a template. Because the enzymatic reaction is really simple and easy to evaluate, we applied **4a**–**d** to TdT. As a primer (P), a 6-(fluorescein-6-ylcarboxamido)hexan-1-yloxyphosphoryl (FAM)-tethered oligonucleotide (5'-FAM-CGCATAACCCTAACC-3') was used for the fluorescent gel imaging. Figure 2a displays a primer elongation scheme of an alkynyl *C*-nucleotide triphosphate by TdT. When the D*-nucleotide triphosphate **4a** was subjected to the enzymatic reaction, a new band assignable to P+1 was observed at a less-mobility region than the primer band in the PAGE analysis after 0.25 h (the top panel in Figure 2b). The new band was indeed identified as the elongated oligomer with the extra single D*-nucleotide residue by MALDI-TOF mass analysis of the band. In addition, time course measurements by MALDI-TOF mass spectrometry also supported the extension reaction of **4a** (Figure 2c). The single-nucleotide elongation for other non-natural *C*-nucleotide triphosphates **4b-d** all successfully progressed at the 3'-termini of the natural oligonucleotide primer (Figure 2b). Regardless of the use of non-natural *C*-nucleotide triphosphates, the single-nucleotide extension seems to be very fast, i.e., the primer oligonucleotide was disappeared completely within 0.5 h for all the cases. The resulting P+1 oligomers, the elongated oligomers with the extra single non-natural *C*-nucleotide, would be further primers for the next enzymatic

elongation. In this reaction, the non-natural *C*-nucleotide residues exist at the reaction point of 3'-end in the primer. Nevertheless, further elongation efficiently progressed between the non-natural triphosphates and the non-natural terminus of the primer to afford P+2 oligomers. This means that the two extra alkynyl *C*-nucleotides were incorporated onto the initial primer. In the case for the D*-nucleotide triphosphate **4a**, the P+3 product was also observed within 0.5 h. Although the second and the third elongation rates seem to be somewhat slow, our non-natural skeleton did participate in natural enzymatic reaction. The success would result from the structural and physicochemical resemblance between our non-natural alkynyl DNAs and natural DNAs. The difference of elongation efficiencies between triphosphates seems to be derived from the difference of heterocyclic skeleton as nucleobases, however, at present, the details have not been clarified. Further studies on this topic by means of *in silico* analysis and by use of another enzymes such as DNA ligases and polymerases are now underway in our laboratory.

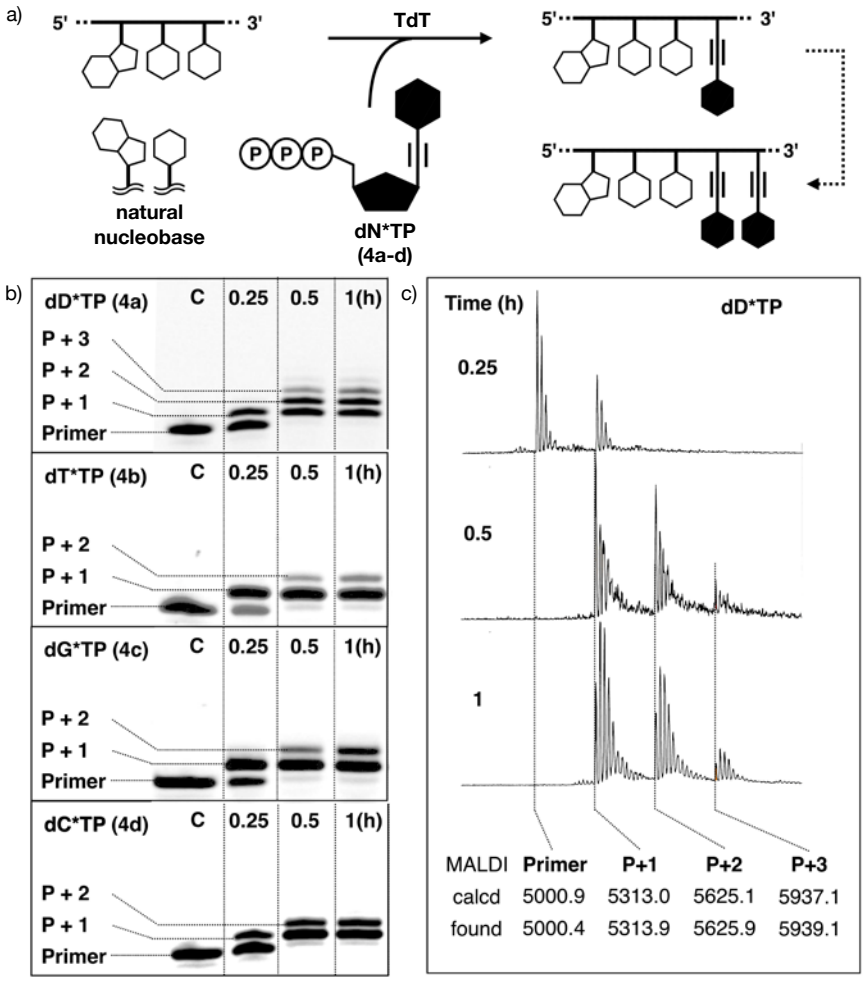



Figure 2. (a) An enzymatic elongation of an alkynyl *C*-nucleotide triphosphate by TdT. (b) Denatured-PAGE analyses of the elongation reaction with different reaction time by dN*TP that indicates each of the four types of the artificial nucleotide triphosphates **4a-d**. (c) Time course measurements by MALDI-TOF mass analysis of the enzymatic elongation of **4a**.

## EXPERIMENTAL

General. $^{1}H$, $^{13}C$, and $^{31}P$ NMR spectra were obtained at 400, 100, and 160 MHz, respectively, on a JEOL ECX400 spectrometer. IR spectra were measured on a JASCO-FT/IR-460 plus spectrometer. ESI-HRMS analyses were carried out on a Thermo LTQ Orbitrap XL ETD mass spectrometer. Melting points were determined with Yanako MP-500D and not corrected. MALDI-TOF mass spectra were recorded on a Bruker-Daltonics-Autoflex mass spectrometer operating under the negative ion mode with 3-hydroxypicolinic acid as a matrix. Gel imaging was carried out on a LAS-4000 (FUJI FILM).

Materials. DMTr- and *N*,*N*-dimethylformamidine (dmf)-protected alkynyl *C*-nucleosides **1a**, **1b**, **1c**, and **1d** were synthesized according to the procedures previously reported.[3] Other materials were all commercially available.

**D*(5'-*O*-DMTr, 3'-*O*-Ac, dmf$_2$)-nucleoside 2a:** A mixture of D*(5'-*O*-DMTr, dmf$_2$)-nucleoside **1a**[3] (100 mg, 0.15 mmol), *N*, *N*-dimethyl-4-aminopyridine (1.8 mg, 0.015 mmol), and acetic anhydride (30 mg, 0.30 mmol) in pyridine (15 mL) was stirred under an argon atmosphere at room temperature for 1 h and concentrated. The residue was diluted with AcOEt (10 mL), and washed with 10% aqueous $Na_2CO_3$ (10 mL) and saturated NaCl (10 mL) aqueous solutions subsequently. The organic phase was dried over $Na_2SO_4$, evaporated, and dried under reduced pressure. The residue consisting of **2a** was used in the next step without further purification.

**D*(5'-OH, 3'-*O*-Ac, dmf$_2$)-nucleoside 3a:** A $CH_2Cl_2$ (10 mL) solution of **2a** (100 mg, 0.14 mmol) and trichloroacetic acid (68 mg, 0.42 mmol) was stirred for 15 min at room temperature. The reaction mixture was quenched by the addition of pyridine (2 mL) and then evaporated. The residue was chromatographed ($SiO_2$; eluent, $CH_2Cl_2$/MeOH = 10 : 1) to give **3a** (57 mg, 59% in 2 steps) as a colorless foam. Mp 48–52 ºC; IR (KBr) 3421, 2925, 2226, 1736, 1628, 1561, 1508, 1437, 1382, 1244 cm$^{-1}$; $^{1}H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.82 (s, 1 H), 8.67 (s, 1 H), 8.33 (s, 1 H), 5.27 (m, 1 H), 5.04 (dd, $J$ = 8.7, 6.4 Hz, 1 H), 4.04 (q, $J$ = 3.2 Hz, 1 H), 3.79 (ddd, $J$ = 21.4, 12.0, 3.8 Hz, 2 H), 3.16 (s, 3 H), 3.14 (s, 3 H), 2.48-2.35 (m, 2 H), 2.08 (s, 3 H) ppm; $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 170.9, 168.9, 165.1, 161.0, 158.6, 157.4, 103.9, 92.8, 85.6, 81.9, 76.5, 69.1, 63.0, 41.3, 41.2, 40.5, 35.3, 35.1, 21.2 ppm; HRMS (ESI) calcd for $MH^+$, $C_{19}H_{27}N_6O_4$: 403.2094; found 403.2079.

**D*-triphosphate 4a:** A mixture of **3a** (70 mg, 0.17 mmol) and 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (52 mg, 0.26 mmol) in MeCN/pyridine (0.7 + 0.4 mL) was stirred under an argon atmosphere at room temperature for 15 min. To the reaction mixture was added a solution of

bis(tri-*n*-butylammonium)pyrophosphate (197 mg, 0.36 mmol) in tributylamine/DMF (0.23 + 0.7 mL). After stirring for 15 min at room temperature, to the reaction mixture was added a 1% iodine/pyridine solution (3.0 mL), and the mixture was stirred at room temperature for 30 min. To the mixture was added water (3.0 mL), and the mixture was further stirred for 30 min at room temperature. After removal of the solvent, the residue was diluted with concentrated aqueous ammonia (10 mL). The mixture was stirred at 40 ºC for 10 min and then evaporated. The resulting residue was purified by reverse phase HPLC using a $5C_{18}$-AR-II column (10 x 250 mm) with an eluent of 100 mM triethylammonium acetate (pH 7.0) and the following MeCN percentages of linear gradient (0–90 min, 0–40%) at a flow rate of 5.0 mL/min under 40 ºC using a column oven, to give **4a** (2.5 mg, 3%) as a colorless solid. $^1$H NMR (400 MHz, $D_2O$) $\delta$ 7.95 (s, 1 H), 5.06 (dd, $J$ = 9.6, 6.4 Hz, 1 H), 4.56 (m, 1 H), 4.11 (m, 1 H), 4.08-4.05 (m, 2 H), 2.42-2.27 (m, 2 H) ppm; $^{31}$P NMR (160 MHz, $D_2O$) $\delta$ -9.48 (d, $J$ = 17.4 Hz), -10.61 (d, $J$ = 17.4 Hz), -22.47 (t, $J$ = 17.4 Hz); HRMS (ESI) calcd for $[M-H]^-$, $C_{11}H_{16}N_4O_{12}P_3$: 488.9983; found 488.9977.

**T*(5'-*O*-DMTr, 3'-*O*-Ac)-nucleoside 2b:** This compound was synthesized from T*(5'-*O*-DMTr)-nucleoside **1b**[3] (100 mg, 0.18 mmol) in a manner similar to that described for **2a**. Title compound was used for the next step without purification.

**T*(5'-OH, 3'-*O*-Ac)-nucleoside 3b:** This compound was synthesized from **2b** (100 mg, 0.16 mmol) in a manner similar to that described for **3a**. The purification was carried out by column chromatography ($SiO_2$; eluent, $CH_2Cl_2$/MeOH = 10 : 1) to give **3b** (35 mg, 69% in 2 steps) as a colorless foam. Mp 69–72 ºC; IR (KBr) 3441, 3207, 3057, 2952, 2850, 2236, 1717, 1685, 1636, 1483, 1457, 1427, 1373, 1334, 1250, 1184, 1097, 1053, 1022, 991 $cm^{-1}$; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 9.55 (s, 1 H), 7.52 (s, 1 H), 5.24 (m, 1 H), 4.92 (dd, $J$ = 8.7, 6.4 Hz, 1 H), 4.02 (dd, $J$ = 6.2, 3.9 Hz, 1 H), 3.79 (qd, $J$ = 12.4, 3.9 Hz, 2 H), 3.41 (s, 3 H), 2.40-2.29 (m, 2 H), 2.08 (s, 3 H) ppm; $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 170.8, 162.3, 150.2, 148.7, 98.8, 92.2, 85.7, 76.7, 76.1, 68.6, 62.7, 40.1, 36.6, 21.2 ppm; HRMS (ESI) calcd for $MNa^+$, $C_{14}H_{16}N_2O_6Na$: 331.0906; found 331.0905.

**T*-triphosphate 4b:** This compound was synthesized from **3b** (30 mg, 0.10 mmol) in a manner similar to that described for **4a**. The purification was carried out by reverse phase HPLC using a $5C_{18}$-AR-II column (10 x 250 mm) with an eluent of 100 mM triethylammonium acetate (pH 7.0) and the following MeCN percentages of linear gradient (0–90 min, 0–40%) at a flow rate of 5.0 mL/min under 40 ºC using a column oven, to give **4b** (13.9 mg, 28%) as a colorless solid. $^1$H NMR (400 MHz, $D_2O$) $\delta$ 7.99 (s, 1 H), 5.02 (dd, $J$ = 9.2, 6.4 Hz, 1 H), 4.53 (m, 1 H), 4.08 (m, 1 H), 4.06-4.03 (m, 2 H), 3.38 (s, 3 H), 2.39-2.25 (m, 2 H) ppm; $^{31}$P NMR (160 MHz, $D_2O$) $\delta$ -8.61 (d, $J$ = 17.4 Hz), -10.51 (d, $J$ = 17.4 Hz) -21.98 (t, $J$ =

17.4 Hz) ppm; HRMS (ESI) calcd for $[M-H]^-$, $C_{12}H_{16}N_2O_{14}P_3$: 504.9820; found 504.9820.

**G*(5'-*O*-DMTr, 3'-*O*-Ac, dmf)-nucleoside 2c:** This compound was synthesized from G*(5'-*O*-DMTr, dmf)-nucleoside **1c**[3] (100 mg, 0.16 mmol) in a manner similar to that described for **2a**. Title compound was used for the next step without purification.

**G*(5'-OH, 3'-*O*-Ac, dmf)-nucleoside 3c:** This compound was synthesized from **2c** (100 mg, 0.15 mmol) in a manner similar to that described for **3a**. The purification was carried out by column chromatography ($SiO_2$; eluent, $CH_2Cl_2$/MeOH = 10 : 1) to give **3c** (50 mg, 90%) as a pale yellow foam. Mp 167–172 ºC; IR (KBr) 3426, 2927, 2217, 1734, 1645, 1561, 1500, 1417, 1388, 1308, 1252, 1117, 1051 $cm^{-1}$; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.54 (s, 1 H), 7.75 (s, 1 H), 5.25 (m, 1 H), 4.96 (t, $J$ = 7.6 Hz, 1 H), 4.02 (q, $J$ = 3.4 Hz, 1 H), 3.78 (qd, $J$ = 12.0, 3.8 Hz, 2 H), 3.34 (s, 3 H), 3.20 (s, 3 H), 2.35 (q, $J$ = 4.0 Hz, 2 H), 2.09 (s, 3 H) ppm; $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 170.9, 163.5, 158.7, 157.4, 151.1, 116.7, 95.6, 91.8, 90.1, 85.5, 81.1, 76.2, 68.9, 63.0, 41.9, 40.5, 35.4, 21.1 ppm; HRMS (ESI) calcd for $MNa^+$, $C_{18}H_{20}N_4O_5Na$: 395.1331; found 395.1324.

**G*-triphosphate 4c:** This compound was synthesized from **3c** (74 mg, 0.20 mmol) in a manner similar to that described for **4a**. The purification was carried out by reverse phase HPLC using a $5C_{18}$-AR-II column (10 x 250 mm) with an eluent of 100 mM triethylammonium acetate (pH 7.0) and the following MeCN percentages of linear gradient (0–60 min, 0–40%) at a flow rate of 5.0 mL/min under 40 ºC using a column oven, to give **4c** (2.0 mg, 2%) as a pale yellow solid. $^1H$ NMR (400 MHz, $D_2O$) $\delta$ 7.86 (s, 1 H), 5.05 (dd, $J$ = 9.8, 6.1 Hz, 1 H), 4.54 (m, 1 H), 4.11 (m, 1 H), 4.08-4.05 (m, 2 H), 2.40-2.26 (m, 2 H) ppm; $^{31}P$ NMR (160 MHz, $D_2O$) $\delta$ -9.67 (d, $J$ = 17.3 Hz), -10.70 (d, $J$ = 25.9 Hz), -22.49 (dd, $J$ = 17.3, 25.9 Hz) ppm; HRMS (ESI) calcd for $[M-H]^-$, $C_{13}H_{15}N_3O_{13}P_3$: 513.9823; found 513.9815.

**C*(5'-*O*-DMTr, 3'-*O*-Ac, dmf)-nucleoside 2d:** This compound was synthesized from C*(5'-*O*-DMTr, dmf)-nucleoside **1d**[3] (100 mg, 0.16 mmol) in a manner similar to that described for **2a**. Title compound was used for the next step without purification.

**C*(5'-OH, 3'-*O*-Ac, dmf)-nucleoside 3d:** This compound was synthesized **2d** (100 mg, 0.15 mmol) in a manner similar to that described for **3a**. The purification was carried out by column chromatography ($SiO_2$; eluent, $CH_2Cl_2$/MeOH = 10 : 1) to give **3d** (28 mg, 52%) as a colorless foam. Mp 102–107 ºC; IR (KBr) 3747, 3410, 2932, 1738, 1628, 1579, 1494, 1418, 1350, 1246, 1120, 1059 $cm^{-1}$; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 8.80 (s, 1 H), 7.47 (s, 1 H), 5.25 (m, 1 H), 4.95 (dd, $J$ = 9.2, 6.4 Hz, 1 H), 4.02 (q, $J$ = 3.2

Hz, 1 H), 3.79 (qd, *J* = 11.9, 3.8 Hz, 2 H), 3.55 (s, 3 H), 3.18 (s, 3 H), 3.13 (s, 3 H), 2.46-2.30 (m, 2 H), 2.07 (d, 3 H) ppm; $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 170.8, 170.1, 159.4, 158.3, 146.6, 104.9, 92.2, 85.8, 79.5, 76.5, 68.8, 62.8, 41.6, 40.2, 39.0, 35.4, 21.2 ppm; HRMS (ESI) calcd for $MNa^+$, $C_{17}H_{22}N_4O_5Na$: 385.1488; found 385.1485.

**C*-triphosphate 4d:** This compound was synthesized from **3d** (30 mg, 0.08 mmol) in a manner similar to that described for **4a**. The purification was carried out by reverse phase HPLC using a $5C_{18}$-AR-II column (10 x 250 mm) with an eluent of 100 mM triethylammonium acetate (pH 7.0) and the following MeCN percentages of linear gradient (0–90 min, 0–40%) at a flow rate of 5.0 mL/min under 40 ºC using a column oven, to give **4d** (1.6 mg, 4%) as a colorless solid. $^1$H NMR (400 MHz, $D_2O$) $\delta$ 7.85 (s, 1 H), 5.03 (dd, *J* = 9.2, 6.4 Hz, 1 H), 4.54 (m, 1 H), 4.11 (m, 1 H), 4.08-4.02 (m, 2 H), 3.51 (s, 3 H), 2.40-2.27 (m, 2 H) ppm; $^{31}$P NMR (160 MHz, $D_2O$) $\delta$ -6.92 (d, *J* = 26.0 Hz), -10.45 (d, *J* = 17.3 Hz), -21.59 (dd, *J* = 17.3, 26.0 Hz) ppm; HRMS (ESI) calcd for $[M-H]^-$, $C_{12}H_{17}N_3O_{13}P_3$: 503.9980; found 503.9979.

**Enzymatic elongation by TdT:** A reaction mixture of a FAM primer (40 pmol, 5'-FAM-CGCATAACCCTAACC-3'), BSA (0.02%, final concentration), 5 x TdT reaction buffer (500 mM HEPES; pH 7.2, 40 mM $MgCl_2$, 0.5 mM DTT), TdT (50 U), dN*TP (10 nmol), and milliQ water for the adjustment to 10 μL of the total volume was incubated at 37 ºC for various time periods (0.25, 0.5, 1 h). The reactions were quenched at the appropriate reaction time by adding 10 μL of gel loading buffer (7 M Urea, 0.1% bromophenolblue, 20 mM EDTA, 1 x TBE buffer) as a stop solution and then TdT was deactivated at 70 ºC for 10 min. The reaction mixtures were analyzed by denatured-PAGE (20% acrylamide, 7 M urea) and MALDI-TOF mass measurements (See Figure 2).

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