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Design and Synthesis of a Photocleavable Fluorescent Nucleotide 3′-*O*-Allyl-dGTP-PC-Bodipy-FL-510 as a Reversible Terminator for DNA Sequencing by Synthesis

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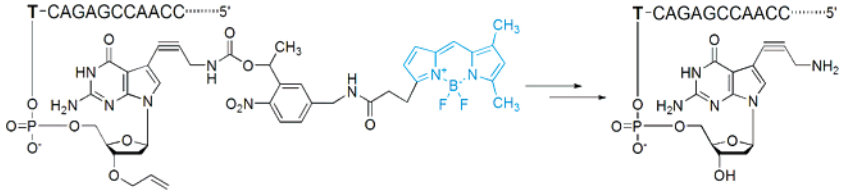

DNA sequencing by synthesis (SBS) using reversible fluorescent nucleotide terminators is potentially an efficient approach to address the limitations of current DNA sequencing techniques. Here, we report the design and synthesis of a 3′-*O*-allyl photocleavable fluorescent nucleotide analogue, 3′-*O*-allyl-dGTP-PC-Bodipy-FL-510, as a reversible terminator for SBS. The nucleotide is efficiently incorporated by DNA polymerase into a growing DNA strand to terminate the polymerase reaction. After that, the fluorophore is photocleaved quantitatively by irradiation at 355 nm, and the allyl group is rapidly and efficiently removed by using a Pd-catalyzed reaction under DNA-compatible conditions to regenerate a free 3′-OH group to reinitiate the polymerase reaction. Two cycles of such steps were successfully demonstrated to sequence a homopolymeric region of a DNA template, facilitating the development of SBS as a viable approach for high-throughput DNA sequencing.

Introduction

DNA sequencing is a fundamental tool for biological research and medical diagnostics, driving disease gene discovery and gene function studies. DNA sequencing by synthesis (SBS) using reversible fluorescent nucleotide terminators[1] is a potentially efficient approach to address the limitations of current DNA sequencing techniques, such as throughput and data accuracy. We have previously reported the design and synthesis of a 3′-*O*-allyl photocleavable (PC) fluorescent nucleotide analogue, 3′-*O*-allyl-dUTP-PC-Bodipy-FL-510, as a reversible terminator for SBS.[2] The nucleotide can be efficiently incorporated by DNA polymerase into a growing DNA strand to terminate the polymerase reaction. After that, the fluorophore can be photocleaved quantitatively by irradiation at 355 nm, and the allyl group is rapidly and efficiently removed by using a Pd-catalyzed reaction in water to regenerate a free 3′-OH group to reinitiate the polymerase reaction.

To the best of our knowledge, using 3′-modified dGTP as a reversible terminator for SBS has not been reported, partly due to the difficulty of modifying the 3′-OH of guanosine by a suitable capping group without protecting the guanine base. Here, we describe the design and synthesis of a 3′-*O*-allyl photocleavable fluorescent 7-deaza-dGTP, 3′-*O*-allyl-dGTP-PC-Bodipy-FL-510 (**10**), with a fluorophore attached to the 7 position of the modified guanine base and its successful application as a reversible terminator to determine two repeated nucleotide sequences in SBS. Previously, the Pd-catalyzed deallylation to regenerate a free 3′-OH of the DNA extension product was carried out in pure water,[2] which can destabilize

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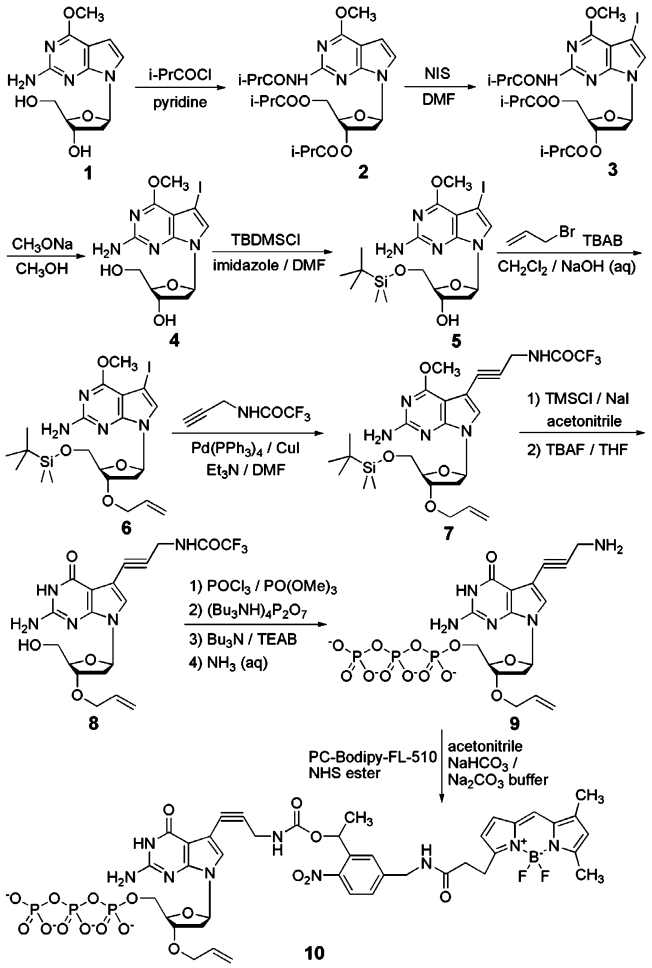
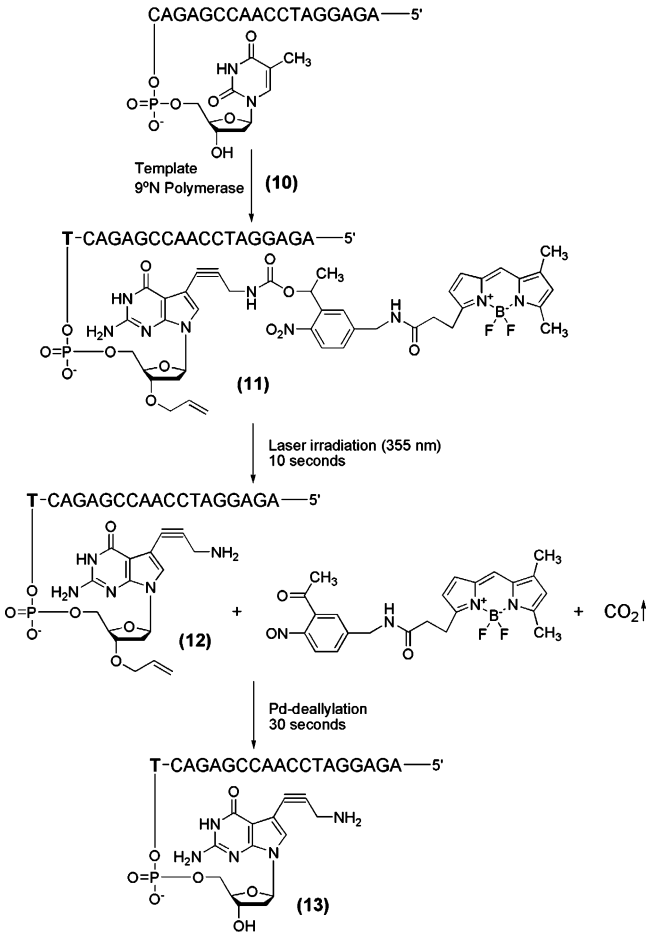
SCHEME 1. Synthesis of 3′-*O*-Allyl-dGTP-PC-Bodipy-FL-510 (10)



SCHEME 2. DNA Polymerase Extension Reaction Using 3′-*O*-Allyl-dGTP-PC-Bodipy-FL-510 (10) as a Reversible Terminator



the primer−template duplex. We report here the identification of a new condition for rapid quantitative deallylation in a buffer solution at pH 8.8, which is commonly used for polymerase reaction. The successful synthesis of compound **10** as a reversible terminator to sequence through a homopolymer sequence and the identification of the new deallylation condition will facilitate the development of SBS as a viable approach for de novo DNA sequencing.

Results and Discussion

2-Amino-6-methoxy-9-(β-D-2′-deoxyribofuranosyl)-7-deazapurine **1** was chosen as the starting material for the synthesis of 3′-*O*-allyl-7-(3-aminoprop-1-ynyl)-7-deaza-dGTP **9** (Scheme 1). Compound **1** was first protected by isobutyryl chloride to yield **2** quantitatively.[3] Compound **2** was iodinated at the 7-position by *N*-iodosuccinimide (NIS) to produce a single product **3** in 90% yield, as the 2-acylamino group in the nucleobase promotes the formation of 7-substituted product.[4] Compound **3** was deprotected by methanolic sodium methoxide to afford **4** in 94% yield. 5′-OH of **4** was protected by *tert*-butyldimethylsilyl chloride to produce **5** in 88% yield.[5] 3′-OH of **5** was subsequently allylated in CH_2Cl_2 and 40% aqueous NaOH solution using tetrabutylammonium bromide as phase-transfer catalyst to give a 92% yield of **6** without N^2-allylated product. Sonogashira cross-coupling reaction of **6** with the terminal alkyne catalyzed by Pd(0)/Cu(I) formed **7** in 94% yield.[6] Next, a one-pot procedure of demethylation[7] and desilylation of **7** gave a moderate 34% yield of **8**. Finally **8** was transformed into the corresponding triphosphate 3′-*O*-allyl-dGTP **9** following established procedures.[8] Coupling **9** with PC-Bodipy-FL-510 NHS ester[9] yielded the target compound, 3′-*O*-allyl-dGTP-PC-Bodipy-FL-510 (**10**).

For 3′-*O*-allyl-dGTP-PC-Bodipy-FL-510 (**10**) to act as a reversible terminator for SBS, it is important to establish that **10** can be used to determine a repeated DNA sequence in a polymerase reaction. To this end, we performed a polymerase DNA extension reaction using **10** as a substrate in solution as shown in Scheme 2. This allows the isolation of the DNA product at each step for detailed molecular structure characterization by using MALDI-TOF mass spectrometry (MS) as shown in Figure 1.

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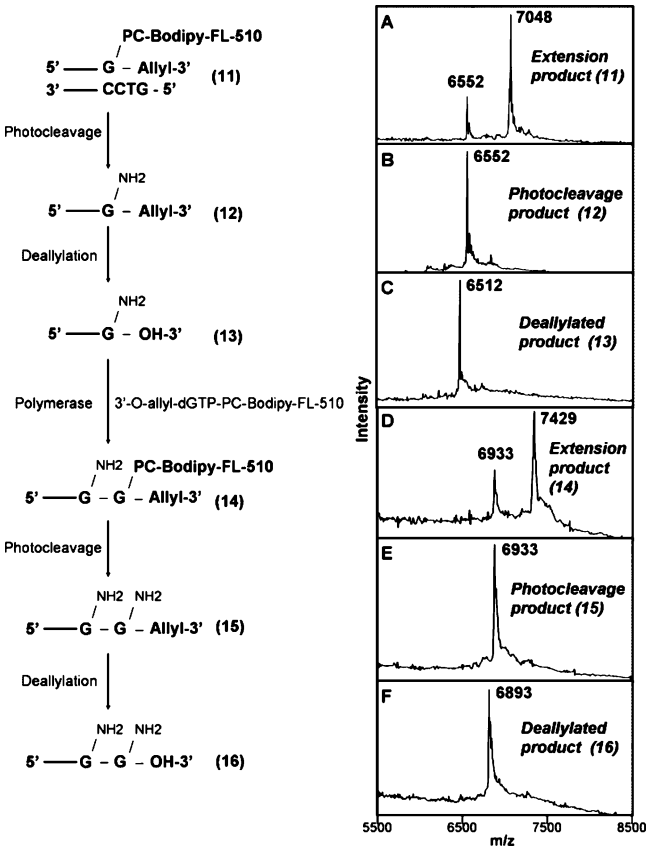

FIGURE 1. Continuous polymerase extension scheme (left) and MALDI-TOF MS spectra of the resulting DNA products (right).

A synthetic 100-mer DNA corresponding to a portion of exon 7 of the human *p53* gene was used as a template to perform the extension. The sequence in the template immediately adjacent to the annealing site of the primer had a repeating sequence of 3′-CC-5′. First, a polymerase extension reaction using **10** as a terminator along with a primer and the above template was performed. After the reaction, a small portion of the DNA extension product was characterized by MALDI-TOF MS. The rest of the product was irradiated at 355 nm for 10 s to cleave the fluorophore from the DNA and then analyzed by MALDI-TOF MS. After photocleavage, the DNA product was added to a deallylation cocktail [1X Thermopol reaction buffer/Na_2PdCl_4/$P(PhSO_3Na)_3$] to remove the 3′-allyl group in 30 s to yield quantitatively deallylated DNA product. The deallylated DNA product with a free 3′-OH group regenerated was then used as a primer to incorporate **10** in a subsequent second extension reaction.

Figure 1 (right panel) shows a sequential mass spectrum at each step of DNA SBS using **10** as a reversible terminator. As can be seen from Figure 1A, the MALDI-TOF MS spectrum consists of a distinct peak at *m*/*z* 7048 corresponding to the single-base DNA extension product **11** with 100% incorporation efficiency, confirming that the reversible terminator **10** can be incorporated base-specifically by DNA polymerase into a growing DNA strand. The small peak at *m*/*z* 6552 corresponding to the photocleavage product is due to the partial cleavage caused by the nitrogen laser pulse (337 nm) used for ionization in MALDI-TOF MS. Figure 1B shows the photocleavage result after 10 s irradiation of the DNA extension product at 355 nm. The peak at *m*/*z* 7048 has completely disappeared, whereas the peak corresponding to the photocleavage product **12** appears as the sole dominant peak at *m*/*z* 6552. Figure 1C shows a single peak at *m*/*z* 6512, which corresponds to a deallylated photocleavage product **13**. The absence of a peak at *m*/*z* 6552 proves that the deallylation reaction was completed with high efficiency. The next extension reaction was carried out by using this deallylated photocleavage product **13** as a primer along with 3′-*O*-allyl-dGTP-PC-Bodipy-FL-510 (**10**) to yield an extension product **14** (Figure 1D). DNA products (**15** and **16**) from photocleavage (Figure 1E) and deallylation (Figure 1F), respectively, were obtained in a similar manner as described previously, thereby completing two entire polymerase extension cycles to sequence a homopolymeric region of a template using **10** as a reversible terminator.

In summary, we have developed a successful strategy for the synthesis of a 3′-*O*-allyl-modified 7-deaza-dGTP bearing a photocleavable fluorophore at the 7 position. This novel nucleotide analogue is shown to be an excellent substrate for 9°N DNA polymerase A485L/Y409V and can be incorporated with high efficiency in a polymerase extension reaction. We have also demonstrated that complete photocleavage to remove the fluorophore is achieved in 10 s on these DNA products. Furthermore, we have shown that deallylation can be swiftly achieved to near completion under mild reaction conditions in an aqueous environment by using a palladium catalyst. Finally, we have established that the deallylated DNA product can be used as a primer to continue the polymerase reaction and that extension, photocleavage, and deallylation can be performed with high efficiency. These results provide further proof of the feasibility of using the allyl group as a reversible capping moiety for the 3′-OH of the photocleavable nucleotide analogues for SBS, validating the approach we had previously proposed.[1] Thus, the successful engineering of the building blocks of DNA with synthetic chemistry will facilitate the development of SBS for high-throughput DNA sequencing and genotyping.

Experimental Section

2-(2-Methylpropanoyl)amino-6-methoxy-9-[3′,5′-bis-*O*-(2-methylpropanoyl)-β-D-2′-deoxyribofuranosyl]-7-deazapurine (2). To a stirred suspension of **1** (1.00 g; 3.57 mmol) in anhydrous pyridine (35 mL) was added slowly isobutyryl chloride (3.40 mL; 32.2 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h. Methanol (2 mL) was then added, and the reaction mixture was stirred for another 10 min. Then most solvent was removed under vacuum. Ethyl acetate (200 mL) and saturated aqueous $NaHCO_3$ (50 mL) were added to the residue. The organic layer was separated, washed with saturated aqueous $NaHCO_3$ and NaCl, respectively, and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the residue was purified by flash column chromatography over silica gel using ethyl acetate−hexane (1:3∼2) as the eluent to afford **2** as white foam (1.75 g; 99% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 7.28 (d, J = 3.7 Hz, 1H), 6.66 (dd, J = 5.9, 8.6 Hz, 1H), 6.51 (d, J = 3.7 Hz, 1H), 5.41 (m, 1H), 4.33−4.36 (m, 2H), 4.22 (m, 1H), 4.08 (s, 3H), 2.83−2.96 (m, 2H), 2.54−2.70 (m, 2H), 2.48−2.54 (ddd, J = 2.0, 5.9, 14.2 Hz, 1H), 1.15−1.23 (m, 18H); ^{13}C NMR (100 MHz, CD_3OD) δ 178.2, 177.7, 177.4, 164.2, 153.4, 152.5, 123.4, 103.5, 100.7, 85.2, 83.0, 75.9, 65.0, 54.4, 37.9, 36.6, 35.0, 34.9, 19.9, 19.3−19.4 (four peaks); HRMS (FAB+) calcd for $C_{24}H_{35}O_7N_4$ ($M + H^+$) 491.2506, found 491.2503.

2-(2-Methylpropanoyl)amino-6-methoxy-7-iodo-9-[3′,5′-bis-*O*-(2-methylpropanoyl)-β-D-2′-deoxyribofuranosyl]-7-deazapurine (3). To a vigorously stirred solution of **2** (1.75 g; 3.57 mmol) in anhydrous DMF (27 mL) was added 95% *N*-iodosuccimide (NIS)

(866 mg; 3.66 mmol). The reaction mixture was stirred at room temperature for 22 h, and then most solvent was removed under vacuum. Diethyl ether (200 mL) and saturated aqueous $NaHCO_3$ (50 mL) were added. The organic layer was separated, washed with saturated aqueous NaCl, and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the residue was purified by flash column chromatography over silica gel using ethyl acetate−hexane (1:3) as the eluent to afford **3** as white solid (1.98 g; 90% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 7.43 (s, 1H), 6.63 (dd, J = 6.0, 8.2 Hz, 1H), 5.41 (m, 1H), 4.33−4.36 (m, 2H), 4.23 (m, 1H), 4.09 (s, 3H), 2.78−2.94 (m, 2H), 2.57−2.70 (m, 2H), 2.50−2.57 (ddd, J = 2.3, 6.0, 14.2 Hz, 1H), 1.17−1.24 (m, 18H); ^{13}C NMR (100 MHz, CD_3OD) δ 178.3, 177.8, 177.5, 164.3, 153.3, 152.8, 128.6, 105.2, 85.3, 83.3, 75.8, 65.0, 54.4, 51.8, 38.2, 36.8, 35.2, 35.1, 19.9, 19.3−19.5 (four peaks); HRMS (FAB+) calcd for $C_{24}H_{34}O_7N_4I$ ($M + H^+$) 617.1472, found 617.1464.

2-Amino-6-methoxy-7-iodo-9-(β-D-2′-deoxyribofuranosyl)-7-deazapurine (4). Compound **3** (1.98 g; 3.21 mmol) was dissolved in 0.5 M methanolic CH_3ONa (50 mL) and stirred at 65 °C for 12 h. Saturated aqueous $NaHCO_3$ (20 mL) was added, and the mixture was stirred for 10 min. Then most of methanol was evaporated, and the residue was extracted by ethyl acetate (150 mL). The organic layer was washed with saturated aqueous $NaHCO_3$ and NaCl, respectively, and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the residue was purified by flash column chromatography over silica gel using $CH_3OH-CH_2Cl_2$ (1:30−15) as the eluent to afford **4** as a white solid (1.23 g; 94% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 7.17 (s, 1H), 6.36 (dd, J = 6.0, 8.4 Hz, 1H), 4.47 (m, 1H), 3.99 (s, 3H), 3.96 (m, 1H), 3.77 (dd, J = 3.4, 12.0 Hz, 1H), 3.70 (dd, J = 3.7, 12.0 Hz, 1H), 2.55−2.64 (ddd, J = 6.0, 8.4, 13.4 Hz, 1H), 2.20−2.26 (ddd, J = 2.4, 5.9, 13.4 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 164.7, 160.6, 154.3, 126.5, 101.6, 88.7, 86.0, 73.0, 63.7, 53.7, 51.3, 41.1; HRMS (FAB+) calcd for $C_{12}H_{16}O_4N_4I$ ($M + H^+$) 407.0216, found 407.0213.

2-Amino-6-methoxy-7-iodo-9-[β-D-5′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyribofuranosyl]-7-deazapurine (5). To a stirred solution of **4** (1.23 g; 3.02 mmol) and imidazole (494 mg; 7.24 mmol) in anhydrous DMF (15 mL) was added *tert*-butyldimethylsilyl chloride (TBDMSCl) (545 mg; 3.51 mmol). The reaction mixture was stirred at room temperature for 20 h. Then most solvent was removed under vacuum, and the residue was purified by flash column chromatography over silica gel using ethyl acetate−hexane (1:2∼0.5) as the eluent to afford **5** as a white foam (1.38 g; 88% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 7.23 (s, 1H), 6.49 (dd, J = 6.1, 7.7 Hz, 1H), 4.46 (m, 1H), 3.99 (s, 3H), 3.94 (m, 1H), 3.79−3.87 (m, 2H), 2.36−2.44 (ddd, J = 5.8, 7.7, 13.3 Hz, 1H), 2.24−2.31 (ddd, J = 3.1, 6.0, 13.3 Hz, 1H), 0.96 (s, 9H), 0.14 (s, 3H), 0.13 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 164.6, 160.7, 154.7, 125.1, 101.0, 88.2, 84.2, 72.7, 64.7, 53.7, 51.7, 41.9, 26.7, 19.4, −5.0, −5.1; HRMS (FAB+) calcd for $C_{18}H_{30}O_4N_4SiI$ ($M + H^+$) 521.1081, found 521.1068.

2-Amino-6-methoxy-7-iodo-9-[β-D-3′-*O*-allyl-5′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyribofuranosyl]-7-deazapurine (6). To a stirred solution of **5** (1.38 g; 2.66 mmol) in CH_2Cl_2 (80 mL) were added tetrabutylammonium bromide (TBAB) (437 mg; 1.33 mmol), allyl bromide (1.85 mL, 21.4 mmol), and 40% aqueous NaOH solution (40 mL). The reaction mixture was stirred at room temperature for 1 h. Ethyl acetate (200 mL) was added, and the organic layer was separated. The aqueous layer was extracted with ethyl acetate (2 × 50 mL). The combined organic layer was washed with saturated aqueous $NaHCO_3$ and NaCl, respectively, and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the residue was purified by flash column chromatography over silica gel using ethyl acetate−hexane (1:3) as the eluent to afford **6** as a white solid (1.37 g; 92% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 7.20 (s, 1H), 6.43 (dd, J = 6.2, 7.9 Hz, 1H), 5.89−5.99 (m, 1H), 5.29−5.35 (dm, J = 17.3 Hz, 1H), 5.16−5.21 (dm, J = 10.5 Hz, 1H), 4.24 (m, 1H), 4.01−4.11 (m, 3H), 3.99 (s, 3H), 3.76−3.84 (m, 2H), 2.32−2.44 (m, 2H), 0.95 (s, 9H), 0.14 (s, 3H), 0.13 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.3, 158.6, 153.6, 134.1, 123.7, 116.9, 100.6, 84.4, 83.0, 79.1, 70.0, 63.6, 53.3, 51.1, 38.1, 26.1, 18.5, −5.1, −5.3; HRMS (FAB+) calcd for $C_{21}H_{34}O_4N_4SiI$ ($M + H^+$) 561.1394, found 561.1390.

2-Amino-6-methoxy-7-{3-[(trifluoroacetyl)amino]prop-1-ynyl]-9-[β-D-3′-*O*-allyl-5′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyribofuranosyl}-7-deazapurine (7). To a stirred solution of **6** (1.37 g; 2.45 mmol) in anhydrous DMF (11 mL) were added tetrakis-(triphenylphosphine)palladium(0) (286 mg; 0.245 mmol) and CuI (101 mg; 0.532 mmol). The solution was stirred at room temperature for 10 min. Then *N*-propargyltrifluoroacetamide (1.12 g; 7.43 mmol) and triethylamine (0.68 mL; 4.90 mmol) were added. The reaction was stirred at room temperature for 13 h with exclusion of air and light. Most DMF was removed under vacuum, and the residue was dissolved in ethyl acetate (100 mL). The solution was washed with saturated aqueous $NaHCO_3$ and NaCl, respectively, and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the residue was purified by flash column chromatography over silica gel using ethyl acetate−hexane (1:3−1.5) and $CH_3OH-CH_2Cl_2$ (1:30), respectively, as the eluent to afford **7** as yellow solid (1.34 g; 94% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 7.34 (s, 1H), 6.42 (dd, J = 6.2, 7.7 Hz, 1H), 5.88−5.99 (m, 1H), 5.28−5.35 (dm, J = 17.3 Hz, 1H), 5.16−5.21 (dm, J = 10.5 Hz, 1H), 4.29 (s, 2H), 4.24 (m, 1H), 4.00−4.09 (m, 3H), 3.98 (s, 3H), 3.76−3.84 (m, 2H), 2.32−2.45 (m, 2H), 0.94 (s, 9H), 0.12 (s, 3H), 0.11 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 165.0, 161.2, 158.1 (q, J = 36 Hz), 154.2, 135.6, 125.0, 117.2 (q, J = 284 Hz), 117.0, 99.2, 97.3, 86.0, 84.6, 84.5, 80.3, 78.0, 71.0, 64.8, 53.8, 39.0, 30.9, 26.5, 19.3, −5.1, −5.2; HRMS (FAB+) calcd for $C_{26}H_{37}O_5N_5F_3Si$ ($M + H^+$) 584.2516, found 584.2491.

3′-*O*-Allyl-7-{3-[(trifluoroacetyl)amino]prop-1-ynyl}-7-deaza-2′-deoxyguanosine (8). To a stirred solution of **7** (1.34 g; 2.30 mmol) in anhydrous CH_3CN (86 mL) were added NaI (363 mg; 2.42 mmol) and chlorotrimethylsilane (TMSCl) (0.306 mL; 2.42 mmol). The reaction was stirred at room temperature for 1 h and then at 50 °C for 12 h. The solvent was evaporated, and the residue was dissolved in anhydrous THF (76 mL). Tetrabutylammonium fluoride (TBAF) (1 M) in THF solution (4.80 mL; 4.80 mmol) was added, and the reaction was stirred at room temperature for 1 h. The solvent was evaporated, and the residue was dissolved in ethyl acetate (150 mL). The solution was washed with saturated aqueous NaCl and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the residue was purified by flash column chromatography over silica gel using CH_3OH−ethyl acetate (1:30) as the eluent to afford **8** as yellow solid (356 mg; 34% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 7.21 (s, 1H), 6.30 (dd, J = 6.0, 8.4 Hz, 1H), 5.88−5.99 (m, 1H), 5.28−5.35 (dm, J = 17.3 Hz, 1H), 5.15−5.20 (dm, J = 10.5 Hz, 1H), 4.29 (s, 2H), 4.23 (m, 1H), 4.00−4.10 (m, 3H), 3.65−3.75 (m, 2H), 2.41−2.49 (ddd, J = 5.8, 8.4, 13.6 Hz, 1H), 2.34−2.40 (ddd, J = 2.3, 6.0, 13.6 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 160.9, 158.0 (q, J = 36 Hz), 154.1, 151.8, 135.6, 124.4, 117.2 (q, J = 284 Hz), 117.0, 101.4, 99.7, 86.4, 85.5, 84.8, 80.7, 78.0, 71.0, 63.7, 38.5, 31.2; HRMS (FAB+) calcd for $C_{19}H_{21}O_5N_5F_3$ ($M + H^+$) 456.1495, found 456.1493.

3′-*O*-Allyl-7-(3-aminoprop-1-ynyl)-7-deaza-2′-deoxyguanosine-5′-triphosphate (9). The procedure is the same as that of preparing 3′-*O*-allyl-5-(3-aminoprop-1-ynyl)-2′-deoxyuridine-5′-triphosphate in ref 2 to yield **9** as a colorless syrup: ^{1}H NMR (300 MHz, D_2O) δ 7.56 (s, 1H), 6.37 (t, J = 7.3 Hz, 1H), 5.89−6.02 (m, 1H), 5.31−5.39 (dm, J = 17.3 Hz, 1H), 5.21−5.28 (dm, J = 10.5 Hz, 1H), 4.49 (s, 2H), 4.32 (m, 1H), 4.06−4.18 (m, 3H), 3.92−3.99 (m, 2H), 2.44−2.60 (m, 2H);^{31}P NMR (121.4 MHz, D_2O) δ −6.1 (d, J = 20.8 Hz, 1P), −10.8 (d, J = 18.9 Hz, 1P), −21.9 (t, J = 19.8 Hz, 1P).

3′-*O*-Allyl-dGTP-PC-Bodipy-FL-510 (10). PC-Bodipy-FL-510 NHS ester (prepared by the same procedure as in ref 9) (7.2 mg, 12 μmol) in 300 μL of acetonitrile was added to a solution of **9** (2 mg, 3.4 μmol) in 300 μL of $Na_2CO_3-NaHCO_3$ aqueous buffer (0.1

M, pH 8.5). The reaction mixture was stirred at room temperature for 3 h. A preparative silica gel TLC plate was used to separate the unreacted PC-Bodipy-FL-510 NHS ester from the fraction containing **10** with $CHCl_3-CH_3OH$ (85:15) as the eluent. The product was concentrated further under vacuum and purified with reversed-phase HPLC on a 150 × 4.6-mm C18 column to obtain the pure product **10** (retention time of 34 min). Mobile phase: A, 8.6 mM triethylamine/100 mM hexafluoroisopropyl alcohol in water (pH 8.1); B, methanol. Elution was performed with 100% A isocratic over 10 min, followed by a linear gradient of 0−50% B for 20 min and then 50% B isocratic over another 20 min. 3′-*O*-Allyl-dGTP-PC-Bodipy-FL-510 **10** was characterized by the following primer extension reaction and characterization by MALDI-TOF MS.

Primer Extension Using 3′-*O*-Allyl-dGTP-PC-Bodipy-FL-510 (10) and Photocleavage of the Extension Product 11. The polymerase extension reaction mixture consisted of 60 pmol of primer (5′-GTTGATGTACACATTGTCAA-3′), 80 pmol of 100-mer template (5′-TACCCGGAGGCCAAGTACGGCGGG-T A C G T C C T T G A C A A T G T G T A C A T C A A C A T-C A C C T A C C A C C A T G T C A G T C T C G G T T G-GATCCTCTATTGTGTCCGGG-3′), 120 pmol of 3′-*O*-allyl-dGTP-PC-Bodipy-FL-510, 1X Thermopol reaction buffer (20 mM Tris-HCl/10 mM $(NH_4)_2SO_4$/10 mM KCl/2 mM $MgSO_4$/0.1% Triton X-100, pH 8.8, New England Biolabs), and 6 units of 9°N Polymerase (exo-)A485L/Y409V in a total volume of 20 μL. The reaction consisted of 20 cycles at 94 °C for 20 s, 46 °C for 40 s, and 60 °C for 90 s. After the reaction, a small portion of the DNA extension product was desalted by using ZipTip and analyzed by MALDI-TOF MS, which shows a dominant peak at *m*/*z* 7048 corresponding to the DNA product **11**. The rest of the product mixture was freeze-dried, resuspended in 200 μL of deionized water, and irradiated at 355 nm for 10 s to cleave the fluorophore from the DNA to yield product **12** and then analyzed by MALDI-TOF MS (*m*/*z* 6552).

Deallyation of Photocleaved DNA Extension Product 12. DNA product **12** (20 pmol) was added to a mixture of degassed 1X Thermopol reaction buffer (20 mM Tris-HCl/10 mM $(NH_4)_2SO_4$/10 mM KCl/2 mM $MgSO_4$/0.1% Triton X-100, pH 8.8, 1 μL), Na_2PdCl_4 in degassed H_2O (7 μL, 23 nmol), and $P(PhSO_3Na)_3$ in degassed H_2O (10 μL, 176 nmol) to perform the deallylation. The reaction mixture was then placed in a heating block and incubated at 70 °C for 30 s to yield quantitatively deallylated DNA product **13** and analyzed by MALDI-TOF MS (*m*/*z* 6512).

Primer Extension Reaction Performed with the Deallylated DNA Product. The deallylated DNA product **13** was used as a primer in a single-base extension reaction. The 20 μL: reaction mixture consisted of 60 pmol of the deallylated product **13**, 80 pmol of the 100-mer template (5′-TACCCGGAGGCCAAGTACGGC-GGGTACGTCCTTGACAATGTGTACATCAACATCACCTACCA-CCATGTCAGTCTCGGTTGGATCCTCTATTGTGTCCGGG-3′), 120 pmol of 3′-*O*-allyl-dGTP-PC-Bodipy-FL-510 (**10**), and 6 units of 9°N Polymerase (exo-)A485L/Y409V in a total volume of 20 μL. The reaction consisted of 20 cycles at 94 °C for 20 s, 46 °C for 40 s, and 60 °C for 90 s. The DNA extension product **14** was desalted by using the ZipTip protocol, and a small portion was analyzed by using MALDI-TOF MS (*m*/*z* 7429). The remaining product was then irradiated with near-UV light (355 nm) for 10 s to cleave the fluorophore from the extended DNA product. The resulting photocleavage product **15** (*m*/*z* 6933) was analyzed by using MALDI-TOF MS. Finally, deallylation of the photocleavage product **15** was performed using a Pd-catalyzed deallylation reaction resulting in a deallylated DNA product **16**, which was then analyzed by MALDI-TOF MS (*m*/*z* 6893).

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Supporting Information Available: Spectral data for **2**−**9** (**^{1}H, ^{13}C, and ^{31}P NMR**) and general experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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