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Synthesis and enzymatic incorporation of photolabile dUTP analogues into DNA and their applications for DNA labeling

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ABSTRACT

Two novel photolabile nucleotide triphosphate (NTP) analogues were synthesized through Sonogashira coupling and their enzymatic incorporation into DNA was evaluated with three different DNA polymerases (Taq, Vent *exo*- and T4) by polymerase chain reaction. Both nucleotide triphosphate analogues were recognized by these DNA polymerases as substrates for primer extension. Light irradiation of PCR products removed the photolabile group and released the amino and carboxyl moieties. Further site-specific dual-labeling for oligodeoxynucleotides (ODNs) and random labeling for a long DNA construct with fluorophores were successfully achieved with incorporation of the photolabile amine modified deoxyuridine triphosphate (dUnTP).

1. Introduction

Enzymatic incorporation of modified nucleotide triphosphates into DNA has a variety of potential applications in modern molecular biology, such as DNA conjugation, sequence analysis, nanotechnology, and DNA sensing.[1–9] In literature, nucleotide triphosphates carrying different functionalities (amine,[10,11] carboxylic acid,[12] alkyne,[2,13–15] azide,[16,17] etc.) have been enzymatically incorporated into DNA strands, followed by covalent derivatization with functional tags, such as biotin,[16] fluorophores[10,18] or peptides.[19] This post-modification approach is particularly useful for large tags that are not suitable substrates for DNA polymerases. Photocaging strategy is widely used as a protecting method for modification of oligonucleotides during solid phase synthesis. Photocaged phosphate, amino, carboxyl, thiol, carbonyl in the middle or at 3′ or 5′ ends of modified oligonucleotides have been previously achieved by solid phase synthesis.[20–24] However, photolabile nucleotide triphosphates were limited to be recoverable chain terminators in applications of DNA enzymetic synthesis.[4,5,25–28] We are interested in developing photolabile deoxyuridine triphosphate analogues for DNA post-modification. Comparing with post-modification methods reported before, its advantages are as follows: (1) when DNA is labeled with multiple functional tags, photocaging strategy may become a good choice in addition to unprotected or other protected functional groups. (2) Unprotected functional groups, especially amino group, may have electrostatic interactions with DNA polymerases, which may lead to the inhibition of some polymerase activities.[1,11,12] (3) Photocaging strategy is widely used in DNA solid phase synthesis, but the length of DNA synthesized by solid phase synthesis is limited. Photolabile nucleotide triphosphates, as substrates of DNA polymerases would open up the possibility of synthesizing longer DNA constructs which are usually inaccessible through chemical synthesis. Here, we synthesized two photolabile nucleotide triphosphate analogues (dUnTP and dUoTP, Scheme 1) that contained functional substitutions (amine and carboxylic acid, respectively) protected by a photocleavable group. The position 5 of deoxyuridine is widely used to introduce new functionalities and substitutions that protrude from major groove of DNA duplex and is not involved in Watson–Crick base-pairing. Nucleotides that modified at this position are usually well accepted by DNA polymerases.[10,12,29] Three different DNA polymerases (Taq, Vent *exo*- and T4) were used to evaluate enzymatic incorporation of these two photolabile nucleotide triphosphate analogues in short oligonucleotides. Further labeling of these oligonucleotides and a long DNA construct were successfully achieved by incorporation of photolabile dUnTP, followed by light irradiation and coupling with a fluorescent tag.

2. Results and discussion

2.1. Synthesis of photolabile dUoTP and dUnTP

α-Methyl substituted *o*-nitrobenzyl moiety has been widely used as a caging group in nucleic acids due to its higher stability in DNA/RNA synthesis and enhanced photochemical cleavage than that of its parent 2-nitrobenzyl group. In our previous study, we applied this photolabile moiety as a temporary protecting group for the introduction of functionality at both 5′ and 3′ of oligonucleotides

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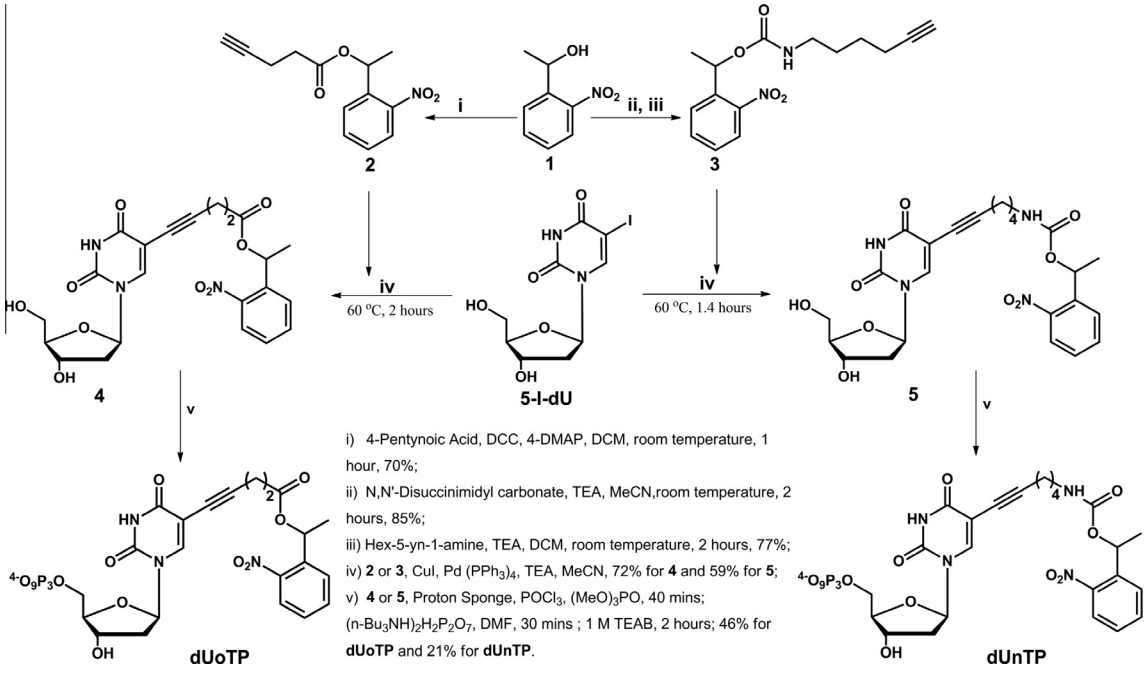


Scheme 1. Synthesis of dUoTP and dUnTP.

in solid phase synthesis.[20] However, for construction of a longer DNA or RNA with the incorporation of functional groups, photolabile NTP analogues are in need. Two photolabile dUTP analogues were then synthesized with 5′-iodo-deoxyuridine as the starting material, as shown in Scheme 1. In order to introduce amine or carboxylic groups to deoxyuridine, we first synthesized photolabile ester (**2**) and carbamate (**3**), followed by coupling to 5′-iodo-deoxyuridine through the palladium-assisted Sonogashira cross-coupling reaction.[24,30] These corresponding nucleoside analogues (**4** and **5**) were obtained with the yields of 72% and 59%, respectively. If the above reactions continued overnight, we got the other two nucleoside analogues which were confirmed to be **6** and **7** (Scheme S1 in Supplementary data) according to NMR spectra, UV–vis spectra (Figure S1) and literature.[31–34]

The resulting nucleoside analogues further reacted with $POCl_3$ to form active intermediates, followed by the addition of tributylammonium pyrophosphate according to the procedure of Yoshikawa reaction.[35,36] The reaction was quenched with the addition of triethylammonium bicarbonate (TEAB), and reaction intermediates were converted to corresponding triphosphate analogues (dUoTP, dUnTP). After reverse phase HPLC purification, the obtained dUTP analogues were then characterized by ^{1}H NMR, ^{31}P NMR and mass spectroscopy (see Supplementary data).

2.2. UV deprotection study of dUoTP and dUnTP

Before enzymatic incorporation of dUoTP and dUnTP into DNA, we evaluated their photocleavable behaviours. The solutions of dUoTP (1 mM, 3 mL) and dUnTP (1 mM, 3 mL) were irradiated with UV light (365 nm, 11 mW/cm^2). At different time point, 200 μL irradiated solution was taken for the analysis by reverse phase HPLC. Figure 1A shows that the peak intensity of photolabile dUnTP (retention time 9.5 min) gradually decreased with almost complete photocleavage in 10 min, while the peak intensity of uncaged product (dUnTP*, retention time 3.2 min) gradually increased. The loss of starting materials and appearance of uncaged products were detected in HPLC traces (Table S1) and were used to quantify the cleavage efficiency (Figure S2). For dUoTP, we observed slower photocleavage rate than that of dUnTP. (Figure S1) Under our photolysis conditions, the half-lives of dUnTP and dUoTP disappearance were 5.0 and 7.5 min, respectively. The uncaged

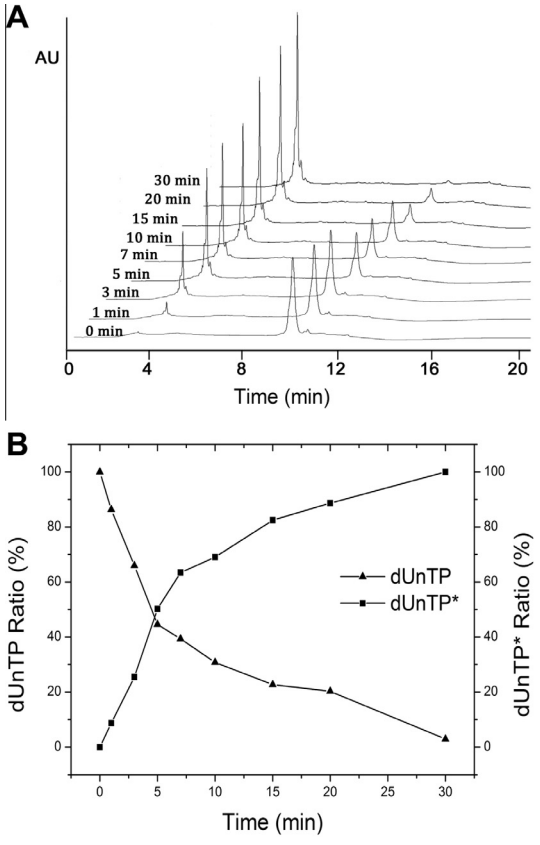


Figure 1. Photolysis of dUnTP. (a) HPLC traces of dUnTP with different irradiation time point; (b) percentages of dUnTP disappearance (9.5 min) and dUnTP* production (3.2 min) after integration of each peak in HPLC traces.

products, dUnTP* and dUoTP*, were characterized and confirmed with ^{1}H NMR, ^{31}P NMR and MS (see Supplementary data).

2.3. Enzymatic incorporation of modified nucleotide triphosphate analogues

Previously, photocaged nucleotide triphosphate analogues were mainly used for strong temporary chain terminators, which the caging groups prevented further addition of dNTP upon the incorporation of caged NTP analogues by polymerases. To study the effect of photocleavable groups on enzymatic incorporation of dUoTP and dUnTP, dUoTP, dUnTP and their corresponding uncaged nucleotides (dUoTP* and dUnTP*) were utilized in primer extension assays. Three commercially available DNA polymerases (Taq, Vent *exo*- and T4) were chosen to evaluate enzymatic incorporation of these modified nucleotide triphosphate analogues. A 33mer template and a 24mer Fam-labeled primer were designed for assay experiments. An adenosine at the 25th position of the template served as the paired base for the above dUTP analogues.

Primer extension assays were analyzed by determining the length of extension products via gel electrophoresis. The gel analyses of dUTP analogue incorporation are summarized in Figure 2. Panel A shows the incorporation of dUnTP and dUnTP*, and panel B shows the incorporation of dUoTP and dUoTP*. For Taq and Vent *exo*-DNA polymerases, lane 'control' shows no extension of primer with the existence of dATP, dGTP and dCTP. But for T4 DNA polymerase, lane 'control' shows the disappearance of 24 nt-long primer and the appearance of a new shorter band due to 3′ → 5′ *exo*nuclease activity of this enzyme. In comparison, lane 'dTTP' corresponds to the use of complete set of dNTPs, which led to the disappearance of 24 nt-long primer due to the full primer extension. Replacement of dTTP with dUnTP, dUnTP*, dUoTP and dUoTP* did not have an obvious effect on the full extension of the primer, which indicates that the enzymatic reaction of dUTP analogues with three different polymerases proceeded successfully under standard conditions of each enzyme. We then manually reduced the polymerase activity (Vent *exo*-polymerase) by lowering the reaction temperature to 35 °C. We did find the fast initial coupling of dTTP than that of dUnTP or dUoTP, however, the coupling efficiency reach to the similar level after 30 min incubation time under our conditions (Figure. S4).

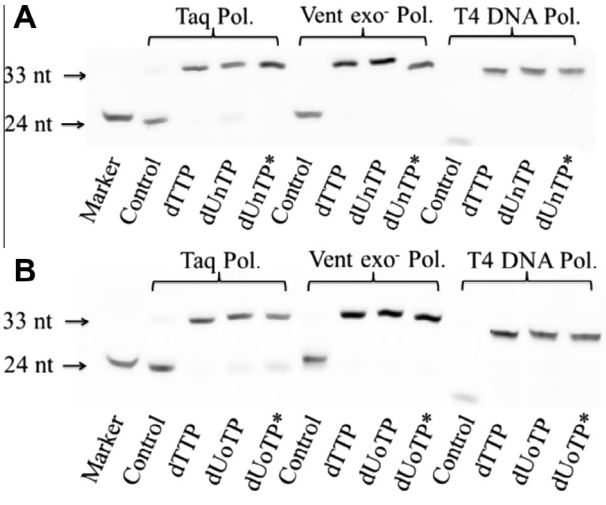


Figure 2. Denaturing PAGE analysis of primer extension reactions with (A) dUnTP, dUnTP* or (B) dUoTP, dUoTP* using Taq, Vent *exo*- or T4 DNA polymerases. Lane 'marker' corresponds to the fluorescence labeled 24 nt-long primer. Lane 'control' corresponds to PCR reaction with the mixture of template, primer and three dNTPs (dATP, dGTP and dCTP).

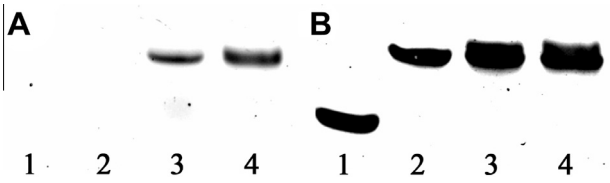
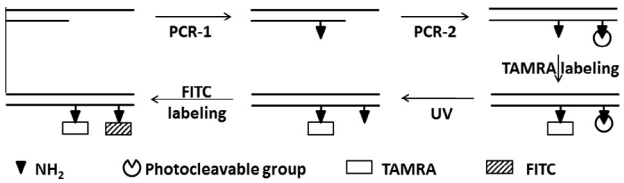


Figure 3. Denaturing PAGE analysis of dual-labeled ODN before and after SYBR Gold DNA stain. Lane 1: Primer only, Lane 2: Primer extension reaction with sequential addition of dUnTP* and dUnTP, Lane 3: ODN synthesized in lane 2 conditions and labeled with TAMRA alone, Lane 4: ODN synthesized in lane 2 conditions and labeled with TAMRA and FITC. (A) Fluorescence image of gel before staining; (B) fluorescence image of gel after staining.

2.4. Site-specific single-labeling and dual-labeling of oligodeoxynucleotides with photolabile deoxyuridine analogues

A preliminary experiment with a short oligonucleotide was first tested with Vent *exo*-polymerase. We applied the same sequences of the template and primer used previously, except that the primer was not labeled with fluorescein. After the completion of primer extension, the reaction solution was irradiated, followed by buffer exchange to remove tris buffer. Further labeling with FITC was successfully achieved. The denaturing PAGE analysis of FITC labeled ODN before and after staining with SYBR™ Gold DNA stain is shown in Figure S5. The extended primer labeled with FITC was observed in gel before DNA staining, which was further confirmed to be the fully extended product by SYBR Gold staining. Furthermore, the absorption and emission spectra of the enzymatically synthesized DNA post-labeled with FITC were shown in Figure 4A and B.

Another template containing two adenine nucleobases was also designed for site-specific dual-labeling with a non-fluorescein labeled primer (Scheme 2). At the initial primer extension reaction, dUnTP* was incorporated, but the reaction was stopped due to no dCTP in solution. After removal of all dNTPs, fresh enzyme with dUnTP and all other three dNTP was added to the recollected primer/template duplex in reaction buffer. dUnTP was then incorporated and fully extended primer was obtained. After buffer exchange, 5/6-carboxytetramethyl-rhodamine, succinimidyl ester (5/6-TAMRA, SE) was added into the oligonucleotide solution for labeling the amine group of dUnTP*, and the obtained TAMRA labeled ODN was then deprotected by UV irradiation to release the other amine group of dUnTP. Further labeling with FITC was successfully achieved. The denaturing PAGE analysis of dual-labeled ODN before (Panel A) and after (Panel B) staining with SYBR™ Gold DNA stain is shown in Figure 3. From the fluorescence images of the gel, ODN labeled with only TAMRA or both TAMRA and FITC showed a bright fluorescence band on gel. And fluorescence signal of dual labeling was more intense than single labeling. Further staining with SYBR™ Gold confirmed full extension of the primer with the incorporation of dUnTP* and dUnTP. The absorption and emission spectra of synthesized ODN with or without dual labeling were also shown in Figure 4C and D. From fluorescence emission spectra, FRET between fluorescein and TAMRA was clearly ob-



Scheme 2. Flowchart of dual-labeling. PCR-1: PCR with dUnTP* and dATP, then buffer and dNTPs were removed; PCR-2: PCR with dUnTP, dATP, dCTP and dGTP.

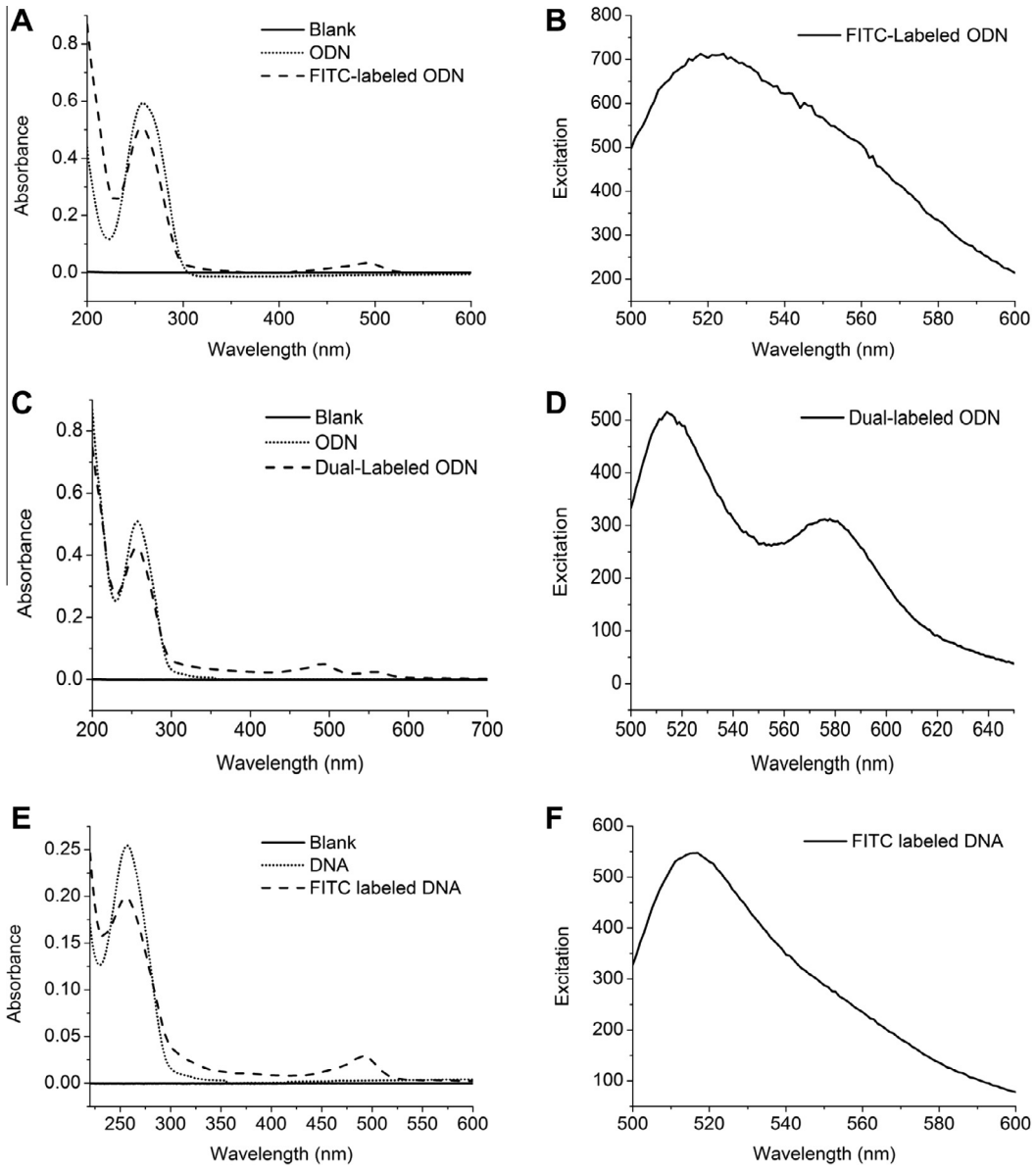


Figure 4. Absorption (A) and emission (B) spectra of FITC-labeled ODN. Absorption (C) and emission (D) spectra of dual-labeled ODN with TAMRA and FITC (λ_{ex} = 488 nm). Absorption (E) and emission (F) spectra of a long multiple FITC-labeled DNA construct.

served, which confirmed the effective uncaging and sequentially labeling of these two fluorophores.

2.5. Random labeling of a long DNA construct

After having successfully achieved the incorporation of dUnTP and post-modification of short oligodeoxynucleotides, we next chose a 539 bp template from PUC18 plasmid to investigate whether dUnTP is capable for effective incorporation of a long DNA construct with Vent *exo*-polymerase. In a typical PCR experiment, 50 μM dUnTP together with 250 μM all four natural dNTPs were added to reaction mixture. After 40 cycles, the reaction mixture was subject to light irradiation and buffer exchange, followed by DNA labeling with FITC. The agarose gel analyses of FITC labeled DNA before (Panel A) and after (Panel B) staining with ethidium bromide (EB) DNA stain are shown in Figure 5. There existed a clear and intense fluorescence band only for the enzymatically synthesized DNA labeled with FITC (Lane 3). This band corresponded to the 539 bp DNA construct as indicated for DNA ladder (Lane 1) and DNA without FITC labeling (Lane 2) in panel B. Comparing Lanes 2 and 3 in panel B, DNA construct without labeling showed

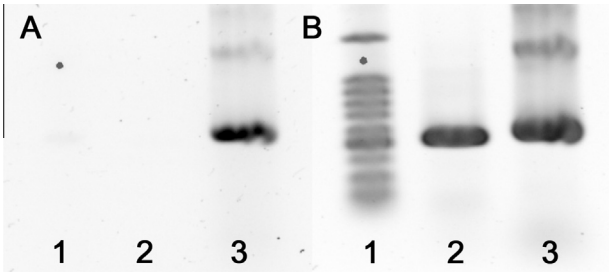


Figure 5. Agarose gel electrophoresis of FITC labeled DNA before and after staining with ethidium bromide DNA stain. Lane 1: 100 bp DNA ladder, Lane 2: 539 bp DNA construct for PUC18 plasmid, Lane 3: 539 bp DNA construct labeled with FITC. (A) Fluorescence image of gel before EB staining; (B) fluorescence image of gel after EB staining.

clear band in the gel, while there existed another faint band of DNA construct after FITC labeling. This may be due to multiple labeling with FITC that disrupted the condensed DNA coil structure. Successful labeling of a long DNA construct with FITC was further confirmed by the absorption and emission spectra of FITC labeled DNA, as shown in Figure 4E and F. By calibration of absorbance of DNA (260 nm) and FITC (488 nm), the overall efficiency of uncaging and labeling [labeled deoxyuracil/(deoxyuracil + thymine)] was determined to be 12%, which was 60% of the ratio of dUnTP/dTTP (20%) that were added to the reaction mixture.

3. Conclusion

In summary, we have synthesized two new photolabile deoxyuridine triphosphate analogues (dUoTP, dUnTP) with temporarily protected functionalities of amine and carboxylic acid moieties. These two triphosphates were then evaluated for their incorporation into oligodeoxynucleotides using three commercially available DNA polymerases (Taq, Vent *exo*- and T4). All three DNA polymerases showed quite effective primer extension with the existence of dUoTP, dUnTP, or their uncaged dUoTP* and dUnTP*. In addition, dUnTP was effectively incorporated in both short oligodeoxynucleotides and a long DNA construct from PUC18 plasmid. Further labeling of the long DNA construct and site-specific dual-labeling of oligodeoxynucleotide were successfully achieved with fluorophores. This work represents the first example of enzymatic introduction of photocaged functional moieties into DNA for further labeling using photolabile nucleotide triphosphate analogues.

4. Experimental section

All chemical solvents used were dried and distilled. Dichloromethane (DCM), triethylamine (TEA), tetrahydrofuran (THF), and acetonitrile were dried over CaH_2 and were freshly distilled before every use. All chemical reagents were purchased from Alfa Aesar, Sigma–Aldrich, or J&K chemicals, and used without further purification. All reactions were monitored by TLC using commercial Merck plates coated with silica gel GF_{254} (0.24 mm thick). Flash column chromatography was performed with silica gel purchased from Qingdao Haiyang Chemical Company (200–300 mesh). ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz) and ^{31}P NMR (162 MHz) spectra were recorded on a Bruker spectrometer at 25 °C. Chemical shifts (δ, ppm) were quoted relative to the residual solvent and coupling constants (J) were corrected and quoted to the nearest 1 Hz. MS spectra were measured on Q-TOF spectrometer using electrospray ionization (ESI) or FT-MS (Bruker APEX IV 7.0T). HPLC were performed with Alliance e2695 and Agilent XDB C-18 column (50 × 4.6 mm, 1.8 μm beads). When necessary, the reactions were conducted in a dark room.

Oligonucleotides were purchased from Shanghai Sangon Biotech. Deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, and dTTP) were purchased from Promega. Vent *exo*-DNA polymerase was purchased from New England Biolabs. T4 DNA polymerase was purchased from Fermantas. Taq DNA polymerase and other biochemical reagent were purchased from TAKARA. G-25 sephadex column (Nap-10 columns sephadex G-25 DNA Grade) was purchased from GE healthcare. Polymerase chain reaction (PCR) was performed on ABI Veriti 96-well Thermal Cycler.

4.1. Synthesis of 1-(2-nitrophenyl)ethyl 4-pentynoate (2)

To a solution of 1-(2-nitrophenyl) ethanol (**1**) (940 mg, 5.6 mmol) in DCM (12 mL) was added 4-pentynoic acid (500 μL, 5.6 mmol), *N*,*N*′-dicyclohexylcarbodiimide (DCC, 2.06 g, 10.0 mmol) and 4-dimethylamiopryidine (240 mg, 2.0 mmol). The whole mixture was stirred at room temperature for 1 h, and was then poured into ethyl acetate (50 mL). The organic layer was washed with 8% citric acid aq (2 × 50 mL), brine (2 × 50 mL), and dried over Na_2SO_4. After removal of ethyl acetate, the organic layer was concentrated in vacuo, and the residue was purified by short silica gel column, eluted with petroleum ether:ethyl acetate (10:1) to give **2** as a clear, yellow oil (970 mg, 3.93 mmol, yield 70%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.95 (dd, J = 8, 1 Hz, 1H), 7.66–7.60 (m, 2H), 7.43 (m, 1H), 6.36 (q, J = 6 Hz, 1H), 2.60–2.55 (m, 2H), 2.51–2.46 (m, 2H), 1.95 (t, J = 3 Hz, 1H), 1.65 (d, J = 6 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.71 147.88, 137.91, 133.65, 128.56, 127.38, 124.60, 82.41, 69.32, 68.67, 33.55, 22.14, 14.43.

4.2. Synthesis of 1-(2-nitrophenyl)ethyl hex-5-yn-1-yl carbamate (3)

To a solution of 1-(2-nitrophenyl)ethyl *N*-succinimidyl carbonate (see Supplementary data) (1.02 g, 3.0 mmol) in DCM (15 mL), was added TEA (1.70 ml, 12.0 mmol) and 5-hexyn-1-amine hydrochloride (see Supplementary data) (400 mg, 3.0 mmol). The mixture solution was stirred at room temperature for 2 h and was then diluted with DCM (100 mL). The organic layer was washed with brine (3 × 50 mL), dried over Na_2SO_4. After concentration in vacuo, the residue was purified by short silica gel column with petroleum ether:ethyl acetate (1:1) to give **3** as a clear, yellow oil (670 mg, 2.3 mmol, yield 77%).^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (d, J = 8 Hz, 1H), 7.61 (d, J = 4 Hz, 2H), 7.44–7.34 (m, 1H), 6.22 (q, J = 6 Hz, 1H), 3.14 (m, 2H), 2.20 (m, 2H), 1.94 (t, J = 6 Hz, 1H), 1.60 (q, J = 6 Hz, 3H), 1.55–1.46 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.32, 147.61, 138.78, 133.48, 128.15, 127.05, 124.39, 83.97, 68.71, 68.52, 40.44, 28.95, 25.47, 22.24, 18.05.

4.3. Synthesis of 4

To a solution of 1-(2-nitrophenyl)ethyl 4-pentynoate (**2**) (750 mg, 3 mmol) in acetonitrile (6 mL), was added 5-iodo-2′-deoxyuridine (710 mg, 2.0 mmol), palladium(0)tetrakis(triphenylphosphine) (115 mg, 0.1 mmol), cuprous iodide (40 mg, 0.2 mmol) and TEA (1.1 mL, 8.0 mmol). The whole mixture was stirred at 60 °C for 2 h under nitrogen atmosphere. The solvents were removed in vacuo, and the remaining crude mixture was purified by silica gel column with 0–5% MeOH in DCM to give **4** as a yellow foamy solid (550 mg, 1.16 mmol, yield 58%). ^{1}H NMR (400 MHz, MeOD) δ 8.17 (d, J = 4 Hz, 1H), 7.97–7.85 (m, 1H), 7.78–7.69 (m, 1H), 7.70–7.62 (m, 1H), 7.53–7.42 (m, 1H), 6.29–6.18 (m, 2H), 4.45–4.33 (m, 1H), 3.93 (q, J = 3 Hz, 1H), 3.84–3.68 (m, 2H), 2.70–2.58 (m, 4H), 2.38–2.12 (m, 2H), 1.63 (d, J = 6 Hz, 3H). ^{13}C NMR (100 MHz, MeOD) δ 172.69, 164.45, 151.11, 149.10, 144.63, 138.58, 134.88, 129.69, 129.69, 128.51, 125.17, 100.64, 93.09, 88.99, 86.91, 73.29, 72.00, 69.63, 69.61, 62.58, 41.54, 34.23, 22.10, 16.00. ESI$^+$ MS: [M+Na]$^+$ 496.28, Calcd [M+Na]$^+$ 496.13. ε_{289} = 11600 L mol^{-1} cm^{-1}.

4.4. Synthesis of 5

To a solution of 1-(2-nitrophenyl)ethyl hex-5-yn-1-yl carbamate (**3**) (667 mg, 2.3 mmol) in acetonitrile (6 mL), was added 5-lodo-2′-deoxyuridine (750 mg, 2.1 mmol), palladium(0)tetrakis(triphenylphosphine) (115 mg, 0.1 mmol), cuprous iodide (40 mg, 0.2 mmol), TEA (1.1 mL, 8 mmol). The mixture solution was stirred at 60 °C for 1.4 h under nitrogen atmosphere. After removal of solvents in vacuo, the remaining residue was purified by silica gel column with 0–5% MeOH in DCM to give **5** as a yellow foamy solid (780 mg, yield 72%). ^{1}H NMR (400 MHz, MeOD) δ 8.20 (d, J = 3.0 Hz, 1H), 7.94 (d, J = 8 Hz, 1H), 7.74–7.66 (m, 2H), 7.51–7.44 (m, 1H), 6.24 (m, 1H), 6.12 (q, J = 6 Hz, 1H), 4.43–4.37

(m, 1H), 3.94–3.91 (m, 1H), 3.83–3.63 (m, 2H), 3.06 (t, J = 6 Hz, 2H), 2.39–2.23 (m, 4H), 1.61 (d, J = 6 Hz, 3H), 1.58–1.50 (m, 4H). ^{13}C NMR (100 MHz, MeOD) δ 164.68, 157.93, 151.21, 149.04, 144.31, 139.98, 134.85, 129.52, 128.16, 125.26, 101.12, 94.72, 89.04, 86.85, 72.94, 72.02, 69.42, 62.58, 41.62, 41.14, 29.85, 26.63, 22.47, 19.79. ESI$^+$ MS: $[M+Na]^+$ 539.62, Calcd $[M+Na]^+$ 539.18. ε_{289} = 12,000 L mol^{-1} cm^{-1}

4.5. Synthesis of dUoTP

To a cooled solution (0 °C) of **4** (120 mg, 0.25 mmol) and 1,8-bis(dimethylamino) naphthalene (proton sponge, 107 mg, 0.50 mmol) in trimethyl phosphate (2 mL), was added phosphorous oxychloride (38 μL, 0.40 mmol) dropwise over 10 min under nitrogen atmosphere. The reaction mixture was stirred for 40 min at 0 °C. A solution of tributylammonium pyrophosphate (250 mg, 0.50 mmol) in dry DMF (2.0 mL) and TEA (140 μL, 1.0 mmol) were added. The reaction mixture was stirred for another 30 min, followed by quenching with triethylammonium bicarbonate (TEAB, 0.1 M, 10 mL). The mixture was left to warm to room temperature whilst stirring for 2 h. Reverse phase HPLC purification (A, 0.05 M TEAA in H_2O; B, acetonitrile; B, 0–60% in 25 min, 60% in 5 min 60–0% in 5 min) yielded dUoTP as the triethylammonium salt, further desalting was performed with ion-exchange resin (H^+ form). (Yield 46%, determined by the absorbance at 260 nm) ^{1}H NMR (400 MHz, D_2O) δ 7.93–7.76 (m, 2H), 7.69 (m, 1H), 7.56 (d, J = 7 Hz, 1H), 7.44–7.31 (m, 1H), 6.26–6.09 (m, 2H), 4.51–4.48 (m, 1H), 4.22–4.01 (m, 3H), 2.69–2.53 (m, 4H), 2.40–2.21 (m, 2H), 1.56 (t, J = 9 Hz, 3H). ^{31}P NMR (162 MHz, D_2O) δ −10.304 (br), −11.012 (br), −22.719 (br). FT-MS$^-$: $[M-H]^-$ 712.0345, $[M-2H+Na]^-$ 734.0167, $[M-H_2PO_3]^-$ 632.0668; Calcd $[M-H]-$ 712.0346, $[M-2H+Na]^-$ 734.0165, $[M-H_2PO_3]^-$ 632.0683.

4.6. Synthesis of dUnTP

To a cooled solution (0 °C) of **5** (80 mg, 0.16 mmol) and 1,8-bis(dimethylamino) naphthalene (proton sponge, 60 mg, 0.20 mmol) in trimethyl phosphate (2 mL) was added phosphorous oxychloride (19 μL, 0.20 mmol) dropwise over 10 min under nitrogen atmosphere. The reaction mixture was stirred for 40 min at 0 °C. A solution of tributylammonium pyrophosphate (160 mg, 0.29 mmol) in dry DMF (2.0 mL) and TEA (69 μL, 0.50 mmol) were then added. The reaction mixture was stirred for another 5 min, followed by quenching with triethylammonium bicarbonate (0.1 M, 10 mL). The reaction solution was left to warm to room temperature whilst stirring for 2 h. RP-HPLC purification (A, 0.05 M TEAA in H_2O; B, acetonitrile; B, 0–60% in 25 min, 60% in 5 min 60–0% in 5 min) yield dUnTP as the triethylammonium salt, further desalting was performed with ion-exchange resin (H^+ form). (Yield 21%, determined by the absorbance at 260 nm) ^{1}H NMR (400 MHz, D_2O) δ 7.99–7.82 (m, 2H), 7.68 (s, 2H), 7.44 (s, 1H), 6.22 (s, 1H), 6.02 (s, 1H), 4.13 (m, 3H), 4.56 (s, 1H), 2.99 (s, 2H), 2.80 (s, 1H), 2.53 (s, 1H), 2.33 (s, 2H), 1.56 (d, J = 6 Hz, 3H), 1.37 (m, 4H). ^{31}P NMR (D_2O, 162 MHz): δ −8.926 (br), −12.115 (br), −22.764 (br). FT-MS$^-$: $[M-H]^-$ 755.0763, $[M-H_2PO_3]^-$ 675.1149; Calcd $[M-H]^-$ 755.0768, $[M-H_2PO_3]^-$ 675.1105.

4.7. Primer extension assay

Template sequence: 5′-TCC TTG TGA TAA GCA AAG ATT AGT TAG TCC CAT-3′.

Primer sequence: 5′-Fam-ATG GGA CTA ACT AAT CTT TGC TTA-3′.

Primer extension reactions were carried out in PCR tubes (200 μL) in a total volume of 10 μL. Each reaction contained 1 μM primer and 1.2 μM template, 50 μM dATP, dGTP and dCTP, 50 μM dUTP analogues, 0.4 U Taq or 0.2 U Vent *exo*- or 0.2 U T4 DNA polymerase in 1× polymerase buffer as given by the suppliers. The incubation procedure included steps of a 4 min denaturation at 94 °C, 8× (94 °C, 30 s; 55 °C, 30 s; 68 °C, 2 min) for Taq and Vent *exo*-polymerases, steps of 37 °C, 20 min and 72 °C, 10 min for T4 polymerase. Reaction temperature was returned to 4 °C after each reaction. Then an aliquot (5 μL) of the unpurified reaction was mixed with 1 μL loading buffer (10× DNA loading buffer: Formamide = 1:4). After denaturation at 92 °C for 2 min, primer extension products were electrophoresed on a denaturing 20% (v/v) polyacrylamide gel containing 7 M urea (1× TBE buffer; pH = 8.2). Gels were imaged with Molecular Imager ChemiDoc™ XRS$^+$ before and after SYBR™ Gold staining.

4.8. Enzymatically synthesis and dual-fluorophore labeling of oligodeoxynucleotide with photolabile dUnTP

Primer extension reactions were carried out in PCR tubes (200 μL) in a total volume of 50 μL. Each reaction contained 1 μM non-Fam labeled primer (5′-ATG GGA CTA ACT AAT CTT TGC TTA-3′) and 1.2 μM template (5′-TCC TAG TTA TAA GCA AAG AAGA TTA GTT AGT CCC AT-3′), 50 μM dATP and dUnTP*, 1 U Vent *exo*- in 1× Vent *exo*-polymerase buffer as given by the suppliers. After the RCR reaction steps of 4 min denaturation at 94 °C and 8× (94 °C, 30 s; 55 °C, 30 s; 68 °C, 2 min), reaction temperature was returned to 4 °C. The mixture was passed through Millipore Amicon™ Ultra (Ultracel-3 Membrane, 3.5 kDa) to remove tris buffer and dNTP, and the detained residue was redissolved in 1× Vent *exo*-polymerase buffer. Then 50 μM dATP, dCTP, dGTP, 50 μM dUnTP and 1 U Vent *exo*- were added. After the RCR reaction steps of 4 min denaturation at 94 °C and 8× (94 °C, 30 s; 60 °C, 30 s; 68 °C, 2 min), reaction temperature was returned to 4 °C. The mixture was passed through Millipore Amicon™ Ultra (Ultracel-3 Membrane, 3.5 kDa) to remove dNTP and tris buffer and the detained residue was redissolved in 50 μL $NaHCO_3$ (0.1 mM, pH = 8.3). 5/6-Carboxytetramethylrhodamine, succinimidyl ester (10 mM in DMF, 5 μL) was added into the mixture and vortexed at 60 °C for 12 h. After sedimentation and desalting (NAP-10), the TAMRA labeled oligonucleotide was redissolved in 50 μL $NaHCO_3$ (0.1 mM, pH = 8.3) and irradiated by UV light (365 nm, 11 mW/cm^2) for 10 min to remove the photolabile group. FITC (10 mM in DMSO, 5 μL) was added into the mixture and it was vortexed at 60 °C for 12 h. After sedimentation, desalting and concentration, the dual-fluorophore labeled oligonucleotide was obtained. The obtained residue was redissolved in 50 μL H_2O, and a aliquot (5 μL) of above mixture was mixed with 1 μL loading buffer and electrophoresed on 20% (v/v) polyacrylamide gel containing 7 M urea (1× TBE buffer, pH = 8.2). The same gel was then imaged before and after SYBR™ Gold staining by Molecular Imager ChemiDoc™ XRS$^+$.

4.9. Synthesis and random labeling of a long DNA construct with photolabile dUnTP

4.9.1. Template preparation

PCR was carried out in PCR tubes (200 μL) in a total volume of 100 μL. Each reaction contained 1 μM forward primer (5′-GAG GCG GTT TGC GTA TTG G-3′), 1 μM reverse primer (5′-GTC GTG TCC TAC GGG GTT GGA-3′), 1 ng PUC18 plasmid, 250 μM dNTP, 2 U Taq in 1× Taq polymerase buffer as given by the supplier. The incubation steps consisted of a 4 min denaturation at 94 °C, and 30× (94 °C, 15 s; 55 °C, 15 s; 72 °C, 1 min). The reaction temperature was returned to 4 °C after the reaction was completed. The obtained DNA was recovered by TAKARA™ PCR product recovery kit,

followed by lyophilisation to remove solvents. The residue was redissolved in PBS buffer with the concentration of 0.2 μM.

4.9.2. Preparation and labeling of a long modified DNA construct

PCR was carried out in PCR tubes (200 μL) in a total volume of 50 μL. Each reaction contained 1 μM forward primer (5′-GAG GCG GTT TGC GTA TTG G-3′), 0.01 μM long DNA template as prepared before, 250 μM dNTP, 50 μM dUnTP, 1 U Vent *exo*- in 1× Vent *exo*-polymerase buffer as given by the supplier. The incubation steps consisted of a 4 min denaturation at 94 °C and 40× (94 °C, 15 s; 55 °C, 15 s; 68 °C, 2 min). The reaction temperature was returned to 4 °C after the reaction was completed. The reaction mixture was irradiated by UV light (365 nm, 11 mW/cm^2) for 10 min to remove the photolabile group. According to the same working procedure as the synthesis and labeling of the above oligodeoxynucleotide, the obtained residue was redissolved in 50 μL H_2O, and a aliquot (5 μL) of above mixture was mixed with 1 μL loading buffer and electrophoresed on 1% agarose gel (1× TAE buffer, pH = 8.0). The same gel was then imaged before and after staining with EB by Molecular Imager ChemiDoc™ XRS$^+$

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Supplementary data

Supplementary data associated (supporting figures, synthetic procedure of 5-hexyn-1-amine and 1-(2-nitrophenyl)ethyl *N*-succinimidyl carbonate and characterization of intermediates and products) with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.04.081.

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