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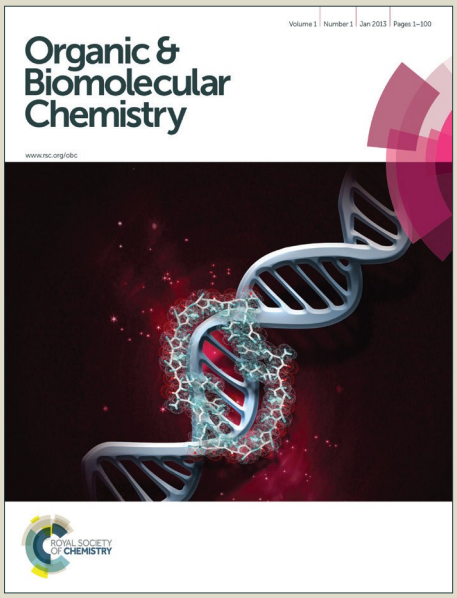

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Photo-cleavable Nucleotides for Primer Free Enzyme mediated DNA synthesis.

Anu Stella Mathews, [a, b] Haikang Yang, [a, b] Carlo Montemagno* [a, b]

Abstract

Synthesis, chatacterization and a potential application of eight 3′ modified 2′-deoxy ribonucleoside triphosphates (dNTPs) is discussed. These nucleotide analogues are modified by capping the 3′-OH by a photolabile protecting group which can temporarily cease DNA strand growth and can smoothly reinitiate the growth by the photo decomposition of protecting group and setting the 3′-OH of dNTPs free to propagate. The synthesis of 3′-O-(2-nitrobenzyl)-2′-deoxy ribonucleoside triphosphates (NB-dNTPs) and 3′-O-(4,5-dimethoxy-2-nitrobenzyl)- 2′-deoxy ribonucleoside triphosphates (DMNB-dNTPs) is discussed in details with structural confirmation using NMR. The UV-cleaving studies is monitored and quantified using LCMS and ^{1}H NMR spectral traces. The synthesised nucleotides are employed for terminating and reinitiating, templateless DNA synthesis, using primer indepentent Terminal Deoxynucleotidyl Transferase (TdT) enzyme. The use of this photolabile nucleotides as one step stop-start DNA synthesis is a novel strategy towards the presice assemply of dNTPs with the potential to reinforce present technologies .

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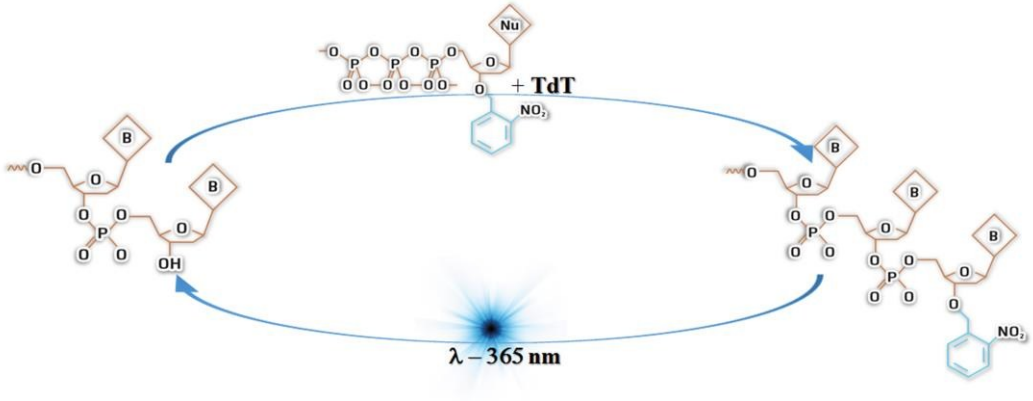

Presented will be the synthesis and characterization of eight 3′ modified 2′-deoxyribonucleoside triphosphates for template less enzyme mediated photo-triggered “stop-start” DNA synthesis.

Introduction

Researches, from Friedrich Miescher’s discovery of phosphorous-rich chemical in the nuclei of leukocytes, which he named nuclein, in 1869[1] to the completion of gold-standard genome sequence in 2003,[2] have set the stage for understanding and exploring the complex macromolecular carriers of inheritance called DNA. Modification of DNA through its basic building blocks 2′ deoxyribonucleoside triphosphates (dNTPs) paves the way for wide applications in modern molecular biology, clinical medicine and health care. dNTPs modified at the 3′ hydroxyl position can act as enzyme mediated DNA synthesis terminators, finding immense applications such as DNA synthesis,[3] DNA and RNA 3′ end labelling,[4] mechanistic probes, antimetabolites and antiviral agents.[5] Thus, 3′ OH modification of dNTPs with photolabile moieties promises a versatile way of terminating or labelling sequence ends with the further possibility of breaking bonds smoothly without the need of any reagent and reinitiating any sort of reaction, leading to the controlled presentation, patterning and release of bioactive sites.[6]

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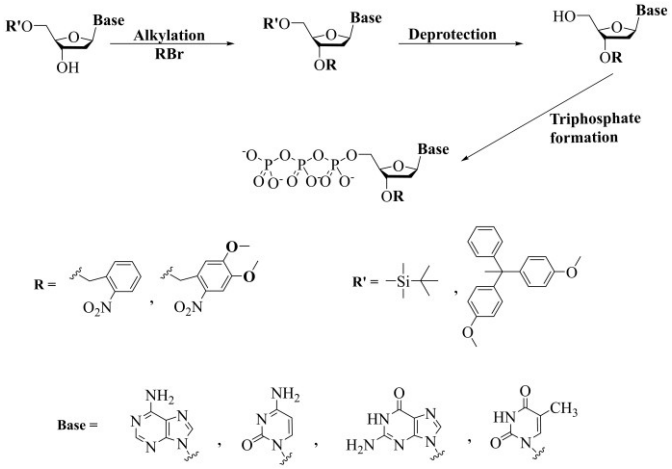
We report the synthesis of eight 3′-O modified dNTPs and their UV response studies. 2-Nitrobenzyl and 4,5-dimethoxy-2-nitrobenzyl groups are used as the photo labile protecting groups due to its high photo cleavable efficiency at near-UV light irradiation.[7] This study is the first of its kind which gives a better understanding of the photo decomposition nature of all the four dNTPs with respect to the base as well as the photolabile moiety attached. Though the termination of DNA synthesis by 3′-O modified dNTPs have been reported earlier,[8,9] template-less precise assembly of nucleotides with terminal deoxynucleotidyl transferase (TdT) enzyme is a novel sequencing strategy. Present work also demonstrates the successful application of synthesized dNTP analogues as DNA sequence terminators during enzymatic synthesis using TdT, which is a template-independent DNA polymerase that catalyses the sequential addition of dNTPs to oligonucleotides.

Results and discussion

The synthesis of eight 3′-O modified dNTPs were done by selective protection strategy.[10] Scheme 1 shows the general synthetic route adapted to make 3′-O-(2-nitrobenzyl)-2′ deoxyribonucleoside triphosphates (NB-dNTPs) and 3′-O-(4,5-dimethoxy-2-nitrobenzyl)-2′-deoxyribonucleoside triphosphates (DMNB-dNTPs)



Scheme 1: General synthetic scheme of NB-dNTPs and DMNB-dNTPs.

The synthesis was started using 5′-O caged deoxyribo purine / pyrimidine compounds. The primary and secondary amines in the base were protected in order to avoid unwanted side reactions which can decrease the yield. Site specific introduction of photolabile group to the 3′- hydroxyl position of the well-sealed starting material in the first step followed by the base and 5′-O deprotection procedures were carried out in dark inert atmosphere. Each intermediate was purified using flash column chromatography and structure was confirmed using ^{1}H NMR. The final triphosphorylation procedure was modified to improve purity and yield. The final products were purified by ion exchange chromatography. The structural confirmation of the modified dNTPs was done using NMR spectra and the purity was assessed using HPLC and LCMS techniques. Table 1 shows the structure, molecular mass and HPLC traces of all the eight dNTPs. The purity of all the compounds was >90%. Molecular mass obtained from negative ion mode of LCMS showed [M+3H] (M is the calculated mass) due to the presence of triphosphate ions.

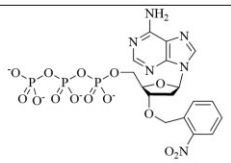
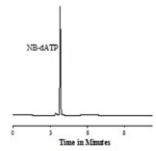
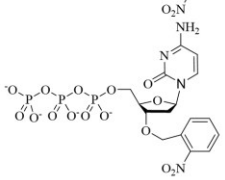
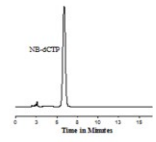
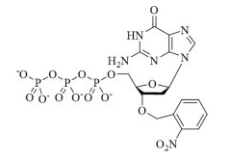
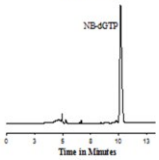
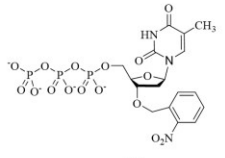
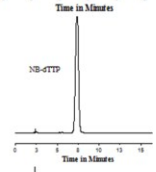
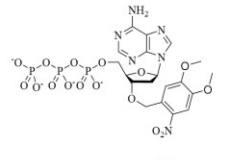
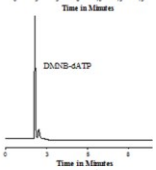
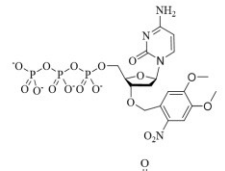
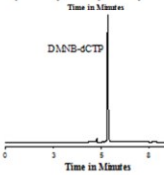
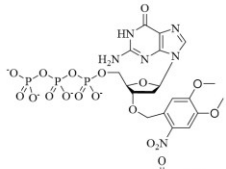
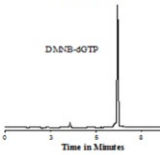
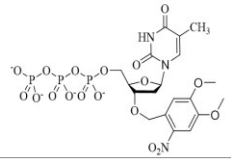
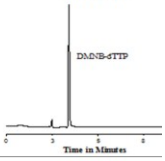
dNTP	Structure	Molecular Mass in Dalton	HPLC Traces
NB-dATP 3′-O-(2-nitrobenzyl)-2′deoxy**adenosine**-5′-triphosphate		622.27	
NB-dCTP 3′-O-(2-nitrobenzyl)-2′deoxy**cytidine**-5′-triphosphate		598.25	
NB-dGTP 3′-O-(2-nitrobenzyl)-2′deoxy**guanosine**-5′-triphosphate		638.27	
NB-dTTP 3′-O-(2-nitrobenzyl)-2′deoxy**thymidine**-5′-triphosphate		613.26	
DMNB-dATP 3′-O-(4,5-dimethoxy-2-nitrobenzyl)-2′deoxy**adenosine**-5′-triphosphate		682.33	
DMNB-dCTP 3′-O-(4,5-dimethoxy-2-nitrobenzyl)-2′deoxy**cytidine**-5′-triphosphate		658.30	
DMNB-dGTP 3′-O-(4,5-dimethoxy-2-nitrobenzyl)-2′deoxy**guanosine**-5′-triphosphate		698.33	
DMNB-dTTP 3′-O-(4,5-dimethoxy-2-nitrobenzyl)-2′deoxy**thymidine**-5′-triphosphate		673.31	

Table 1: Structure and molecular mass of 3′-O-modified dNTPs confirmed using 1H NMR, HPLC and LCMS analysis.

Biochemical and chemical researches, employing various photo-active groups and irradiation protocols, show that the photoreactions carried out in the body tend to use photo source with comparable long wavelength (λ>300 nm) to minimize damage to tissue.[11] Therefore, we choose nitrobenzyl derivatives as the caging moiety of 3′OH of dNTPs. Photo-decomposition reactions were carried out by irradiation using 365 nm UV source (1.4 mW/cm^2 and 10 mW/cm^2) with a working distance of 5 cm. The

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concentration and volume of all samples in water were fixed to 1 mg/mL and 1 mL respectively.

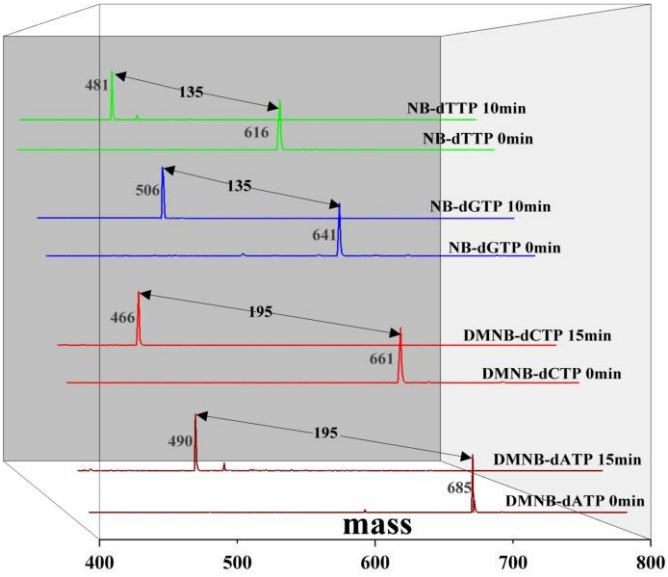


Figure 1: 3D Mass traces of 3′-O-caged-*d*NTPs before and after UV irradiation.

The photo removability of the protecting groups was first verified using the mass changes in LCMS spectra. Figure 1 shows the 3D mass traces quantified using LCMS for four model dNTPs when irradiated. 365 nm 10 mW/cm^2 light source was used for complete photo cleavage of 1 mL of 1 mg/mL solutions of samples in water. A subtraction of mass ~ 135 and 195 was determined after irradiation proving the exact detachment of the nitrobenzyl and dimethoxynitrobenzyl groups respectively from 3′-O position leaving the base and sugar structures of dNTPs intact and 3′-OH available for further reactions.

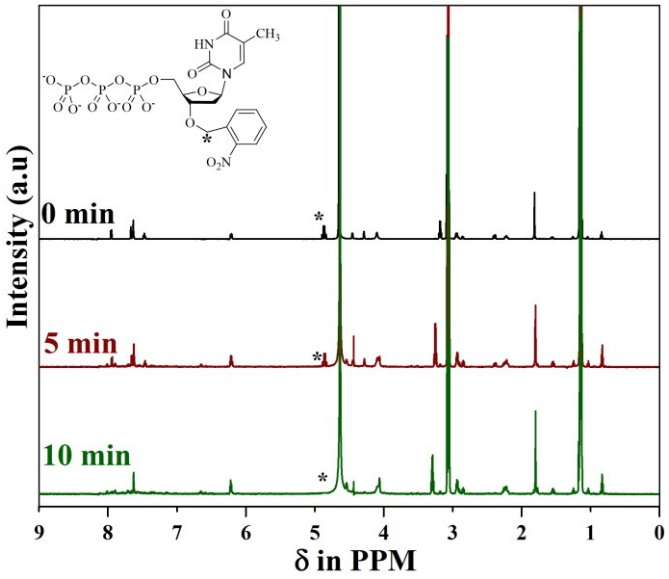


Figure 2: ^{1}H NMR spectra of NB-dTTPs at 0, 5 and 10 minutes of UV irradiation

When dealing with the dNTP molecules which have several functional groups, it is important to study the structural changes during deprotection to get an insight into the background activities that can arise from the contamination by the deprotected materials. ^{1}H NMR monitored the structural changes of the dNTPs at regular intervals of time. Base and sugar structures of the modified dNTPs exhibited no changes in ^{1}H NMR after irradiation while the 2H peaks of 3′ O-C**H$_2$**-nitrobenzyl, around 5.02-4.90 ppm, showed integration decrease. The aromatic region corresponding to nitrobenzyl group and O-CH$_3$ of DMNB-dNTPs in NMR spectra was also altered while no new peaks were formed proving the absence of formation of new bonds resulting from contamination reactions with deprotected group and dNTPs. Figure 2 shows the photo decomposition of NB-dTTP at 0, 5 and 10 minutes, of 1 mL of 1 mg/mL solutions of samples in D_2O, with 365 nm 10 mW /cm^2. The ^{1}H NMR spectra showed that all the peaks of pyrimidine and sugar in thymidine remained intact, subjected to small peak shift as a result of change in hydrogen interaction, while the 3′ O-CH$_2$ peak gradually disappeared showing complete decomposition.

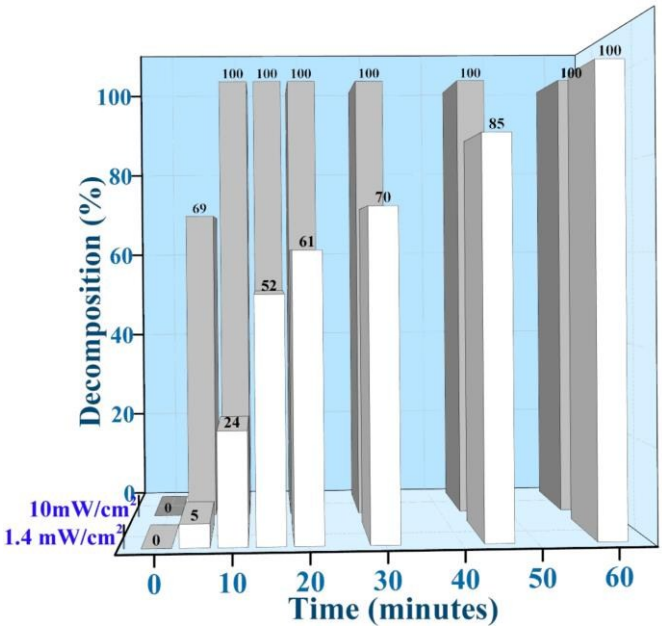


Figure 3: Decomposition - irradiation time histograms of NB-dTTP using 1.4 mW/cm^2 and 10 mW/cm^{2}power at 365 nm.

Prolonged UV exposure at 365 nm can damage biomolecules.[12] To decrease the exposure time, we have used two different strategies i.e. 1. increase the power of UV source and 2. change the protecting group. Integration changes of the 3′ O-CH$_2$ peak in comparison with the 1H of 1′H of the sugar ring, fixed as 1, in ^{1}H NMR spectral analysis of the dNTPs in D_2O were calculated as degree of decomposition. To quantify the photo lability of NB-dNTPs and DMNB-dNTPs the decomposition percentage was plotted as a function of time (Figure 3 and 4). Figure 3 shows the decomposition degree as a function of power of UV light. The photo decompositions were carried out with all 8 dNPTs with two different power (1.4 mW/cm^2 and 10 mW/cm^2) for 60 minutes under fixed conditions of concentration and solvent. Faster dissociation kinetics was obtained at elevated power of light. More than 50% of deprotection was observed within 5 minutes of irradiation for 10 mW/cm^2 power source while only 5% cleavage was observed for 1.4 mW/cm^2 source. The complete deprotection occurred within 10 minutes for 10 mW /cm^2 and within 55 minutes

for 1.4 mW/cm^2 power source. Major concern was the distortion of dNTP molecule by the use of high power UV. This was ruled out as the ^{1}H NMR spectra shows the dNTP structure to be very well stable after UV exposure till completion reaction. The result was confirmed by the Mass obtained by LCMS analysis.

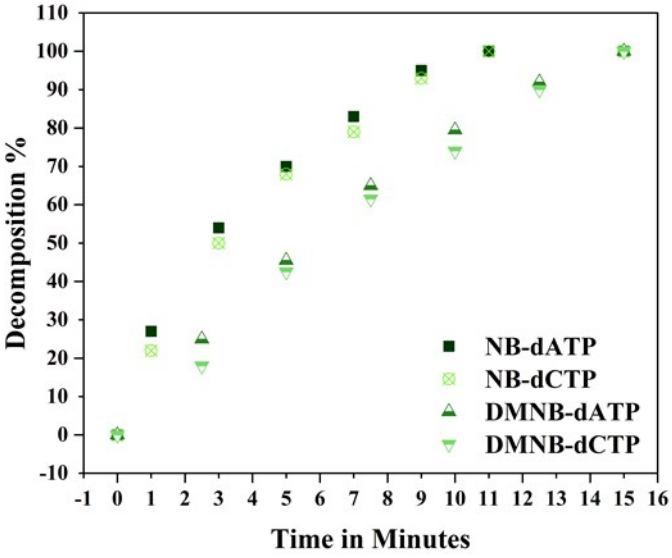


Figure 4: Decomposition - irradiation time plots of selected 3′-O-caged-dNTPs at 365 nm 10 mW/cm^2.

The impact of the structure of protecting group on the time of decomposition is portrayed in figure 4. It was observed when the 4,5-dimethoxy-2-nitrobenzyl moiety was replaced by 2-nitrobenzyl photo-liable group the rate of decomposition was enhanced. 50% photodecomposition was noted at 3 minutes for NB-dNTPs and at 5 minutes for DMNB-dNTPs. All the NB-dNTPs were completely cleaved in 10 minutes of irradiation with 365 nm 10 mW/cm^2 light while it took 15 minutes for the complete deprotection of DMNB-dNTPs. The presence of electron donating –OCH_3 groups in the 4, 5 position of the nitrobenzyl ring have significantly reduced the rate of photo decomposition showing that photo lability of the nucleotides can be tailored according to the substitution in the nitrobenzyl ring.

The photodecomposition of 2-nitrobenzyl compounds occurs via electronic excitation generating aci-nitro tautomers.[13,14] The rate of formation, stability and dissociation of this intermediates play a significant role in the rate of this photo reactions. Higher power source can accelerate the formation of the aci-nitro transient resulting in the increased rate of photo decomposition. The effect of -OCH_3 substitution can be attributed to the difference in the photo tautomerisation mechanism[15] leading to the aci-nitro intermediate imparted by the electron donating methoxy substitution of nitro benzene. The substitution can either slow down the rate of tautomerization or can stabilize the aci-nitro intermediate therefore decreasing the dissociation rate of the intermediate to form final product.

Figure 5 plots the decrease in 3′-O-CH_2 peak integration of all four NB-dNTPs, showing the effect of base structure can on photo decomposition rate. Purine based adenosine and guanine are decomposed slightly faster than pyrimidine based cytosine and thymine. The stability of the azi-nitro intermediate imparted by the base can contribute to the difference in decomposition rates.[7] Since the base is well isolated from the aci-nitro intermediate forming photo-tautomerization occurring in the 3′-O position of the sugar ring due to the absence of any pi electrons on its way, we attribute the difference in the dissociating rate to the spatial orientation of the bulky imidazole fused pyrimidine on adenosine and guanine base containing dATPs and dGTPs.

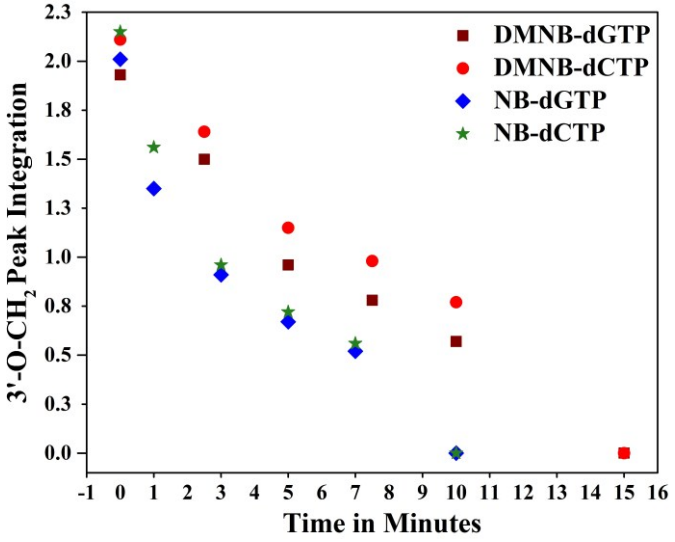


Figure 5: Peak Integration - irradiation time plots of NB-dCTP, NB-dGTP, DMNB-dCTP and DMNB-dGTP in D_2O at 365 nm 10 mW/cm^2.

The behaviour of the dNTP analogues in one step spot-start DNA synthesis was also evaluated. Studies show that 3′-O modification of dNTPs possess great challenge for incorporation by natural polymerase, especially when the 3′-O cage is a bulky one as nitro benzyl moiety.[16] Keeping this in mind we selected terminal deoxynucleotidyl transferase (TdT) enzyme to catalyse the DNA symthesis. TdT belongs to a class of DNA polymerase with relatively simple mechanism for catalysis due to the template free polydeoxynucleotide direction for DNA synthesis. Together with the efficiency at catalysing the growth of DNA from a DNA initiator, TdT can also incorporate multiple unnatural fluorescent nucleotides.[17] Enzymatic, one step off-on, DNA extension was performed by incubating 92.5 ng/μL of a blunt-end 750 base-pair DNA strand (750bp-DNA) with TdT at 20 U/μL in presence of cobalt containing buffer and 1mM of mono 3′-O protected dNTP at 37 ºC for 1 hour. Half the volume of samples was withdrawn after 1 hour and subjected to UV light at 365 nm 10 mW/cm^2 for 15 minutes and was again treated with TdT and unprotected mono dNTP and incubated at 37 ºC for another 1 hour. Figure 6 Shows the agarose gel electrophoresis image of termination and reinitiation of the DNA sequencing before and after UV irradiation. The caged dNTPs terminated the addition of more nucleotides after initial addition of the first NB-dNTP / DMNB-dNTP as indicated by bands 3, 4, 5, and 6. The original activity was found to be restored after photolysis as indicated by bands 8, 9, 10 and 11. The result also shows a pronounced difference between purine and pyrimidine triphosphates which can be attributed to the difference photodecomposition depending on the purine, pyrimidine stereochemistry[14] and binding

pattern depending on TdT enzyme base preference while adding different dNTPs to a DNA strand.[18]

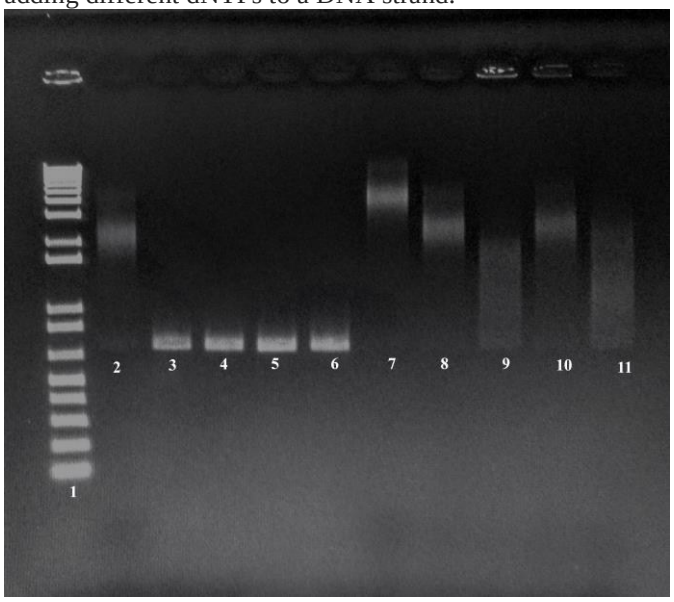


Figure 6: Gel electrophoresis showing the incorporation of NB-dNTPs by TdT to 750bp-DNA causing the temperarory termination of sequencing and the reinitiation after photo-cleaving the 3′-O-2′-nitrobenzyl group and incorporating nonprotected dNTP. (1. Standard Ladder, 2. Commercial dNTP mix, 3. NB-dATP, 4. NB-dCTP,5. NB-dGTP,6. NB-dTTP, and samples after UV irradiation and reintiation with 7. Commercial dNTP mix, 8. dATP, 9. dCTP, 10. dGTP, and 11. dTTP). (Figure 16 in *ESI* shows the gel electrophoresis data for DMNB-dNTPs)

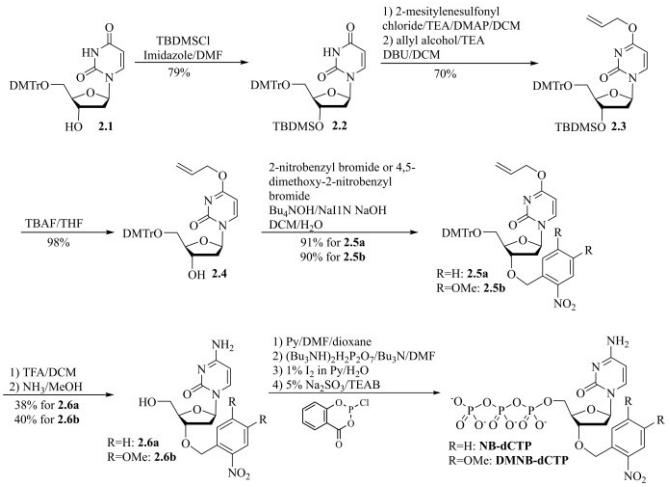
Conclusions

Photoselective template independent enzymatic DNA synthesis was adapted and developed to give a novel strategy for precise stacking of single nucleotides. Photo release of 2-nitrobenzyl and 4,5-dimethoxy-2-nitrobenzyl groups from dNTPs was quantified as a function of UV source power, caging moiety and base. The rate of photo decomposition was perceived to be dependent on the aci-nitro transients, as observed form the altered release of photo labile group, in accordance with the power of irradiation and substitution on the nitrobenzyl group. Purine and pyrimidine base structure was found to affect the photodecomposition. The study was well-applied for termination and reinitiation of DNA synthesis with the merit of enzyme specificity and non-chemical reaction. Ours is the first example of "stop-start" DNA synthesis allowing the efficient use of the unique template independent nature of TdT enzyme in a controlled manner, which opens a new direction of research and potentially new applications for photo labile nucleotides.

Experimental

Synthesis of NB-dNTPs and DMNB-dNTPs: Experimental procedures were done according to previous literature,[10] with some modifications. The repeated reactions are explained in *Electronic Supplementary Information (ESI).* The ^{1}H NMR, ^{31}P NMR, LCMS of all 3'-O-modified dNTPs and ^{13}C NMR and HRMS of novel compounds DMNB-dNTPs are given in *ESI.* The modified synthetic procedures are explained below.

1. Synthesis of 3′-O-(2-nitrobenzyl)-2′-deoxyadenosine-5′-triphosphate (NB-dATP) and 3′-O-(4,5-dimethoxy-2-nitrobenzyl)-2′-deoxyadenosine-5′-triphosphate (DMNB-dATP).



Scheme 2: Synthesis of NB-dATP and DMNB-dATP.

1.3b. Synthesis of 9-[β-D-5'-O-(tert-butyldimethylsilyl)-3'-O-(4,5-dimethoxy-2-nitrobenzyl)-2'-deoxyribofuranosyl]-6-chloropurine.

To a stirred solution of 9-[β-D-5'-O-(tert-butyldimethylsilyl)-2'-deoxyribofuranosyl]-6-chloropurine **1.2**[10](1.73 g, 4.48 mmol) in DCM (135 mL) were added tetrabutylammonium bromide (0.722 g, 2.24 mmol), 4,5-dimethoxy-2-nitrobenzyl bromide (3.09 g, 11.2 mmol) and 40% aq. NaOH (65 mL). The reaction mixture was stirred at room temperature for 1 h, diluted with EtOAc (300 mL) and then the organic layer was separated. The aqueous layer was extracted with EtOAc (2x125 mL). The combined organic layers were washed with sat. $NaHCO_3$ (80 mL), brine (80 mL) and dried over Na_2SO_4. After concentration of the filtrate, the residue was purified by flash column chromatography over silica gel using EtOAc/hexanes (1:2) to give compound **1.3b** was obtained as a yellow solid (2.22 g, 85%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.74 (s, 1H, 2-H), 8.51 (s, 1H, 8-H), 7.73 (s, 1H, one of C_6H_2), 7.26 (1H, one of C_6H_2), 6.59 (dd, J = 6.0, 7.8 Hz, 1H, 1′-H), 5.03-4.94 (m, 2H, $OCH_2C_6H_2$), 4.49-4.45 (m, 1H, 3'-H), 4.38-4.33 (m, 1H, 4′-H), 4.04 (s, 3H, OCH_3), 3.97 (s, 3H, OCH_3), 3.94 (dd, J = 3.6, 11.4 Hz, 1H, one of 5′-H), 3.86 (dd, J = 3.0, 11.4 Hz, 1H, one of 5′-H), 2.82-2.68 (m, 2H, 2′-H), 0.90 (s, 9H, $C(CH_3)_3$), 0.11 (s, 3H, one of $SiCH_3$), 0.10 (s, 3H, one of $SiCH_3$).

1.4b. Synthesis of 3′-O-(4,5-dimethoxy-2-nitrobenzyl)-2′-deoxyadenosine.

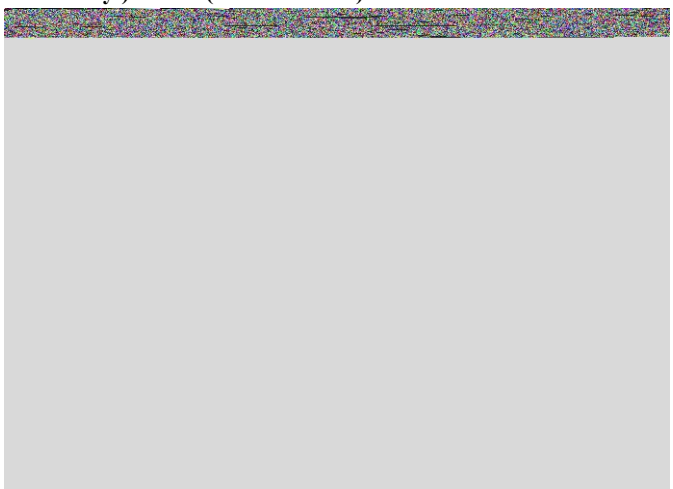
To a stirred solution of 9-[β-D-5′-O-(tert-butyldimethylsilyl)-3′-O-(4,5- dimethoxy -2- nitro benzyl) -2′- deoxyribo furanosyl]-6-chloropurine **1.3b** (2.22 g, 3.83 mmol) in THF (40 mL) was added 1.0 M tetrabutylammonium fluoride (TBAF) in THF solution (4.20 mL, 4.20 mmol) dropwise over 5 min. The reaction mixture was stirred at room temperature for 1.5 h. After concentration, the residue was dissolved in a mixture of dioxane (20 mL) and 7 N NH_3 in MeOH (40 mL). The solution was stirred in a sealed flask at 85-90 °C for another 18 h. After evaporation, the residue was purified by flash column chromatography over silica gel using MeOH/DCM (1:20) to compound **1.4b** as a pale yellow solid (0.953 g, 56%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.34 (s, 1H, 2-H), 8.13 (s, 1H, 8-H), 7.69 (s, 1H,

one of C_6H_2), 7.32 (brs, 3H, 6-NH_2 and one of C_6H_4), 6.35 (dd, J = 6.0, 8.4 Hz, 1H, 1′-H), 5.34 (t, J = 5.4 Hz, 1H, 5′-OH), 4.96-4.88 (m, 2H, OC$H_2C_6H_2$), 4.38-4.35 (m, 1H, 3′-H), 4.16-4.12 (m, 1H, 4′-H), 3.94 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 3.66-3.53 (m, 2H, 5′-H), 2.90-2.84 (m, 1H, one of 2′-H), 2.58-2.52 (m, 1H, 2′-H).

DMNB-dATP. Synthesis of 3′-O-(4,5-dimethoxy-2-nitrobenzyl)-2′-deoxyadenosine-5'-triphosphate.

To a suspension of 3′-O-(4,5-dimethoxy-2-nitrobenzyl)-2′-deoxyadenosine (134 mg, 0.300 mmol) in anhydrous pyridine (1.0 mL) and DMF (1.0 mL) was added a solution of 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (85.0 mg, 0.405 mmol) in anhydrous dioxane (1.0 mL) over 1 min at room temperature, leading to a clear solution. After 20 min, a mixture of tributylammonium pyrophosphate (380 mg, 0.690 mmol) in anhydrous DMF (1.5 mL) and tributylamine (0.350 mL, 1.47 mmol) was added over 20 seconds. After 20 min, a solution of iodine (105 mg, 0.410 mmol) and water (0.16 mL) in pyridine (8 mL) was added over 5 min. After 20 min, the reaction was quenched by addition of 5% Na_2SO_3 until excess iodine disappeared. After 10 min, 2.0 mL of 1.0 M triethyl ammonium bicarbonate solution (TEAB) was added. After stirring at room temperature for 1 h, the solvents were removed in vacuo. Water (20 mL) was added, and the mixture was filtered. The filtrate was then purified by anion exchange chromatography on DEAE-Sephadex A-25 using a gradient of TEAB (pH 8, 0.1-1.0 M) to give compound **DMNB-dATP** as a pale yellow gum of TEA salt (144 mg, 44%) after lyophilization. ^{1}H NMR (600 MHz, D_2O) δ 8.60 (brs, 1H, 2-H), 8.25 (brs, 1H, 8-H), 7.75 (s, 1H, one of C_6H_2), 7.35 (s, 1H, one of C_6H_2), 6.52-6.47 (m, 1H, 1′-H), 5.04-4.94 (m, 2H, OC$H_2C_6H_2$), 4.73-4.69 (m, 1H, 3′-H), 4.57-4.52 (m, 1H, 4′-H), 4.29-4.15 (m, 2H, 5′-H), 4.00 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 2.85-2.73 (m, 2H, 2′-H); ^{13}C NMR (150.8 MHz, D_2O) δ 165.8, 156.4, 155.2, 154.5, 152.8, 151.4, 146.8, 138.7, 128.8, 110.4, 107.6, 83.9, 83.8, 80.5, 67.9, 58.5, 56.1, 55.8, 36.6. ^{31}P NMR (242.7 MHz, D_2O) δ -10.0 (brs, 2P), -21.7 (brs, 1P); HRMS (ES^-) calcd for $C_{19}H_{24}N_6O_{16}P_3^-$ [(M+3H)]: 685.0467, found: 685.0459; HPLC: 89.8%.

2. Synthesis of 3′-O-(2-nitrobenzyl)-2′-deoxycytidine-5′-triphosphate (NB-dCTP) and 3′-*O*-(4,5-dimethoxy-2-nitrobenzyl)-dCTP (DMNB-dCTP)



Scheme 3: Synthesis of NB-dCTP and DMNB-dCTP

2.5b. Synthesis of 4-O-allyl-5′-O-(4,4′-dimethoxytrityl)-3′-O-(4,5-dimethoxy-2-nitrobenzyl)-2′-deoxyuridine.

To a vigorously stirring mixture of 4-O-allyl-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyuridine **2.4**[10] (1.91 g, 3.35 mmol), Bu_4NOH (tetrabutylammonium hydroxide) (1.5 mL, 55-60% in water) and NaI (50.0 mg, 0.335 mmol) in DCM/water (10 mL/10 mL) was added 1.0 M NaOH solution (10 mL, 10 mmol). The mixture was stirred for 10 min at room temperature, a solution of 4,5-dimethoxy-2-nitrobenzyl bromide (1.84 g, 6.70 mmol) in 10 mL of DCM was added over 5 min and the resulting reaction mixture was stirred for another 7 h at room temperature. The reaction mixture was diluted with DCM (150 mL) and then washed with brine (20 mL). The organic layer was dried over anhydrous Na_2SO_4. After evaporation, the crude product was then purified by flash column chromatography over silica gel using hexanes/ethyl acetate (3:1~1:1) to give compound **2.5b** as a pale yellow solid (2.31 g, 90%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.05 (d, J = 7.2 Hz, 1H, 6-H), 7.72 (s, 1H, one of C_6H_2), 7.39-7.36 (m, 2H, two aromatic protons of DMTr), 7.30-7.20 (m, 7H, seven aromatic protons of DMTr), 7.19 (s, 1H, one of C_6H_2), 6.84-6.79 (m, 4H, four ortho protons to CH_3O of DMTr), 6.37 (t, J = 6.6 Hz, 1H, 1′-H), 6.07-5.99 (m, 1H, CH_2=$CH$$CH_2$), 5.70 (d, J = 7.2 Hz, 1H, 5-H), 5.38 (dd, J = 1.2, 18.0 Hz, 1H, one of CH_2=$CHCH_2$), 5.28 (dd, J = 1.2, 10.2 Hz, 1H, one of CH_2=$CHCH_2$), 4.93-4.82 (m, 4H, CH_2=$CHCH_2$ and OC$H_2C_6H_2$), 4.33-4.26 (m, 2H, 3′-H and 4′-H), 3.96 (s, 6H, two CH_3O), 3.78 (s, 6H, two CH_3O), 3.51 (dd, J = 3.6, 10.8 Hz, 1H, one of 5′-H), 3.42 (dd, J = 3.6, 10.8 Hz, 1H, one of 5′-H), 2.82-2.76 (m, 1H, one of 2′-H), 2.22-2.16 (m, 1H, one of 2′-H).

2.6b. Synthesis of 3′-O-(4,5-dimethoxy-2-nitrobenzyl)-2′-deoxycytidine

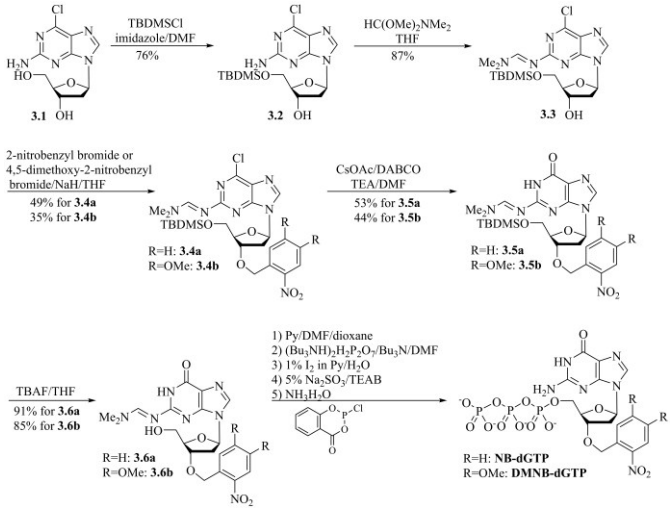
To an ice cold solution of 4-O-allyl-5′-O-(4,4′-dimethoxytrityl)-3′-O-(4,5-dimethoxy-2-nitrobenzyl)-2′-deoxyuridine **2.5b** (2.30 g, 3.00 mmol) in DCM (45 mL) was added trifluoroacetic acid (2.70 mL, 35.0 mmol) was added slowly over 5 min. The resulting red solution was stirred for 15 min at room temperature and then quenched with saturated aqueous $NaHCO_3$ solution until pH = 8-9. The mixture was diluted with DCM (150 mL). The organic layer was separated, washed with saturated $NaHCO_3$ solution (2x20 mL) and dried over anhydrous Na_2SO_4, and then concentrated. The residue was dissolved in 7 N NH_3 in methanol (55 mL) and stirred in a sealed tube for 20 h at 55 °C. After evaporation, the crude product was then purified by flash column chromatography over silica gel using MeOH/DCM (1:10) to afford compound **2.6b** as a pale yellow solid (0.51 g, 40%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.75 (d, J = 7.8 Hz, 1H, 6-H), 7.67 (s, 1H, one of C_6H_2), 7.26 (s, 1H, one of C_6H_2), 7.15 and 7.09 (s, 2H, NH_2), 6.17 (dd, J = 6.0, 8.4 Hz, 1H, 1′-H), 5.71 (d, J = 7.8 Hz, 1H, 5-H), 5.04 (t, J = 5.4 Hz, 1H, OH), 4.86 (s, 2H, $OCH_2C_6H_2$), 4.19-4.16 (m, 1H, 3′-H), 4.03-4.00 (m, 1H, 4′-H), 3.90 (s, 3H, one of CH_3O), 3.84 (s, 3H, one of CH_3O), 3.58-3.53 (m, 2H, 5′-H), 2.36-2.31 (m, 1H, one of 2′-H), 2.06-1.99 (m, 1H, one of 2′-H).

DMNB-dCTP. Synthesis of 3′-O-(4,5-dimethoxy-2-nitrobenzyl)-2′-deoxycytidine-5′-triphosphate.

Using the same preparation procedure of **DMNB-dATP**, **3′-O-(4,5-dimethoxy-2-nitrobenzyl)-dCTP** was obtained as a pale yellow gum of TEA salt (90 mg, 28%). ^{1}H NMR (600 Hz, D_2O) δ 8.26 (d, J = 7.2 Hz, 1H, 6-H), 7.73 (s, 1H, one of C_6H_2), 7.21 (s, 1H, one of

C_6H_2), 6.36 (d, J = 7.8 Hz, 1H, 5-H), 6.28 (dd, J = 6.0, 8.4 Hz, 1H, 1′-H), 4.97-4.87 (m, 2H, $OCH_2C_6H_2$), 4.60-4.57 (m, 1H, 3′-H), 4.54-4.51 (m, 1H, 4′-H), 4.32-4.21 (m, 2H, 5′-H), 3.98 (s, 3H, one of CH_3O), 3.93 (s, 3H, one of CH_3O), 2.74-2.69 (m, 1H, one of 2′-H), 2.39-2.32 (m, 1H, one of 2′-H); ^{13}C NMR (150.8 MHz, D_2O) δ 159.0, 152.8, 148.0, 146.7, 144.2, 138.6, 128.9, 110.1, 107.5, 95.0, 86.8, 84.1, 80.6, 67.6, 58.4, 56.0, 55.7, 37.3. ^{31}P NMR (242.7 MHz, D_2O) δ -10.6 (brs, 2P), -23.0 (brs, 1P); HRMS (ES^-) calcd for $C_{18}H_{24}N_4O_{17}P_3^-$ [(M+3H)]: 661.0355, found: 661.0356; HPLC: 93.3%.

3. Synthesis of 3′ -*O*- (2-nitrobenzyl) -2′- deoxyguanosine -5′-triphosphate (NB-dGTP) and 3′-*O*-(4,5-dimethoxy-2-nitrobenzyl)- 2′-deoxyguanosine-5′-triphosphate (DMNB-dGTP)



Scheme 4: Synthesis of NB-dGTP and DMNB-dGTP

3.4b. Synthesis of 6-Chloro-2-[(dimethylaminomethylene) amino] -9- [β-D -5′ -O- (tert-butyldimethylsilyl) -3′- O- (4,5-dimethoxy -2-nitrobenzyl)-2′-deoxyribofuranosyl] purine.

To an ice cold solution of 6-chloro-2-[(dimethylaminomethylene)amino]-9- [β-D-5′-O-(tert-butyldimethyl silyl) -2′-deoxyribofur anosyl] purine **3.3**[10] (2.50 g, 5.50 mmol) in anhydrous THF (25 mL) was added 95% NaH powder (0.306 g, 12.1 mmol) in portions. After stirring for 50 min at room temperature, a solution of 4,5-dimethoxy-2-nitrobenzyl bromide (3.04 g, 11.0 mmol) in THF (5.0 mL) was added, and then the reaction mixture was stirred for another 2.5 h at room temperature with exclusion of air and light. After concentration, the resulting residue was dissolved in ethyl acetate (250 mL), washed with saturated aqueous $NaHCO_3$ (20 mL), brine (20 mL) and dried over anhydrous Na_2SO_4. After concentration, the crude product was purified by flash column chromatography over silica gel using EtOAc/hexanes (1:1-2:1) to afford compound **3.4b** as a pale yellow foam (1.25 g, 35%). ^1H NMR (600 MHz, $CDCl_3$) δ 8.74 (s, 1H, C*H*N$(CH_3)_2$), 8.28 (s, 1H, 8-H), 7.73 (s, 1H, one of C_6H_2), 7.27 (s, 1H, one of C_6H_2), 6.66 (dd, J = 6.0, 8.4 Hz, 1H, 1′-H), 4.98-4.92 (m, 2H, OC*H*$_2C_6H_2$), 4.42-4.36 (m, 1H, 3′-H), 4.32-4.27 (m, 1H, 4′-H), 4.05 (s, 3H, one of CH_3O), 3.97 (s, 3H, one of CH_3O), 3.93-3.84 (m, 2H, 5′-H), 3.18 and 3.17 (s, 6H, N$(CH_3)_2$), 2.78-2.73 (m, 1H, one of 2′-H), 2.50-2.44 (m, 1H, one of 2′-H), 0.92 (s, 9H, C$(CH_3)_3$), 0.13 (s, 3H, one of SiCH_3), 0.12 (s, 3H, one of SiCH_3).

3.5b. Synthesis of 5′-O-(t-butyldimethylsilyl)-N^2-[(dimethylamino)methylene]-3′-O-(4,5-dimethoxy-2-nitrobenzyl) -2′-deoxyguanosine

To a solution of 6-Chloro-2-[(dimethylaminomethylene)amino]-9-[β-D-5′-O-(tert–butyl dimethyl silyl) - 3′ - O - (4,5-dimethoxy -2-nitrobenzyl)-2′-deoxyribofuranosyl]purine **3.4b** (1.23 g, 1.89 mmol) in anhydrous DMF (12.0 mL) were added cesium acetate (1.09 g, 5.68 mmol), 1,4-diazabicyclo[2.2.2]octane (DABCO) (0.212 g, 1.89 mmol) and triethylamine (0.790 mL, 5.68 mmol) under argon and stirred overnight at room temperature with exclusion of air and light. Ac_2O (5.4 mL) was added to the above reaction mixture and stirred for another 30 min. The reaction mixture was then quenched with water (20 mL) and extracted with ethyl acetate (3x50 mL). The organic layer was dried over Na_2SO_4. After evaporation, the crude product was purified by flash column chromatography over silica gel using MeOH/DCM (1:20) to afford compound **3.5b** as a pale yellow solid (0.52 g, 44%). ^1H NMR (600 MHz, $CDCl_3$) δ 8.65 (s, 1H, NH), 8.61 (s, 1H, C*H*N$(CH_3)_2$), 7.90 (s, 1H, 8-H), 7.73 (s, 1H, one of C_6H_2), 7.25 (s, 1H, one of C_6H_2), 6.39 (dd, J = 5.4, 8.4 Hz, 1H, 1′-H), 5.05-4.92 (m, 2H, OC*H*$_2C_6H_2$), 4.45-4.42 (m, 1H, 3′-H), 4.29-4.26 (m, 1H, 4′-H), 4.02 (s, 3H, one of CH_3O), 3.97 (s, 3H, one of CH_3O), 3.87-3.79 (m, 2H, 5′-H), 3.18 (s, 3H, one of N$(CH_3)_2$), 3.10 (s, 3H, one of N$(CH_3)_2$), 2.70-2.65 (m, 1H, one of 2′-H), 2.60-2.54 (m, 1H, one of 2′-H), 0.91 (s, 9H, C$(CH_3)_3$), 0.10 (s, 6H, two of SiCH_3).

3.6b. Synthesis of N^2-[(dimethylamino)methylene]-3′-O-(4,5-dimethoxy-2-nitrobenzyl)-2′-deoxyguanosine.

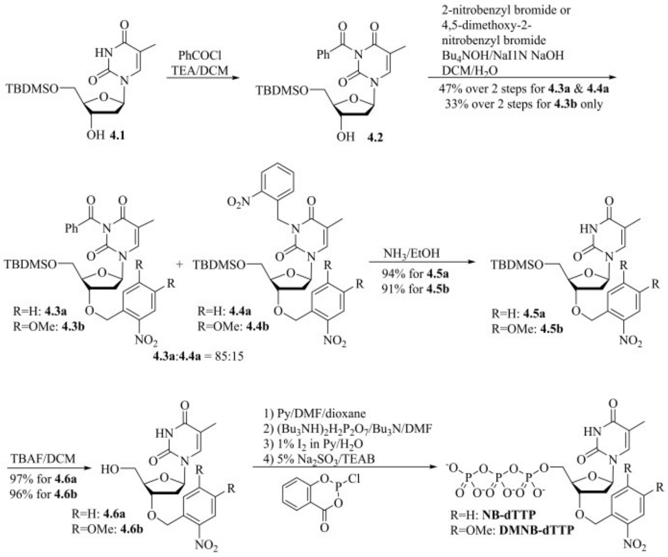
To an ice cold solution of 5′-O-(t-butyldimethylsilyl)-N^2-[(dimethylamino)methylene]-3′-O-(4,5-dimethoxy-2-nitrobenzyl)-2′-deoxyguanosine **3.5b** (0.51 g, 0.70 mmol) in THF (6.0 mL) was added 1.0 M TBAF in THF solution (1.6 mL, 1.6 mmol) over 1 min. The reaction mixture was allowed to warm to room temperature and stirred for 2.5 h with exclusion of air and light, diluted with EtOAc (100 mL), washed with water (15 mL), brine (15 mL) and dried over anhydrous Na_2SO_4. After concentration of the filtrate, the residue was purified by flash column chromatography over silica gel using MeOH/DCM (1: 20) to afford **3.6b** (0.350 g, 85%) as yellow solid. ^1H NMR (600 MHz, DMSO-d_6) δ 11.3 (s, 1H, NH), 8.56 (s, 1H, C*H*N$(CH_3)_2$), 8.04 (s, 1H, 8-H), 7.67 (s, 1H, one of C_6H_2), 7.29 (s, 1H, one of C_6H_2), 6.25 (dd, J = 6.0, 8.4 Hz, 1H, 1′-H), 5.04 (t, J = 6.0 Hz, 1H, OH), 4.94-4.86 (m, 2H, OC*H*$_2C_6H_2$), 4.35-4.31 (m, 1H, 3′-H), 4.10-4.06 (m, 1H, 4′-H), 3.92 (s, 3H, one of CH_3O), 3.85 (s, 3H, one of CH_3O), 3.60-3.52 (m, 2H, 5′-H), 3.12 (s, 3H, one of N$(CH_3)_2$), 3.01 (s, 3H, one of N$(CH_3)_2$), 2.76-2.60 (m, 1H, one of 2′-H), 2.54-2.48 (m, 1H, one of 2′-H overlapped with DMSO signal).

DMNB-dGTP. Synthesis of 3′-O-(4,5-dimethoxy-2-nitrobenzyl)-2′-deoxyguanosine-5′-triphosphate.

Using the same preparation procedure of **DMNB-dATP,** compound **3′-O-(4,5-dimethoxy-2-nitrobenzyl)-dGTP** was obtained as a pale yellow gum of TEA salt (118 mg, 35%). ^1H NMR (600 MHz, D_2O) δ 8.11 (s, 1H, 8-H), 7.71 (s, 1H, one of C_6H_2), 7.23 (s, 1H, one of C_6H_2), 6.29-6.25 (m, 1H, 1′-H), 5.02-4.89 (m, 2H, OC*H*$_2C_6H_2$), 4.69-4.64 (m, 1H, 3′-H), 4.53-4.47 (m, 1H, 4′-H), 4.25-4.13 (m, 2H, 5′-H), 3.99 (s, 3H, one of CH_3O), 3.93 (s, 3H, one of CH_3O), 2.87-2.71 (m, 2H, 2′-H); ^{13}C NMR (150.8 MHz, D_2O) δ 158.1, 153.4, 152.9, 146.8, 146.7, 138.6, 138.4, 129.1, 129.0, 110.2, 107.5, 83.9, 83.6, 80.6, 67.8, 58.5, 56.1, 56.8, 36.2. ^{31}P NMR (242.7

MHz, D_2O) δ -1.31 (brs, 1P), -11.7 (brs, 1P), -22.9 (brs, 1P); HRMS (ES$^-$) calcd for $C_{19}H_{24}N_6O_{17}P_3^-$ [(M+3H) $^-$]: 700.0576, found: 700.0581; HPLC: 76.2%.

4. Synthesis of 3'-O-(2-nitrobenzyl)- thymidine-5′-triphosphate. (NB-dTTP) and 3'-O-(4,5-dimethoxy-2-nitrobenzyl) thymidine-5′-triphosphate (DMNB-dTTP).



Scheme 5: Synthesis of NB-dTTP and DMNB-dTTP

4.2. Synthesis of 3-N-benzoyl-5′-O-(tert-butyldimethylsilyl) thymidine.

To a cooled (0 °C) solution of 5′-*O*-(*tert*-butyldimethyl)-thymidine **4.1** (1.00 g, 2.81 mmol) in DCM (15 mL) was added triethylamine (0.470 mL, 3.37 mmol) followed by benzoyl chloride (0.36 mL, 3.09 mmol). The reaction mixture was stirred at 0 °C for 6 h, and then warmed to room temperature overnight, diluted with DCM (50 mL), washed with water (20 mL). The organic layer was dried over anhydrous Na_2SO_4. Evaporation of the filtrate gave compound **4.2** as a crude product (1.33 g, quantitative yield) without further purification, directly used in the next step. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.93 (d, J = 7.8 Hz, 2H, two of C_6H_4), 7.64 (d, J = 7.8 Hz, 1H, one of C_6H_4), 7.61 (s, 1H, 6-H), 7.52-7.47 (m, 2H, two of C_6H_4), 6.35 (dd, J = 6.0, 8.4 Hz, 1H, 1′-H), 4.51-4.48 (m, 1H, 3′-H), 4.05-4.02 (m, 1H, 4′-H), 3.94-3.92 (m, 1H, one of 5′-H), 3.88-3.85 (m, 1H, one of 5′-H), 2.39-2.35 (m, 1H, one of 2’-H), 2.20-2.15 (m, 1H, one of 2′-H), 1.97 (s, 3H, 5-CH_3), 1.83 (d, J = 3.6 Hz, 1H, OH), 0.95 (s, 9H, C(CH_3)$_3$), 0.15 (s, 3H, one of SiCH_3), 0.14 (s, 3H, one of SiCH_3).

4.3b. Synthesis of 3-N-benzoyl-5′-O-(tert-butyldimethylsilyl)-3′-O-(4,5-dimethoxy -2-nitrobenzyl) thymidine.

To an ice cold mixture of 3-N-benzoyl-5′-O-(tert-butyldimethylsilyl) thymidine **4.2** (crude 1.33 g, 2.81mmol) in DCM (10 mL), aqueous Bu_4NOH (60%, 0.9 mL), NaI (84 mg, 0.56 mmol), water (10 mL) and aqueous NaOH solution (1.0 M, 10 mL) was added a solution of 2-nitrobenzyl bromide (0.630 g, 2.92 mmol) in DCM (10 mL) dropwise over 10 min. The reaction mixture was stirred at 0°C for 2 h and then stirred at room temperature for 6 h with exclusion of light. The reaction mixture was diluted with water (20 mL) and then extracted with DCM (3x50 mL). The organic layer was washed with brine (30 mL) and dried over anhydrous Na_2SO_4. After filtration and concentration, the resulting crude product was purified by flash column chromatography over silica gel using 10-25% ethyl acetate in hexanes to afford compound **4.3b** as a pale yellow foam (0.60 g, 33% over two steps). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.94-7.90 (m, 2H, two of C_6H_5), 7.80 (s, 1H, one of C_6H_2), 7.65-7.61 (m, 2H, one of C_6H_5 and 6-H), 7.51-7.46 (m, 2H, two of C_6H_5), 7.20 (s, 1H, one of C_6H_2), 6.38 (dd, J = 5.4, 9.0 Hz, 1H, 1′-H), 4.92(s, 2H, OC$H_2$$C_6H_2$), 4.31-4.29 (m, 1H, 3′-H), 4.26-4.24 (m, 1H, 4′-H), 3.98-3.90 (m, 1H, one of 5’-H), 3.95 (s, 3H, one of CH_3O), 3.94 (s, 3H, one of CH_3O), 3.87-3.84 (m, 1H, one of 5′-H), 2.59-2.54 (m, 1H, one of 2′-H), 2.14-2.08 (m, 1H, one of 2′-H), 1.97 (s, 3H, 5-CH_3), 0.95 (s, 9H, C(CH_3)$_3$), 0.15 (s, 3H, one of SiCH_3), 0.14 (s, 3H, one of SiCH_3).

4.5b. Synthesis of 5′-O-(tert-butyldimethylsilyl)-3′-O-(4,5-dimethoxy-2-nitrobenzyl) thymidine.

To a mixture of 3-N-benzoyl-5′-O-(tert-butyldimethylsilyl)-3′-O-(4,5-dimethoxy-2-nitrobenzyl) thymidine **4.3b** (0.600 g, 0.920 mmol)) in ethanol (10 mL) was added 30% ammonium hydroxide solution (1.50 mL). The reaction mixture was stirred for 3 h at room temperature with exclusion of light, and then subjected to evaporation. The residue was extracted with DCM (3x50 mL). The organic layers were combined and washed with brine (20 mL) and dried over anhydrous Na_2SO_4. After concentration, the residue was purified by flash column chromatography over silica gel using ethyl acetate/hexanes (1:2) to afford compound **4.5b** as a pale yellow solid (0.46 g, 91%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.18 (s, 1H, NH), 7.72 (s, 1H, one of C_6H_2), 7.51 (s, 1H, 6-H), 7.25 (s, 1H, one of C_6H_2), 6.38 (dd, J = 5.4, 9.0 Hz, 1H, 1′-H), 4.98-4.90 (m, 2H, OC$H_2$$C_6H_2$), 4.31-4.28 (m, 1H, 3′-H), 4.25-4.22 (m, 1H, 4′-H), 4.02 (s, 3H, one of CH_3O), 3.96 (s, 3H, one of CH_3O), 3.95-3.91 (m, 1H, one of 5′-H), 3.85-3.82 (m, 1H, one of 5′-H), 2.55-2.50 (m, 1H, one of 2′-H), 2.08-2.03 (m, 1H, one of 2′-H), 1.94 (s, 3H, 5-CH_3), 0.94 (s, 9H, C(CH_3)$_3$), 0.13 (s, 6H, two of SiCH_3).

4.6b. Synthesis of 3′-O-(4,5-dimethoxy-2-nitrobenzyl) thymidine.

To a cooled (0 °C) solution of 5′-O-(tert-butyldimethylsilyl)-3′-O-(4,5-dimethoxy-2-nitrobenzyl) thymidine **4.5b** (0.460 g, 0.835 mmol) in THF (8 mL) was added 1.0 M TBAF in THF solution (1.67 mL, 1.67 mmol) over 2 min. The solution was allowed to warm to room temperature and continue stirring for 2 h with exclusion of air and light. The mixture was poured into cold water (30 mL), and the resulting mixture was extracted with ethyl acetate (3x40 mL). The organic layers were combined, washed with brine (20 mL) and dried over anhydrous Na_2SO_4. After concentration, the residue was purified by flash column chromatography over silica gel using 3-5% MeOH in DCM to afford compound **4.6b** as a pale yellow solid (0.35 g, 96%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.26 (s, 1H, NH), 7.71 (s, 1H, one of C_6H_2), 7.41 (s, 1H, 6-H), 7.20 (s, 1H, one of C_6H_2), 6.22 (dd, J = 6.0, 8.4 Hz, 1H, 1′-H), 4.98-4.90 (m, 2H, OC$H_2$$C_6H_2$), 4.42-4.39 (m, 1H, 3′-H), 4.24-4.21 (m, 1H, 4′-H), 4.01 (s, 3H, one of CH_3O), 4.01-3.96 (m, 1H, one of 5′-H), 3.96 (s, 3H, one of CH_3O), 3.88-3.83 (m, 1H, one of 5′-H), 2.52-2.47 (m, 1H, one of 2′-H), 2.42-2.35 (m, 1H, one of 2′-H), 1.93 (s, 3H, 5-CH_3).

DMNB-dTTP. Synthesis of 3′-O-(4,5-dimethoxy-2-nitrobenzyl) thymidine-5′-triphosphate.

Using the same preparation procedure of **DMNB-dATP**,

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compound **3′-O-(4,5-dimethoxy-2-nitrobenzyl)-dTTP** was obtained as a pale yellow gum of TEA salt (100 mg, 31%). ^{1}H NMR (600 MHz, D_2O) δ 7.81 (s, 1H, one of C_6H_2), 7.29 (s, 1H, one of C_6H_2), 6.37 (dd, *J* = 5.4, 9.6 Hz, 1′-H), 5.03-4.93 (m, 2H, $OCH_2C_6H_2$), 4.64-4.61 (m, 1H, 3′-H), 4.47-4.43 (m, 1H, 4′-H), 4.29-4.21 (m, 2H, 5′-H), 4.01 (s, 3H, one of CH_3O), 3.96 (s, 3H, one of CH_3O), 2.59-2.54 (m, 1H, one of 2′-H), 2.41-2.34 (m, 1H, one of 2′-H), 1.94 (s, 3H, 5-CH_3); ^{13}C NMR (150.8 MHz, D_2O) δ 166.1, 152.9, 151.3, 147.0, 139.0, 137.0, 129.1, 111.6, 110.5, 107.8, 85.1, 83.3, 80.6, 67.8, 58.3, 56.1, 55.9, 36.0, 11.6. ^{31}PNMR (242.7 MHz, D_2O) δ -10.1 (brs, 1P), -10.6 (brs, 1P), -22.7 (brs, 1P); HRMS (ES^-) calcd for $C_{19}H_{25}N_3O_{18}P_3^-$ [$(M+3H)^-$]: 676.0351, found: 676.0339; HPLC: 95.5%.

Photodecomposition Studies: The photo decay of each dNTP was done with 1 mg/mLsolution of compound in water / D_2O. 1 mL of the solution was irradiated with 365 nm UV light and samples were withdrawn at regular intervals for ^{1}H NMR and LCMS analysis.

Stop-Start DNA synthesis: For a standard reaction, 5 μL of DNA (92.5 ng/μL, 750bp) was mixed with 5μL of 5XTdT Buffer, $CoCl_2$ solution (100mM, 0.5 μL), dNTP (10 mM, 1 μL) and TdT (20 un/μL) in a micro centrifuge tube and the final volume was made up to 50ml by adding milliQ water. The mixture was then incubated at 37^{o}C for 60 minutes. After completion of DNA end caging, 25 μL was loaded into 2% Agarose gel and remaining 25 μL of the mixture was taken out and subjected to UV irradiation (365 nm, 10 mW/cm^2) for 15 minutes. After UV irradiation the mixture was again treated with dNTP (10 mM, 1 μL) and TdT (15 un) and incubated for another 60 minutes and loaded on gel.

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