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Dideoxy nucleoside triphosphate (ddNTP) analogues: Synthesis and polymerase substrate activities of pyrrolidinyl nucleoside triphosphates (prNTPs)

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ABSTRACT

The dideoxynucleoside triphosphates (ddNTPs) terminate the bio-polymerization of DNA and become essential chemical component of DNA sequencing technology which is now basic tool for molecular biology research. In this method the radiolabeled or fluorescent dye labeled ddNTP analogues are being used for DNA sequencing by detection of the terminated DNA fragment after single labeled ddNTP incorporation into DNA under PCR conditions. This report describes the syntheses of rationally designed novel amino-functionalized ddNTP analogue such as Pyrrolidine nucleoside triphosphates (prNTPs), and their polymerase activities with DNA polymerase by LC–MS and Gel-electrophoretic techniques. The Mass and PAGE analyses strongly support the incorporation of prNTPs into DNA oligonucleotide with Therminator DNA polymerase as like control substrate ddNTP. As resultant the DNA oligonucleotide are functionalized as amine group by prNTP incorporation with polymerase. Hence prNTPs provide opportunities to prepare demandable conjugated DNA with other biomolecules/dyes/fluorescence molecule without modifying nucleobase structure.

1. Introduction

The dideoxynucleoside triphosphate (ddNTP) is structurally modified derivative of deoxynucleoside triphosphate (dNTP), and also known as Sanger's nucleotide which contains dideoxygenated ribose ring, nucleobase (A/T/G/C) and triphosphate residues (Fig. 1). In late seventies, Sanger et al. established the function of ddNTPs as termination of DNA polymerization after single incorporation, enzymatically, into DNA primer (at 3′-OH), and laid down the foundation of the DNA sequencing technology.[1] In DNA sequencing, the labeled ddNTP analogues have been employed to terminate the DNA synthesis from DNA primer under PCR condition, and make detectable primer with inclusion of one labeled-ddNTP residue. The labeling of ddNTPs are being performed by introducing the radioactive isotope or chromophores into the chemical structure of ddNTPs such as α-^{32}P-radioisotope for radioactive ddNTPs,[2] and big-dyes for fluorescent ddNTPs.[3] Due to cost and safety reasons, the radioactive labeling are not being encouraged for practical application in sequencing. The dye/fluorophore-labeled ddNTP, however, are appreciated to employ in DNA sequencing and their synthesis are accomplished by attaching dye/fluorophore, via linker, at the nucleobase region of modified ddNTP which are obtained by functionalization-such as amine or alcohol functional group-at purine/pyrimidine carbon atom of nucleobases. Mostly the purine 7-deazapurine and C-5-substituted pyrimidine containing ddNTP analogues have been used for functionalization with amino group which coupled with carboxylate group of dyes via amide bond. Recently, four different colors dyes are being employed to prepare four different colors of ddNTPs such as red-ddTTP, blue-ddATP, green-ddCTP, and yellow-ddGTP and then employed in DNA sequencing technologies.[4]

In addition, the 3′-hydroxyl group protected dNTP derivatives have also been synthesized, and explored their ability to terminate the DNA synthesis as like ddNTP (Fig. 1). Though only the unhindered protecting groups show remarkable characters in DNA sequencing as terminators. If 3′-OH protecting groups of dNTPs are linear and softly cleavable with physiological pH condition or UV-light then these dNTPs are being used as reversible terminators. A variety of 3′-OH protected/modified dNTP terminators, reversible or irreversible, have been explored in DNA sequencing under.[5–7] Moreover the sugar ring modified dNTPs as cyclopentane ring containing dNTPs are also explored and found as antiviral characters.[8] Recently pyrrolidine ring containing non-sugar nucleoside derivatives, including PNA, have also been studied.[9–11]

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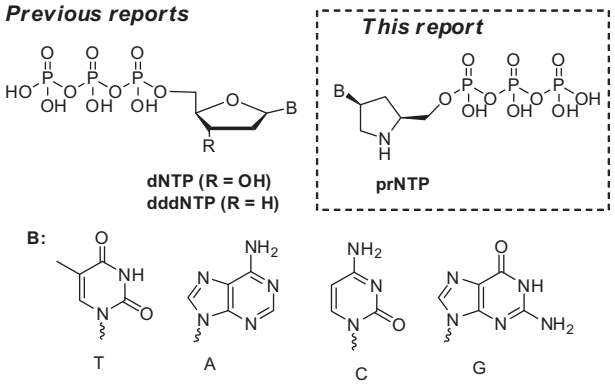


Figure 1. Chemical structure of dNTP, ddNTP and pr-NTP.

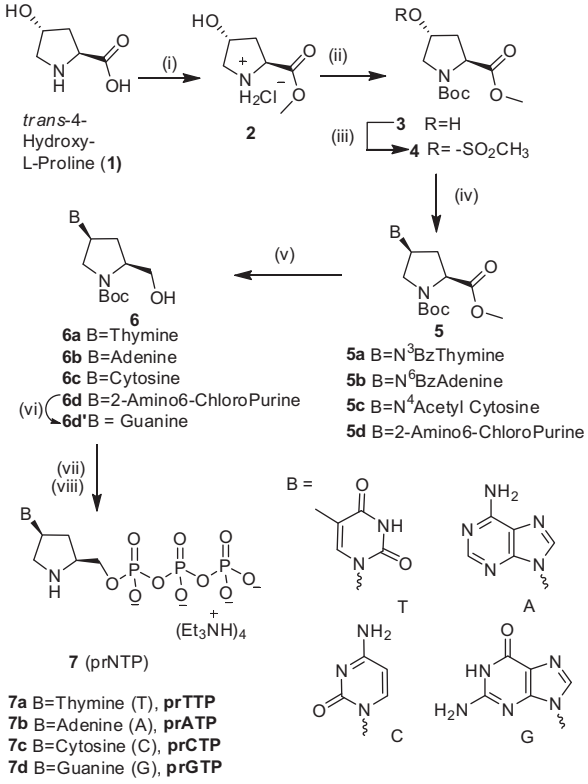
Interestingly, one of such pyrrolidine nucleoside analogue has shown antiviral activities.[12]

Further Metzker and co-workers have also explored unique reversible terminators from dNTPs by hydroxyl functionalization of purine/pyrimidine ring structure with chemical synthesis which contains free 3′-OH.[13,14] Recently, a few other 3′OH modified reversible ddNTP analogues are also reported.[15–18] For an example, Ju and co-workers developed a series chemo labile/photolabile reversible 3′O-modified dNTP analogues for DNA sequencing technologies.[4,19–22]

In repertoire of the labeled ddNTP analogues, the amino functionalized sequencing terminators are in demand to prepare the potential labeled ddNTP analogues without modifying the structure of nucleobase. Benner and co-workers, however, have developed 3′-O-NH_2 containing dNTP analogues and used as reversible terminators.[23,24] Moreover other amino protected analogues such as *acyclic achiral*-ddNTP analogues,[25]/allylic adenine triphosphate are also explored as DNA sequencing terminators.[26] Apart from terminator, other functions of amino functionalized ddNTP analogues are employed such as synthesis of the synthetic lipid membrane channels by conjugation with lipid structural moieties.[27] Thus we hypothesized to prepare the amino functionalized ddNTP analogues without altering nucleobase structure. So far no report is available which contain free-NH/NH_2 at sugar ring along with unmodified nucleobases. We have rationally designed new ddNTP analogue from pyrrolidine nucleoside derivatives as pyrrolidinyl nucleoside triphosphate (prNTP) (Fig. 1). Though the pyrrolidine ring containing DNA analogues as peptide nucleic acid (PNA) analogues are reasonably explored.[28–31] Herein, we report the syntheses of all four derivatives of prNTPs as prTTP/prATP/prCTP/prGTP, and their DNA polymerase activities.

2. Results and discussion

We began the syntheses of all four derivatives of pyrrolidinyl nucleoside triphosphates (prNTPs)-prTTP, prATP, prGTP and prCTP-from 4-hydroxy-L-Proline (**1**) following the synthetic Scheme 1. First of all, the functional groups (–NH– and –COOH) of amino acid (**1**) were protected and converted into pyrrolidine derivatives (**2** & **3**) in two steps by following known procedure.[36] The C4-hydroxyl group of compound **3** was then converted into reactive mesylate pyrrolidine derivative (**4**) for N-alkylation of nucleobases: Adenine, Guanine and cytosine. While N-alkylation of thymine with hydroxyl derivative (**3**) was performed directly from N^{3Bz}Thymine (N^{3Bz}T) under Mitsunobu conditions by following the reported procedure which produced pyrrolidine thymine ester derivative (**5a**) in good yield.[32] For other prolyl nucleosides derivative, the mesylate derivative (**4**) was treated with respective



Scheme 1. Synthesis of pyrrolidinyl nucleoside triphosphate (prNTP). *Reagents and conditions:* (i) $SOCl_2$, MeOH, 0 °C-reflux, 98%; (ii) H_2O:CH_3CN (1:1), TEA, $(Boc)_2O$; (iii) mesylchloride, pyridine, quantitative yield; (iv) N^3Bz-thymine, $(Ph)_3P$, DIAD, THF, 50% (for **5a**)/N^6Bz-adenine, DMF, K_2CO_3, 18-crown-6, 75 °C, 12 h, 65% (for **5b**)/N^4Ac-cytosine, DMF, K_2CO_3, 18-Crown-6, 75 °C, 12 h, 60% (for **5c**)/2-amino-6-chloropurine, DMF, K_2CO_3, 18-Crown-6, 75 °C, 12 h, 50% (for **5d**); (v) $LiBH_4$, THF, 0 °C-rt, 87% (**6a**)/75% (**6b**), 80% (**6c**)/70% (**6d**); (vi) 1.0 N LiOH, dioxane, H_2O, 50%. (vii) (a) salicylchlorophosphidite, dioxane, pyridine; (b) $(((Bu)_3NH)_2H_2P_2O_7^{2-})$ (TBAPP), DMF, TBA; (c) I_2/Py/H_2O; (d) aq NH_4OH. (vii) (a) 1.0 N HCl; (b) aq NH_4OH.

nucleobase derivatives-N^6-benzoyladenine (N^{6Bz}A), N^4-acetyl cytosine (N^{4Ac}C) and 2-amino-6-chloropurine (precursor of Guanine) via N-alkylation. As resultant the other protected pyrrolidine nucleosides ester derivatives: pyrrolidine adenine ester (**5b**), pyrrolidine cytosine ester (**5c**) and pyrrolidine2-amino-6-chloro purine ester (**5d**), were achieved in good yields. All these four nucleosides esters derivative (**5a**–**5d**) were reduced into respective pyrrolidine nucleoside alcohols (**6a**–**6d**) with versatile reducing agent, $LiBH_4$ under anhydrous conditions. The precursor of guanine, 2-amino-6-chloro purine derivative **6d**, converted into respective pyrrolidine guanine alcohol (**6d′**) with LiOH (1.0 N) with good yield. These pyrrolidinyl nucleoside alcohols (**6**) were employed for phosphorylation by following the Eckstein–Ludwig procedure.[33] In this methods, the following reactions and reagent were employed: (i) *cyclic*-triphosphite ester formation with phosphorylating agents-Salicylchlorophosphidite in pyridine and dioxane followed by treatment with bis-tributylammonium pyrophosphate $((Bu)_3NH^+)_2H_2P_2O_7^{2-}$ (TBAPP) in presence of tributylamine in DMF, (ii) oxidation of that cyclic phosphite into cyclic-phosphate with oxidizing agents I_2/Py/H_2O, and (iii) hydrolysis of cyclic triphosphate into linear triphosphate with alkaline hydrolysis. As resultant, the pyrrolidine alcohols (**6a**–**6c** & **6d′**) were converted into respective nucleoside triphosphate derivatives (**7a**–**7d**) with moderate yield after lengthy purification steps: (i) anion exchange chromatography and (ii) HPLC. Moreover, the mechanism of that phosphorylation reaction, for Thymine analogue, is demonstrated by ESI-Mass method (see in SI). The mass results support the formation of cyclic triphosphite intermediate

Table 1
Sequence of primer and template

Entry	DNA Oligo nucleotides
1	Self-priming template (hairpin DNA Oligonucleotide) (**ON1**)5'-GTCA*GCGCCGCGCC-T 3'-CGCGGCGCGG-T
2	Self-priming template (**ON2**) 5'-GACT*GCGCCGCGCC-T 3'-CGCGGCGCGG-T
3	Self-priming template (**ON3**) 5'-ATCG*GCGCCGCGCC-T 3'-CGCGGCGCGG-T
4	Self-priming template (**ON4**) 5'-GATC*GCGCCGCGCC-T 3'-CGCGGCGCGG-T
5	Primer (**P1**): 5'TGTAAAACGACGGCCAGT-3' Template (**T1**):3'ACATTTTGCTGCCGGTCAA*GTCGAGGCAT 5'
6	**FAM-P1**: FAM-5'TGTAAAACGACGGCCAGT-3' Template (**T1**):3'ACATTTTGCTGCCGGTCAA*GTCGAGGCAT 5'
7	**FAM-P1**: FAM-5'TGTAAAACGACGGCCAGT-3' Template (**T2**): 3'ACATTTTGCTGC CGG TCAT*GTCGAGGCAT
8	**FAM-P1**: FAM-5'TGTAAAACGACGGCCAGT-3' Template (**T3**): 3'ACATTTTGCTGC CGG TCAC*GTCGAGGCAT-5'
9	**FAM-P1**: FAM-5'TGTAAAACGACGGCCAGT-3' Template (**T4**): 3'ACATTTTGCTGC CGG TCAG*GTCGAGGCAT-5'

*Incorporation of complimentary **dNTP/ddNTP/pr-NTP** into primer.

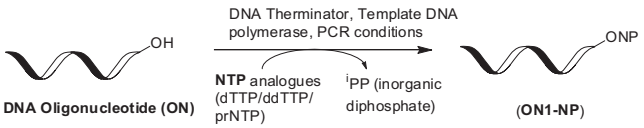
from **6a** and then oxidized into cyclic-triphosphate before hydrolysis of cyclic-phosphoester bond into linear triphosphate with ammonium hydroxide. This triphosphate derivative was further treated with aq. HCl (1.0 N) for removal of N-Boc group. As resultant the desired triphosphate compound as **7a** was prepared with moderate yield. Similarly all other three prNTPs (**7b**–**7d**) were synthesized and purified. Finally all amino functionalized pyrrolidinyl nucleoside triphosphates (prNTP): prTTP (**7a**), prATP (**7b**), prCTP (**7c**), and prGTP (**7d**), were characterized by NMR/Mass. Their analytical data (^{1}H NMR, ^{13}C NMR, ^{31}P NMR, ESI-HRMS and HPLC) provided in Supporting information (SI). For control studies, we purchased all four ddNTP (ddTTP, ddATP, ddCTP, ddGTP) from commercial suppliers.

These four prNTPs (**7a**–**7d**), along with control ddNTP, were examined for polymerase activities by primer extension reaction in presence of DNA primer/template/polymerase enzyme (Table 2). We employed the designed DNA primer/template with DNA polymerases (*Taq*, *Klenowexo*, *Ventexo*, *Bst*, and *Therminator*) in primer extension reaction and analyzed by LC–MS/Gel-electrophoretic methods (Table 1).[34,35] The summary of mass results are given Table 2, while the mass spectral data are provided in Supporting information. The mass value of DNA self-priming template (**ON1**), Table 2 (Entry 1), remained same after incorporation assay of prTTP with *Taq*, *Klenowexo*, *Ventexo*, and *Bst*, unlike natural substrate dTTP with relatively more efficient enzyme, DNA *Therminator* polymerase, the mass value of DNA **ON1** increased after primer extension reaction with prTTP (Table 2, Entry 2) and matched to the mass value of one-prTTP incorporated DNA **ON1**. For control studies, the similar Primer extension reaction was performed with known substrate ddTTP in presence of the same polymerase. The deconvoluted mass spectra of primer (**ON1**), after enzymatic primer extension reaction with prTTP or ddTTP (control), are depicted in Figure 2, while their full range mass spectra are provided in Supporting information.

The molecular mass of DNA (**ON1**) was observed remained same after primer extension reaction with *Therminator* in absence of prTTP/ddNTP (Fig. 2A) and considered as blank (Table 2, entry 1). With prTTP, The molecular mass of DNA (**ON1**) was significantly increased after primer extension reaction of DNA (**ON1**):prTTP with Therminator polymerase (Fig. 2B). That observed mass value is matched with the single prTTP incorporated DNA (**ON1**) molecular mass (Table 2, Entry 2). With ddTTP (control studies), the molecular mass of DNA (**ON1**) was expectedly increased after primer extension reaction of DNA (**ON1**):ddTTP with *Therminator* polymerase (Fig. 2C). That observed mass value is matched with the single ddTTP incorporated DNA (**ON1**) molecular mass (Table 2, Entry 3).

The similar primer extension reactions, with *Therminator* polymerase, were also performed with other prNTP:**DNA** samples: prATP:**ON2**, prCTP:**ON3**, and prGTP:**ON4**, and studied by LC–MS. Their mass results strongly support that one unit incorporation

Table 2
LC–MS studies of DNA primer after primer extension reactions



Entry	DNA primer	Nucleoside triphosphate analogues (**NTP**)	Calculated molecular mass of DNA primer $[M]_{calcd}$	Observed molecular mass of DNA primer $[M]_{obs+}$*	Analysis
1	**ON1**	*None*	7966.181	7961.604	$[M-5H]^-$; No change
2	**ON1**	*prTTP*	8254.379	8250.485	$[M-4H]^-$; One extension
3	**ON1**	*ddTTP*	8252.363	8249.331	$[M-3H]^-$; One extension
4	**ON2**	*none*	7966.181	7961.604	$[M-5H]^-$; No change
5	**ON2**	*prATP*	8262.379	8257.313	$[M-5H]^-$; One extension
6	**ON3**	*none*	7966.181	7961.604	$[M-5H]^-$; No change
7	**ON3**	*prCTP*	8256.451	8250.241	$[M+NH_4-5H]^-$; One extension
8	**ON4**	*none*	7966.181	7961.604	$[M-5H]^-$; No change
9	**ON4**	*prGTP*	8270.160	8275.313	$[M-5H]^-$; One extension
10	**P1**	*none*	5532.676	5528.984	$[M-3H]^-$; No change
11	**P1**& **T1**	*pr-TTP*	5819.743	5816.052	$[M-3H]^-$; One extension
12	**P1**& **T1**	*pr-NTPs*	5819.743	5816.052	$[M-3H]^-$; One extension
13	**P1**& **T1**	*ddTTP*	5820.727	5817.126	$[M-3H]^-$; One extension
14	**P1**& **T1**	*ddNTPs*	5820.727	5817.024	$[M-3H]^-$; One extension

* The mass value extracted from LC–MS data of respective reaction mixture (see in SI).

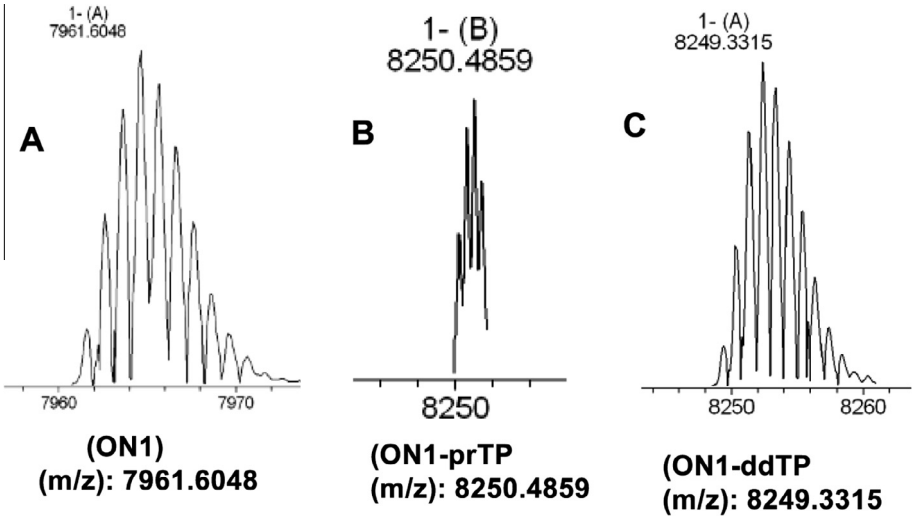


Figure 2. Deconvoluted ESI-Mass (LC–MS) spectra of primer and extended and terminated products with *Therminator* polymerase enzyme:m (A) self-priming template DNA oligonucleotide (**ON1**); (B) extended DNA ON-1 with prTTP; (C) extended DNA **ON1** with ddTTP (control). Full range of LC–MS are given in SI.

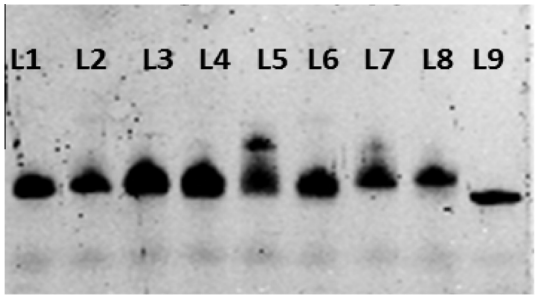


Figure 3. PAGE (20% denaturating Urea) Image of DNA primer (FAMP1) after incorporation assay of prNTP/ddNTP with DNA Therminator polymerase: ddTTP (L1); prTTP (L2); ddATP (L3); prATP (L4); ddGTP (L5); prGTP (L6); ddCTP (L7); prCTP (L8); and Primer 5′-FAM-P1 ((L9).

of prATP/prCTP/prGTP into respective DNA **ON2/ON3/ON4** (Table 2, Entry 4–6). Further, to ensure the incorporation of prTTP (or control ddTTP) into a typical DNA primer (**P1**) guided by template (**T1**), the similar incorporation assay was performed DNA **P1**:**T1** with *Therminator* polymerase and studied by LC–MS (SI). Again, the mass results confirm the single incorporation prTTP into DNA **P1** as like control substrate ddTTP (Table 2, Entry 7–8).

To examine any further incorporation of **prNTP** at free amine group (–NH–) of its pyrrolidine ring, the similar primer extension reactions were performed with all four prNTPs, DNA **P1**, and DNA **T1** with *Therminator* polymerase, and subsequently studied by LC–MS. The mass results support only one-prTTP was incorporated into DNA **P1** (Table 2, Entry 11). In control experiment too, only ddTTP was incorporated into DNA primer **P1** after similar primerextension reactions of control substrate ddNTP, DNA primer/template (**P1/T1**) with *Therminator* polymerase (Table 2, Entry 12). No further incorporation of prNTP was observed after one extension of DNA **P1** as like control substrate ddNTPs. Thus prNTPs are emerged as true analogues of ddNTPs. Furthermore the incorporation of prNTPs into DNA primer was attempted to visualize at PAGE-Gel. First we optimized conditions using **FAMP1/T1** for prTTP along with control ddTTP using *Therminator* polymerase (detailed conditions and gel picture provided in SI) and employed the similar studies for other prNTP and **ddNTP** analogues, **FAM-P1**, *Therminator* polymerase and DNA template (**T1/T2/T3/T4**), employed to incorporate respective **prNTP/ddNTPs** (**TTP/ATP/GTP/CTP**) into labeled primer (**FAM-P1**). Their gel bands are depicted in Figure 3. The gel band of DNA **FAM-P1** (L9) is significantly shifted with control substrates such as ddTTP (L1), ddATP (L3), ddGTP (L5) and ddCTP (L7) after primer extension reactions. The shifting of gel bands of DNA FAM-P1 (L9) with prTTP (L2), prATP (L4), prGTP (L6) and prCTP (L8) are also observed equally to respective control substrates under similar primer extension reactions.

Hence our PAGE analyses also support that **prNTPs** are quantitatively incorporated into primer like **ddNTP** by *Therminator* DNA polymerase.

3. Conclusions

We have successfully synthesized amino functionalized all four prNTPs analogues, and demonstrated their polymerase activities with *Therminator* DNA polymerase by LC–MS/PAGE analyses. Our results confirm the single incorporation of prNTPs into DNA primer in presence of DNA template as like known sequence terminator ddNTPs. The major advantage of prNTP over ddNTP is to prepare the amino functionalized DNA sequence after termination of DNA synthesis with *Therminator* DNA polymerase. The synthesis amino functionalized DNA terminated products are in demand for various reasons including labeling at 3′-DNA after DNA synthesis. Hence prNTP analogues are potential ddNTP analogues for synthesis of amino functionalized DNA oligonucleotide without altering nucleobase structure. Our future works are in progress to synthesize labeled prNTP analogues and employ in DNA sequencing.

4. Experimental

Material and methods All chemicals, solvents and reagents were purchased from commercial suppliers and used as received. DMF distilled over CaH_2 and stored over 4 Å molecular sieves, THF and dioxnae distilled over Na/benzophenone and stored over sodium wire, pyridine distilled over anhydrous potassium hydroxide and stored over CaH_2 and tributyl amine distilled over potassium hydroxide and stored over 4 Å molecular sieves. DNA oligos and FAM labeled primers purchased from IDT. All enzymes and buffers for primer extension reactions were purchased from New England Bio labs.

Compounds **2–4** were synthesized by following literature report.[36]

4.1. (2*S*,4*S*)-4-N^{3}benzoyl thymidyl Boc prolyl methyl ester (5a)

NBoc **1**-hydroxyl proline ester (0.5 g, 2.04 mmol) was dissolved in anhydrous tetrahydrofuran (10 ml) and stirred under nitrogen atmosphere for 10 min, N^{3}benzoyl thymine (0.47 g, 2.04 mmol) and triphenyl phosphine (0.642 g, 2.448 mmol) were added and the resulting suspension stirred at 0 °C for 10 min under nitrogen atmosphere. Diisopropyl azo dicarboxylate (0.485 ml, 2.44 mmol) was added drop wise at 0 °C and the reaction was warmed to stir at room temperature over night. The solvents were evaporated and the crude reaction mixture purified by column chromatography using methanol and dichloromethane on silica gel to obtain 466 mg of title compound as white foam in 50% yield. ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) (9H, s) 1.44, (3H, s) 1.96, (1H, m, br) 2.14, (1H, m, br) 2.76, (1H, m) 3.55–.367, (3H, s) 3.78, (1H, t J = 8 Hz) 3.95, (1H, m, br) 4.33–4.39, (1H, m.br) 5.21, (1H, s) 7.37, (2H, m) 7.46–7.50, (1H, t, J = 8 Hz) 7.64, (1H, d, J = 8 Hz. ^{13}C NMR ($CDCl_3$, 100 MHz); δ (ppm) 12.76, 28.31, 35.69, 49.38, 52.63, 57.98, 70.13, 81.39, 111.72, 129.27, 130.56, 131.59, 132.50, 135.20, 136.15, 150.06, 162.54, 168.93, 173.20. HRMS (ESI-TOF): calcd for [$C_{23}H_{27}N_3O_7$Na, M+Na] m/z 480.1741 found m/z 480.1743.

4.2. General procedure for nucleosidation

Mesyl compound (1.0 g, 3.07 mmol), one of the protected nucleobases (N^{6}benzoyl adenine or N^{4}acetyl cytosine or 2-amino 6-chloropurine (1.0 equiv)), anhydrous potassium carbonate (0.636 g, 4.6 mmol) and a catalytic amount of 18-crown-6 (0.162 g, 0.614 mmol, 0.2 equiv, 0.5 equiv in case of 2-amino 6-chloro purine) were stirred at 75 °C for 12 h in 20 ml of anhydrous DMF under nitrogen atmosphere. After completion of reaction indicated by TLC, DMF removed in rota vapor under reduced pressure and purified on silica gel using dichloromethane and methanol.

4.3. Adenine methyl ester (5b)

0.932 g of white solid obtained in 65% yield. ^{1}H NMR 400 MHz$CDCl_3$: (9H, s) 1.46, (1H, m, br) 2.55, (1H, m) 2.97–2.99, (3H, s) 3.71, (1H, m) 3.94–4.07, (1H, m) 4.18–4.22, (1H, m) 4.45–4.52, (1H, m) 5.26, (2H, t J = 8 Hz) 7.53, (1H, m) 7.60–7.63, (1H, d, J = 8 Hz) 8.03, (1H, s) 8.21, (1H, s) 8.79, (1H, s, br) 9.05. ^{13}C NMR 100 MHz $CDCl_3$: 28.23, 35.09, 36.16, 50.12, 50.92, 52.12, 52.45, 57.61, 81.26, 123.18, 128.01, 128.79, 132.76, 133.65, 141.18, 149.76, 152.00, 152.53, 153.31, 164.97, 172.34. HRMS (ESI-TOF): calcd for [$C_{23}H_{26}N_6O_5$ (M+H)] m/z 467.2037, found m/z 467.2044.

4.4. Cytosine methylester (5c)

0.700 g of cytosine ester obtained as white solid in 60% yield. ^{1}H NMR (400 MHz, $CDCl_3$): (9H, s) 1.38 ma, 1.42 mi, (3H, s) 2.18, (1H, m) 2.34–2.37, (2H, m), 2.56–2.61, (3H, s) 3.65 mi, 3.67 ma, (1H, m) 4.35–4.38 ma, 4.57–4.60 mi, (1H, m) 5.35–5.41, (1H, m) 7.74–7.76, (1H, m) 8.29–8.32, (1H, s, br) 8.89 ma, 8.95 mi. ^{13}C NMR (100 MHz, $CDCl_3$): 24.71, 28.29, 28.42, 35.45, 36.18, 51.77, 52.25, 52.39, 57.54, 57.82, 74.10, 75.33, 80.36, 80.42, 104.2, 114.10, 153.87, 154.38, 159.40, 160.30, 163.51, 170.08, 172.60. HRMS: calcd for [$C_{17}H_{24}N_4O_6$ (M+H)] m/z 381.1769 found m/z 381.1743.

4.5. (2*S*,4*S*) 2-Amino 6-chloro purine ester (5d)

0.609 g of obtained as white solid in 50% yield. ^{1}H NMR (400 MHz, $CDCl_3$): (9H, s) 1.43, (1H, m, br) 2.48, (1H, m, br) 2.86, (3H, s) 3.72, (1H, m) 3.77–3.91, (1H, m) 4.09–4.15, (1H, m) 4.40–4.47, (1H, m) 4.96–7.97, (1H, m, br) 5.23–5.28, (1H, s) 7.88. ^{13}C NMR (100 MHz, $CDCl_3$): 28.31, 34.87, 35.81, 49.94, 50.49, 51.98, 52.52, 57.41, 57.70, 81.28, 125.50, 140.29, 151.65, 153.36, 153.81, 159.10, 159.16, 172.38. HRMS, (ESI-TOF): calcd for [$C_{16}H_{21}N_6O_4Cl$ (M+H)] m/z 397.1386, found m/z 397.1341.

4.6. (2*S*,4*S*)-N-Boc-4-thyminyl prolinol (6a)

Anhydrous THF (10 ml) was added to $LiBH_4$ (0.038 g, 1.7 mmol) and cooled to 0 °C and stirred under nitrogen atmosphere for 15 min followed by addition of compound **5a** (0.2 g, 0.437 mmol dissolved in tetrahydrofuran) at 0 °C under nitrogen atmosphere, the reaction was warmed to room temperature, and then stirred over night at room temperature. After completion the reaction was quenched with ammonium chloride solution and the reaction mixture concentrated to dryness and the residue dissolved in water, extracted with ethylacetate. Ethylacetate layer washed with water and brine solution, dried with anhydrous sodium sulfate and concentrated followed by purification on silica gel using dichloromethane and methanol afforded 123 mg of alcohol **6a** as white solid in 87% yield. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) (s, 9H) 1.47, (s, 3H) 1.94, (m, 1H) 1.98–2.0, (m, 1H) 2.39–2.46, (m, 1H) 3.23–3.28, (m, 1H) 3.65–3.69, (m, 1H) 3.89–3.92, (m, 2H) 3.99–4.01, (s, br 1H) 4.30, (m, 1H) 5.02–5.11, (s, 1H) 7.13, (s, 1H) 8.73. ^{13}C NMR ($CDCl_3$, 100 MHz); δ (ppm) 12.94, 28.22, 32.03, 31.67, 49.75, 51.40, 58.32, 66.63, 81.21, 112.03, 135.49, 151.06, 163.74. HRMS, (ESI-TOF): calcd for [$C_{15}H_{23}N_3O_5$Na (M+Na)] m/z 348.1530 found m/z 348.1535.

4.7. Adeninealcohol (6b)

0.5 g of N^{6}benzoyl adenine methyl ester was reduced to Adenine alcohol by following procedure as described for the synthesis of compound **6a**. 0.284 g of 5b obtained as white powder in 75% yield. ^{1}H NMR (400 MHz, $CDCl_3$) (9H, s) 1.43, (1H, m) 2.30–2.41, (1H, m, br) 2.65, (2H, m) 3.63–3.72, (1H, m, br) 3.89, (3H, m) 4.1–4.18, (1H, t J = 8 Hz) 5.0, (2H, s, br) 6.55, (1H, s) 7.93, (1H, s) 8.26. ^{13}C NMR (100 MHz, $CDCl_3$): 28.47, 33.56, 51.34, 52.22, 58.91, 66.30, 81.02, 114.34, 119.77, 124.05, 138.76, 139.34, 149.85, 152.88, 155.92, HRMS, ESI-TOF calcd for [$C_{15}H_{22}N_6O_3$ (M+H)] m/z 335.1826, found m/z 335.1873.

4.8. Cytosine alcohol (6c)

0.5 g of N^{4}acetyl cytosine methyl ester was reduced to cytosine alcohol by following procedure as described for the synthesis of compound **6a**. 0.326 g of **5c** obtained as white powder in 80% yield. ^{1}H NMR (400 MHz, $CDCl_3$): (9H, s) 1.43, (2H, m) 1.95–2.04, (1H, s) 2.35–2.36, (1H, s) 3.53–3.56, (2H, s) 3.63–3.72, (1H, s) 3.89–3.94, (1H, s) 4.06–4.12, (1H, s, br) 5.36, (2H, s, br) 5.49, (1H, d J = 4 Hz) 6.08–6.09, (1H, d J = 4 Hz) 7.92–7.93. ^{13}C NMR (100 MHz, $CDCl_3$): 28.5, 34.37, 53.33, 59.54, 60.50, 67.50, 74.46, 80.70, 100.09, 156.82, 157.04, 164.17, 165.15, 17.31, 175.20. HRMS, (ESI-TOF) calcd for [$C_{14}H_{24}N_4O_4$ (M+H)] m/z 311.1714 found m/z 311.1726.

4.9. (2*S*,4*S*)2-Amino 6-chloro purine alcohol (6d)

0.5 g of 2-amino 6-chloro purine ester was reduced to 2-amino 6-chloro purine alcohol by following procedure as described for the synthesis of compound **6a**. 0.326 g of compound **6d** obtained in 70% yield as a white foam. ^{1}H NMR (400 MHz, $CDCl_3$ (9H, s) 1.45, (1H, m) 2.02–2.07, (1H, m) 2.30–2.37, (1H, m) 2.60–2.65, (1H, m) 3.62–3.66, (1H, m) 3.71–3.75, (1H, m) 3.85–3.87, (2H, m) 4.08–4.10, (1H, m) 4.82–4.90, (2H, s, br) 5.39, (1H, s) 7.87 ^{13}C NMR 100 MHz $CDCl_3$: 28.47, 31.99, 33.25, 50.77, 52.22, 59.04, 62.71,

66.07, 81.33, 99.04, 114.15, 125.53, 139.37, 140.67, 151.72, 153.68, 159.06, 174.45. HRMS: calcd for [$C_{15}H_{21}N_6O_3Cl$ (M+H)] *m*/*z* 369.1436 found *m*/*z* 369.1406.

4.10. (2*S*,4*S*) 2Amino 6 oxo purine alcohol (6d′)

0.2 g of (2*S*,4*S*) 2-amino 6-chloro purinyl prolyl alcohol (**6d**) was dissolved in 4 ml of 1,4 dioxane and 4 ml of 1 N LiOH solution was added and stirred at room temperature over night solvents were removed under reduced pressure and the reaction mixture dried in roto followed by purification on silicagel obtained 0.095 g of title compound as white powder in 50% yield. ^{1}H NMR (400 MHz, CD_3-OD): (9H, s) 1.49, (1H, m) 2.01–2.13, (1H, m) 2.55–2.61, (1H, m) 3.19–3.24, (1H, m) 3.57, (2H, m) 3.72–3.81, (1H, m) 4.00, (1H, m) 4.16, (1H, s) 7.89. ^{13}C NMR (100 MHz, CD_3OD): 28.69, 30.73, 33.06, 34.88, 53.40, 59.14, 63.90, 81.69, 114.68, 128.54, 128.79, 129.03, 140.14, 155.22. HRMS, ESI-TOF calcd for [$C_{15}H_{22}N_6O_4$ (M +H)] *m*/*z* 351.1775 found *m*/*z* 351.1771.

4.11. (2*S*,4*S*)-Pyrrolidine thymidine triphosphate (prTTP) (7a)

Triphosphorylation was performed by following Eckstein–Ludwig's procedure.[33]

(2*S*,4*S*) N-Boc-4-thyminyl prolinol (**6a**) (0.035 g, 0.107 mmol) dried by co-evaporation using anhydrous pyridine, followed by drying under high vacuum over P_2O_5 in a vacuum desiccator for one hour at ambient temperature. Desiccator opened under nitrogen atmosphere and round bottom flask closed with a septum and a nitrogen balloon attached. Alcohol **6a** dissolved in 0.5 ml anhydrous pyridine and 1.0 ml anhydrous dioxane. Salicylchlorophosphidite (0.023 g, 0.117 mmol) dissolved in anhydrous dioxane (1.0 ml) was added and stirred at room temperature. After 10 min a well vertexed mixture of tri-butylammonium pyrophosphate (0.088 g, 0.161 mmol) and tri-butylamine (0.177 ml, 0.749 mmol) in anhydrous dimethylformamide (DMF) (1 ml) added. After 10 min, Iodine solution (1% in pyridine/water 98/2) added till the brown color remained. After 10 min concentrated ammonia (5 ml) was then added and reaction was monitored for complete hydrolysis of cyclic triphosphate. Hydrolysis step was monitored by mass spectrometry. After 3 h complete hydrolysis of cyclic triphosphate was observed, then solvents were lyophilized, the residue again dissolved in 10 ml distilled water and then washed with ethyl acetate (15 ml × 3). The aqueous layer was loaded to anion-exchange column (DEAE Sephadex A-25) column, and then eluted with triethyl ammonium bicarbonate (TEAB) buffer (50–1000 mM). Compound was identified by UV spectroscopy and mass spectrometry. Again compound was dissolved in 5.0 ml Milli-Q-water, cooled to 0 °C before adding 0.5 ml (11.0 N HCl) and warmed to room temperature to remove Boc group. After one hour complete deprotection was observed, and then quenched with dilute ammonia at 0 °C. This solution was further lyophilized and purified by HPLC to obtain the pure compound **7a** in form of triethylammonium salt. ^{1}H NMR (D_2O, 400 MHz): δ (ppm) 1.89 (s, 3H), 2.23–2.31 (m, 1H), 2.62–2.70 (m, 1H), 3.66–3.73 (m, 2H), 4.04–4.06 (m, 1H), 4.21–4.28 (m, 1H), 4.41–4.43 (m, 1H), 5.04–5.08 (m, 1H), 7.56 (s, 1H). ^{31}P NMR (162 MHz, H_3PO_4); as external standard at δ (0.0), δ (ppm) 6.95 (d, J = 19.44 Hz, 1P, P_γ), 10.65 (d, $J_{\beta\gamma}$ = 21.0 Hz, 1P, P_α), 21.32 (t, 1P, P_β) $J_{\alpha\beta}$ = 19.5 Hz, $J_{\beta\gamma}$ = 21.06 Hz), HRMS (ESI-TOF) calcd for [$C_{10}H_{17}N_3O_{12}P_3$ (M−H)] *m*/*z* 464.0020, found *m*/*z* 464.0037.

prATP (**7b**), prGTP (**7c**) and prCTP (**7d**) (all 100 μmol scale) were also synthesized by following the above procedure.

4.12. Adenine triphosphate (7b)

^{1}H NMR (400 MHz, D_2O): (1H, m) 1.79, (1H, m) 2.39–2.44, (2H, m) 2.91–3.11, (1H, m) 3.82–3.96, (1H, m) 4.11–4.21, (1H, m) 4.31–4.62, (1H, m) 5.46–5.51, (1H, s) 8.29, (1H, s) 8.32. ^{31}P NMR (162 MHz, D_2O): (−21.11, t, 1P, P_β), (−10.52, −10.64, d, 1P, P_γ), (−5.32, −5.50, d, 1P, P_α) ($J_{\alpha\beta}$ = 29.16 Hz, $J_{\beta\gamma}$ = 19.44 Hz) HRMS, ESI-TOF calcd for [$C_{10}H_{16}N_6O_{10}P_3$ (M−H)] *m*/*z* 473.0135, found *m*/*z* 473.0144.

4.13. Cytosine triphosphate (7c)

^{1}H NMR (400 MHz, D_2O): (1H, m) 2.15–2.18, (1H, m) 2.66–2.74, (1H, m) 3.57–3.62, (1H, m) 3.73–3.76, (3H, m) 4.10–4.30, (1H, s, br) 5.69, (1H, d J = 8 Hz) 6.36–6.38, (1H, d J = 4 Hz) 7.90–7.91. ^{31}P NMR (162 MHz, D_2O): (−21.87, t 1P, P_β), (−11.01–11.03 1P, P_α) (−7.59–7.72, 1P, P_γ), HRMS: calcd for [$C_9H_{16}N_4O_{11}P_3$ (M−H)] *m*/*z* 449.0023 found *m*/*z* 449.0095.

4.14. Guanine triphosphate (7d)

^{1}H NMR (400 MHz, D_2O): (1H, m) 2.20–2.28, (1H, m) 2.86–2.94, (1H, m) 3.76–3.90, (1H, m) 4.07–4.08, (2H, m) 4.38–4.42, (1H, m) 5.34–5.38, (1H, s) 7.78 ^{31}P NMR (162 MHz, D_2O): (−21.17, t, 1P, P_β), (−10.63, −10.76, d, 1P, P_α) (−0.32, −0.44P, P_γ) ($J_{\alpha\beta}$ = 21.06 Hz, $J_{\beta\gamma}$ = 19.44 Hz) HRMS, (ESI-TOF) calcd for [$C_{10}H_{15}N_6O_{11}P_3$ (M−2H)] *m*/*z* 488.0006 found *m*/*z* 488.0079.

4.15. Primer extension reactions and LC–MS procedures

4.15.1. Primer extension experiments for LC–MS analysis

For all enzymatic reactions with TTP analougues self priming DNA oligomer (ON-1). 5′-GTC AGC GCC GCG CCT TGG CGC GGC GC-3′ used.

4.15.2. Ventexo—DNA polymerase

Self priming DNA template (5 μM), **dTTP** or **prTTP** (100 μM), 10× thermopol buffer (2.5 μl) and Ventexo DNA polymerase (1 unit) in 25 μl reaction mixture incubated at 60 °C for 60 min followed by LC–MS analysis.

4.15.3. Klenowexo

Self priming DNA template (5 μM), **dTTP** or prTTP (100 μM), 10× NEB2 buffer (2.5 μl) and Klenowexo DNA polymerase (25 units) in 25 μl reaction mixture incubated at 37 °C for 60 min followed by LC–MS analysis.

4.15.4. Taq DNA polymerase

Self priming DNA template (5 μM), dTTP or prTTP(100 μM), 10× standard Taq buffer (2.5 μl) and Taq DNA polymerase (10 units) in 25 μl reaction mixture incubated at 72 °C for 60 min followed by LC–MS analysis.

4.15.5. Bst DNA polymerase

Self priming DNA template (5 μM), dTTP or pr-TTP (100 μM), 10× Thermopol buffer (2.5 μl) and Bst DNA polymerase (16 units) in 25 μl reaction mixture incubated at 65 °C for 60 min followed by LC–MS analysis. Boricacid and EDTA pH 8.0) buffer and run in 1× TBE at 90 V for 3 h. Images recorded using Bio Rad Gel doc system.

4.15.6. Therminator DNA polymerase

Self priming DNA oligomer (**ON-1**) or **FAM-ON1** (5 μM), ddTTP or prTTP (100 μM), 10× thermopol buffer (2.5 μl) and *Therminator* DNA polymerase (2 units) in 25 μl reaction mixture incubated at 72 °C for 60 min followed by LC–MS analysis or denaturating PAGE analysis.

For primer extension reaction of other prNTPs following self-priming DNA templates were used**:** prTTP: **ON1**; **prATP**:**ON2**; prCTP:**ON3**; prGTP:**ON4**.

Gel experiments were performed using 5′FAM labeled primer (FAM-P1) in combination with suitable template for each **NTP** (prNTP/ddNTP).

A reaction mixture containing 5′FAM labeled Primer **P1 FAM P1** (1 μmol) and suitable template (1 μmol) **T1** for **prTTP** and ddTTP, **T2** for prATP and ddATP, **T3** for prGTP and ddGTP, **T4** for prCTP and ddCTP, 10× Thermo pol buffer (1.0 μl), NTPs (prNTP or ddNTP) (100 μmol) and 1 unit of *Therminator* polymerase in total volume of 10 μl were incubated at 55 °C for 30 min and subsequently reactions were quenched with 10 μl of 2× loading dye (contains 90% formamide, 0.5% EDTA, 0.1% xylene cyanol and 0.1% bromphenol blue) by heating at 85 °C for 5 min followed by cooling at 4 °C. Primer extension reactions were analyzed by 20% denaturating urea PAGE. Gels prepared and ran in 1× TBE (Tris Boricacid EDTA, pH 8.0) buffer.

4.15.7. LC–MS experiments

LC–MS experiments performed using BrukermicroTOF-Q II coupled with a Waters Acquity UPLC auto sampler. primerextension reaction products diluted with 25 μl Milli-Q water and 10.0 μl from this mixture injected by auto sampler.

LC–MS experiments performed using 5 mM ammoniumacetate (bufferA pH 7.0) and methanol (buffer B) with the following programme at a flow rate 0.2 ml/min linear gradient. 0–10 min 80% A gradient, 10–12 min 30% A gradient, at 12–13 min 30% A isocratic, 13–14 min 100% A gradient, at 14–15 min 100% A isocratic. A C-18 RP UPLC column (2.1 × 5.0 mm, 1.7 μm, Acquity Waters) used for LC–MS experiments. Column temperature maintained at 30 °C during the experiments.

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Supplementary data

Supplementary data (the NMR (^{1}H $^{13}C/^{31}P$ NMR), HRMS of compounds (**2**–**7**), HPLC chromatogram, LC–MS data of primer extension reaction products, and PAGE-image) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2016.06.043.

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