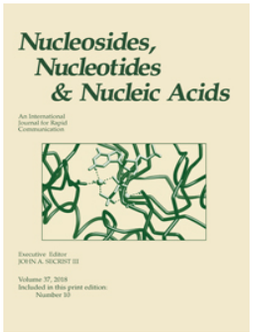

Nucleosides, Nucleotides and Nucleic Acids

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Synthesis and biochemical evaluation of *Aminopropanolyl*-Thymine *tri*-Phosphate (*ap*-TTP)

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ABSTRACT

Deoxyribonucleoside triphosphates (dNTPs) are building blocks for the biosynthesis of DNA. Various modified dNTPs' analogs have synthesized by structural changes of nucleoside's susgar and nucleobases and employed for synthesis of modified DNA. A very few modified dNTPs have prepared from non-sugar nucleoside analogs. This report describes the synthesis of acyclic nucleoside triphosphate (NTP) analog from amino acid L-Serine as aminopropanolyl-thymine triphosphate (*ap*-TTP) and demonstrate its biochemical evaluation as enzymatic incorporation of *ap*-TTP into DNA with DNA polymerases with primer extension methods. Alanyl peptide nucleicacids (Ala-PNA) are the analogs of DNA which contains alanyl backbone. Aminopropanolyl – analogs are derivatives of alanyl back bone. Ap-TTP analog is nucleoside triphosphate analog derived from Ala-PNA. Importantly, this report also sheds light on the crystal packing arrangement of *alaninyl* thymine ester derivative in solid-state and reveals the formation of self-duplex assembly in *anti*-parallel fashion via reverse Watson-Crick hydrogen bonding and π–π interactions. Hence, *ap*-TTP is a useful analog which also generates the free amine functional group at the terminal of DNA oligonucleotide after incorporation.

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Aminopropanolyl-thymine triphosphate; DNA polymerase; primer extension reaction; LC-MS; gel electrophoresis

Introduction

Deoxyribonucleotide triphosphates (dNTPs) such as dTTP, dATP, dCTP & dGTP are building blocks of deoxyribose nucleic acid (DNA) biosynthesis (Figure 1).[1] Since 1953, after discovery of DNA duplex structure, structure of dNTPs are modified to tune the functional and structural properties of DNA.[2–4] As resultant, various kind of modified dNTPs and their respective DNA analogs are accomplished, and a few of them have shown astonishing applications. There are two practical methods to modify the structure of

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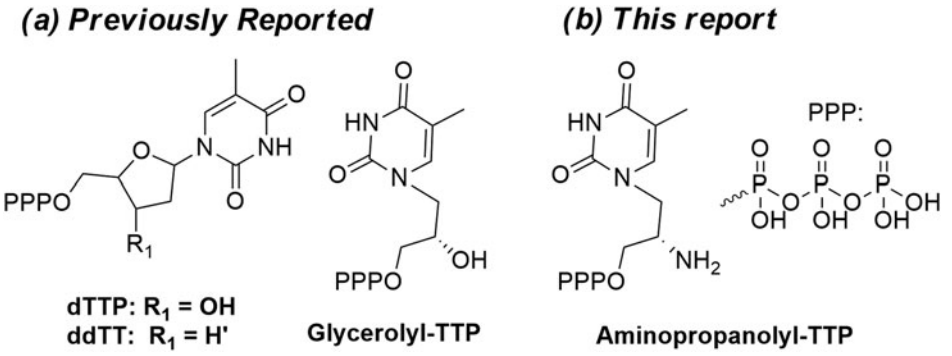
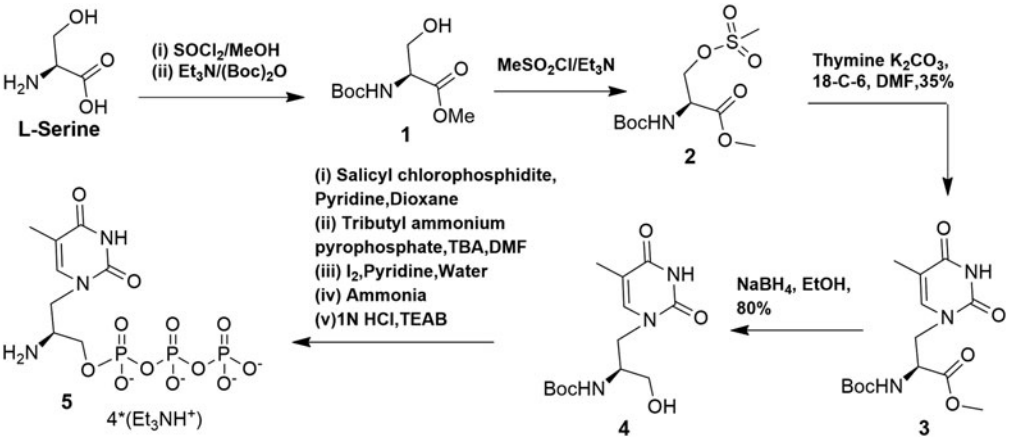


Figure 1. Structure of dTTP/ddTTP, glycerol TTP and *Aminopropanolyl*-TTP

DNA: (a) enzymatic incorporation of modified dNTPs into DNA primer with DNA ploymerase, and (b) chemical incorporation of modified nucleoside phosphoramidites at DNA synthesizer. The chemically modified dNTPs are prepared by structural changes of sugar ring/nucleobases residues in target-specific nucleosides, and incorporated into sequence-specific DNA primer for various motives.[5–10] For an example, glycerol nucleoside triphosphate (gNTP), non-deoxyribose ring acyclic dNTP, is substrate of DNA polymerase and successfully incorporated only at the terminal of DNA primer, however, the further extension of gNTP incorporated DNA primer are reportedly unsuccessful.[11,12] It has been explained that the failure of multiple incorporations of gNTP into DNA primer is possibly due to following reasons: (i) The loss of contact between the single incorporated DNA primer-template complex with polymerase enzyme; (2) The disturbance of gNTP's hydroxyl group alignment for nucleophilic attack at phosphate group of next gNTP (or dNTP) because of smaller size, greater flexibility, or increased rotational freedom; (ii) destabilization duplex structure after incorporation of gNTP. Further, the conformationally constrained synthetic cyclic analog of *g*NTP as threose nucleoside triphosphate (tNTP) has been prepared which are also substrate of DNA polymerase.[13] Another modified nucleoside analog as 2′-amino threose nucleoside, bearing activated monophosphate ester, is capable for template guided extension on primer without DNA polymerase.[14] Similarly, 5′-amino and 3′-activated monophosphate esters are also proficient for the template guided DNA synthesis without DNA polymerase enzyme.[15,16] To explore the DNA duplex/triplex structure stabilizing candidate, various the backbone-modified nucleic acid analogs are prepared and studied. For example, peptide nucleic acid (PNA) is a potential DNA analog that has acyclic and achiral backbone, aminoethyl glycinyl (*aeg*), in place of sugar-phosphate backbone of native DNA. Nevertheless, PNA has considered as gene-based therapeutic drug candidates.[17] The synthesis of *aeg*-PNA oligomers, now, are customized using solid phase synthesis methods. Importantly, the



Scheme 1. Synthesis of AminopropanolylThymine triphosphates (*ap*-TTP).

triphosphate derivatives of *aeg*-PNA are also synthesized and incorporated into the DNA primer with the help of DNA polymerase.[18,19] Further the achiral *aeg*-backbone of PNA is also modified by replacing with another amino acid. For example, *alanyl*-PNA, bearing alanine amino acid residue, is emerged as a potential PNA analog and studied the formation of stable duplex, triplex, and tetraplex (*i-motif*) PNA:DNA (or PNA:PNA) structures.[20–22]

These reports inspired us to design the amino-functionalized dNTP analogs from naturally ocurring chiral amino acids, and to demonstrate their substrate activity with DNA polymerase. Recently, we have reported the amino functionalized ddNTP analogs as *pr*-NTP from amino acid L-Proline, and found that *pr*-NTPs are the substrate of *Therminator* DNA polymerase.[23] We, herein, describe the synthesis of rationally designed amino-functionalized aminopropanolyl-Thymine triphosphate (*ap*-TTP) from naturally occuring L-Serine amino acid and study their enzymatic incorporations into DNA primer with DNA polymerase by primer extension reactions (PExR). The results of our studies would be significant for the generation of amino-functionalized DNA primer.

Results and discussion

Synthesis of ap-TTP. We derivatized L-Serine into *N*-Boc-Serine methylate (**1**), and converted into another reactive intermediate (**2**) as *O*-mesylate compound (Scheme 1). The compound (**2**) was treated with nucleobase Thymine (T) for *N*-alkylation under basic conditions which produced chiral *alanyl*-PNA monomer as *N*-Boc-alanyl thymine methyl ester derivative (**3**). The ester group of *N*-Boc-alanyl thymine ester (**3**) was reduced into hydroxyl group as nucleoside derivative (**4**) with versatile reducing agents

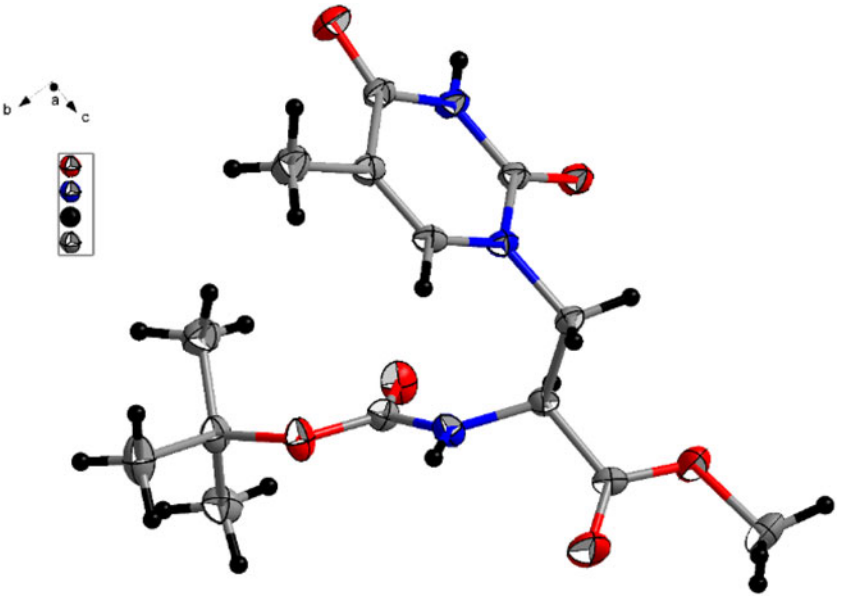


Figure 2. ORTEP diagram and Phase data of *N*-Boc-alanyl thymine methyl ester (**3**): Space-group P 1 21/c 1 (14) – monoclinic; Cell $a = 5.2456(3)$ Å, $b = 26.0994(15)$ Å, $c = 12.1838(7)$ Å $\beta = 99.467(4)^{\circ}$; $V = 1645.33(16)$ Å^3; $Z = 4$.

($NaBH_4$). The hydroxyl group of nucleoside (**4**) was phosphorylated with phosphorylating reagents by following the Ludwig-Eckstein method.[24] Finally, the desired product *aminopropanolyl* thymine triphosphate (**5**) was isolated after purification by HPLC. The synthetic derivatives (**1–4**) and desired compound *ap*-TTP (**5**) are characterized by NMR (^{1}H/^{13}C/^{31}P), and HRMS techniques, their spectral data are provided in Supplemental File.

Structural studies of N-Boc-alanyl thymine Ester (3). We crystalized *N*-Boc-alanyl thymine ester (**3**) and obtained its single crystal. Our X-ray diffractometer studies have confirmed the chemical structure of the compound (**3**), and X-ray data are deposited to Cambridge crystallographic data center (CCDC) with reference number CCDC 1568315. The ORTEP diagram of compound **3** is depicted in Figure 2.

Since DNA/RNA nucleobases have strong abilities to exhibit non-covalent interactions such as hydrogen bonding and π–π interactions. Thus we extracted the crystal packing arrangement of compound (**3**) using software (Diamond 3.2) to explore the supramolecular self-assembly structure (Figure 3). The packing arrangement of thymine ester (**3**) (along c-axis) exhibits the formation of Thymine-Thymine dimer via hydrogen bonding and π–π interactions. The N^3-H (Thymine residue) of compound **3** is hydrogen bonded with $O{=}C^2$ (another thymine residue) of the same compound as two N^3-H—$O{=}C^2$ at the distance ∼2.0 Å. Importantly, these hydrogen bond interactions are reversely different from natural DNA Watson-Crick hydrogen bonding (N^3-H—$O{=}C^4$). In literature, such type

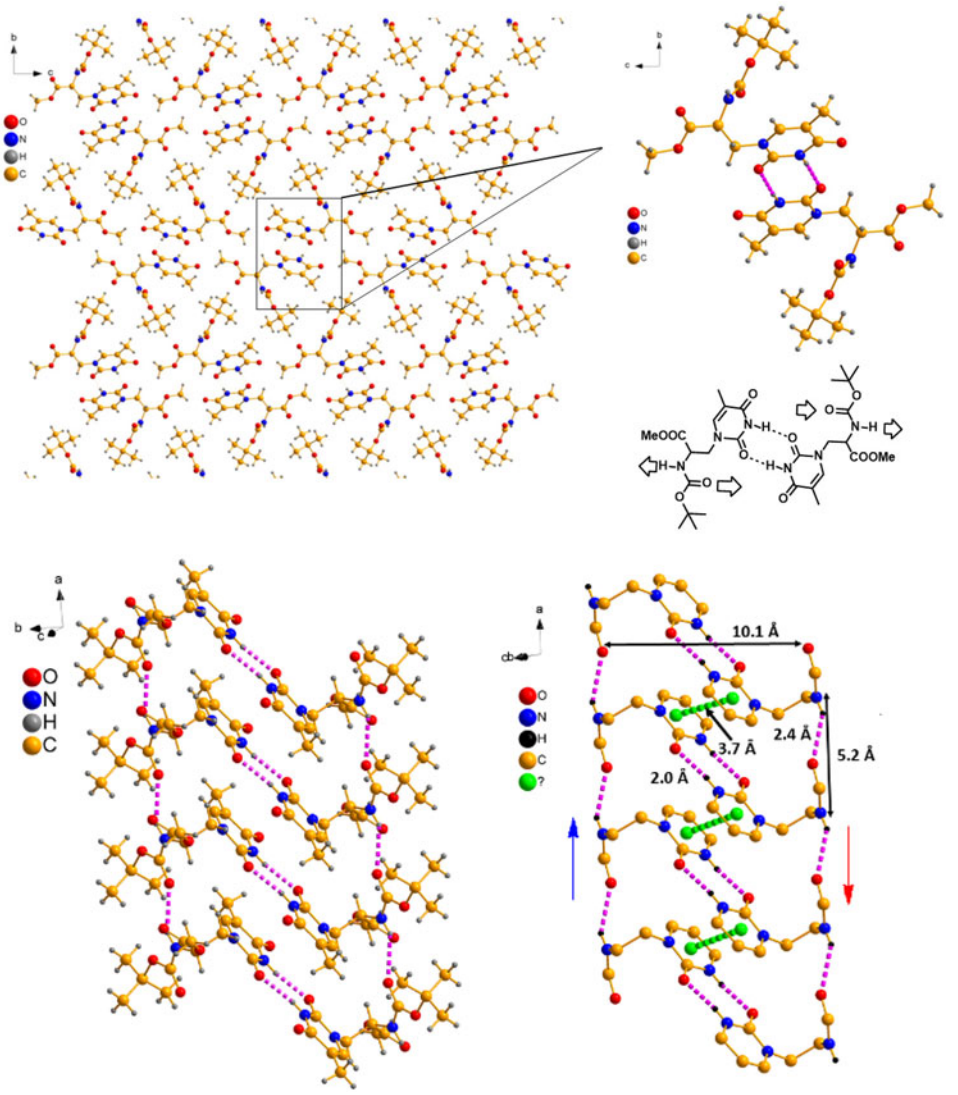


Figure 3. Structural studies of single crystal of *N*-Boc-alanyl thymine methyl ester (**3**) by X-analyse: (a) Packing diagram (top left); (b) diagram of selected region (top right); (c) Hydrogen bonding pattern (bottom left) and (d) Detail of distance for hydrogen bonding and π–π interaction (Bottom right).

of hydrogen bonding, also called *reverse* Watson-Crick hydrogen bonding, are reported in the supramolecular assemblies structure of ferrocene linked thymine/uracil conjugated compounds.[25,26] Generally, Watson-Crick and Hoogsteen types of hydrogen bonding are most common in nucleobase supramolecular self-assembly structure by modified nucleosides.[27–31] Recently we reported the formation of helical supramolecular self-assembly by prolamide thymidine/uridine analogs.[32] Besides, the hydrogen bonding, the side view of compound **3**'s packing diagram shows the formation of the

anti-parallel self-duplex type of structure. The strand of that duplex structure is formed by hydrogen bonding between C = O and N–H of two adjacent Boc residue of compound **3** as C = O—H–N at a distance 2.4 Å. Also, unique π–π interactions are noticed at a distance 3.7 Å, between two thymine residues such as thymine of one stand with the thymine of another. We also extracted the diameter of the self-assembled duplex as 10 Å, and pitch value 5.2 Å (Figure 3). Overall, non-covalent interactions firmly stabilized the self-duplex structure of aminopropanol thymine ester (**3**) in the solid-state crystal (Figure 3, bottom right).

*Biochemical Evaluation of ap-TTP (**5**).* After structural studies of protected *ap*-nucleoside ester, its triphosphate was attempted to incorporate into DNA primer using a primer extension reaction method. For comparative studies, we used known substrates of DNA polymerase (dTTP & ddTTP) as control studies with the various commercially available DNA polymerases: *Taq, Vent exo⁻, Deepvent exo⁻, Bst, and Therminator*. The DNA primers (**P1/FAM-P1)** and DNA Templates (**T1/T2**) were used for incorporation of *ap*-TTP/control templates are following

Primers: (**P1**) 5′-TGTAAAACGACGGCCAGT-3′;

(**Fam-P1**)Fam-5′TGTAAAACGACGGCCAGT-3′

Template (the underlined sequence is complementary to the primers):

(**T1**) 3′ACATTTTGCTGCCGGTCAA*GTCGAGGCAT-5’

(**T2**) 3′ACATTTTGCTGCCGGTCAAAAAAAAAAAA-5

For the gel-electrophoretic studies, the FAM-labeled primer (5′**Fam-P1: FAM**-5′TGTAAAACGACGGCCAGT-3′) was treated with *ap*-TTP in the presence of DNA polymerase and DNA template **(T1)** at temperature 55 °C for primer extension reactions. Then the reaction mixture was separated and visualized with the desaturating PAGE gel electrophoretic technique for compression with labeled primer. The similar experiments were performed with control substrates (dTTP/ddTTP). Their gel images are depicted in Figure 2. In Figure 2a, the gel-band of lane L1 belongs to the labeled DNA primer (5′-FAM-P1), while the gel-bands of L2/L3/L4 belong to the primer extension reaction with respective dTTP/ddTTP/*ap*-TTP with *therminator* DNA polymerase (Figure 2a). The gel shifts show that *ap*-TTP has incorporated into primer (**5′-FAM-P1**) as like control substrate ddTTP. It has known that ddTTP is a terminator of DNA synthesis after single incorporation into DNA primer.[33] These results strongly support the extension of labeled primer with *ap*-TTP after single incorporation as like ddTTP. However, the gel band shifts with both *ap*-TTP and ddTTP are lower than another control natural substrate dTTP under similar conditions. It shows

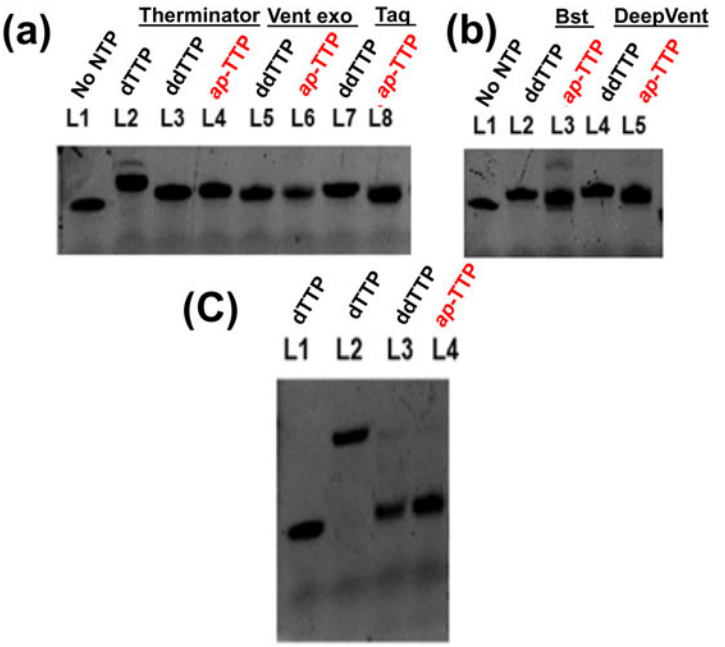


Figure 4. Gel-shift images of DNA primer (Fam-P1) after dTTP/ddTTP/*ap*-TTP incorporation with different DNA polymerase Enzymes and DNA template (T1): (a) the only primer no dNTPs or its analogs (L1); with enzyme Therminator (L2–L4)/Vent-Exo (L5 and L6)/Taq (L7 and L8). (b) Only primer no dNTPs or its analogs (L1); with enzyme Bst (L2 and L3)/Deepvent (L4 and L5). (c) Full-length extension of primer Fam-P1 other three dNTPs and respective TTP with Therminator DNA polymerase and DNA template (T2)

that more than one dTTP (control) have incorporated into the DNA primer (**5′-FAM-P1**). In literature, the multiple incorporations of dNTP into primer are reported with high fidelity DNA polymerase enzyme.[34] We tested *ap*-TTP with other DNA polymerases such as *Vent-exo, Taq, Bst, or Deepvent* and then compared with control ddTTP by the visualization of respective gel shifts under similar primer extension reaction conditions (Figure 4a,b). In Figure 4a, the gel shifts of *ap*-TTP and ddTTP are depicted in respective lane L4 & L5 for Vent-exo polymerase, L5 & L6 for Taq polymerase. However, the gel shift of *ap*-TTP and ddTTP, with *Bst/Deepvent polymerase,* are provided in lane L2-L4 in Figure 4b, and then compared with DNA primer L1 of Figure 4b. The gel shifts of *ap*-TTP consistently match with the gel-shift of ddTTP (control) in the presence of other given DNA polymerases. These gel studies strongly support the single incorporation of *ap*-TTP into DNA primer as like control ddTTP with various DNA polymerases. The incorporation of dTTP/ddTTP/*ap*-TTP into labeled primer with DNA polymerase has also summarized in Table 1. Hence *ap*-TTP is also substrate of DNA polymerase as like as like dTTP and ddTTP.

Further, to investigate the multiple incorporations of *ap*-TTP into the primer, we have designed a template (**T2**) that could incorporate multiple

Table 1. DNA polymerase vs. *aminopropanolyl*-TPP & control (dNTP &ddNTP) reaction results.

Sr. No	DNA Polymerase	dTTP	ddTTP	*Ap*-TTP
1	Bst	✓	✓	✓
2	Deepventexo-	✓	✓	✓
3	Taq	✓	✓	✓
4	Vent exo-	✓	✓	✓
5	Therminator	✓	✓	✓

dTTP. Herein, we have performed primer extension reactions with designed primer/template, *ap*-TTP and control dTTP by gel-electrophoretic methods, and their gel-shifts are depicted in Figure 4c. In case of dTTP, we noticed the multiple incorporations of dTTP into the DNA primer up to almost full-length of DNA sequence (Figure 4c, L2). However, with *ap*-TTP, the gel-shift band (Figure 4c, L3) is almost equal to that of terminator ddTTP (Figure 3, L4). Herein, *ap*-TTP could not get incorporated into DNA after single incorporation, unlike dTTP. The similar results have reported with gNTP. Hence *ap*-TTP is another terminator of DNA biosynthesis after single incorporation.

Liquid Chromatography-Mass Spectrometry (LC-MS) Studies. To ensure the incorporation of *ap*-TTP into DNA primer, we analyzed the reaction PCR mixture by LC-ESI-Mass methods. We recorded the mass spectra of DNA primer (**P1**) and Template (**T1**) before and after treatment with *ap*-TTP in the presence of DNA polymerase (*Vent-exo*). A similar experiment was repeated with the ddTTP (control) for comparative studies. The ESI-Mass spectra of primer/extended primer with *ap*-TTP and control ddTTP are provided in the supplemental file. The selected region of mass spectra for the primer extension reaction of *ap*-TTP is depicted in Figure 5. The mass spectrum of DNA primer **P1** exhibits a prominent peak at 1381.99 (*m*/*z*) which belongs to molecular mass ion (M_p) of primer as $[M_p\text{-}4H]^{4-}$, while the mass of template (**T1**) has appeared at 1454.16. In the presence of *Ventexo-* DNA polymerase and *ap*-TTP, the mass of DNA primer (P1) appeared at 1447.28 (*m*/*z*), higher than lone primer, which belong to the molecular mass ion of single *ap*-TTP extended DNA primer (M_p*) as $[M_p{*}\text{-}4H]^{4-}$. Similarly, the mass of DNA primer **P1** was appeared at 1447.28 (*m*/*z*) which belongs to $[Mp{**}\text{-}4H]^{4-}$, the mass of single ddTTP extended DNA primer (Mp**). This mass is also higher than primer under similar primer extension reaction conditions as like *ap*-TTP. Hence, these mass results confirm only single incorporation of *ap*-TTP into DNA primer in the presence of DNA Template and DNA polymerase.

Conclusion

In summary, we have described the synthesis, characterization, and biochemical evaluation of *ap*-TTP with various DNA polymerases. We have also described the supramolecular self-assembly duplex structure in

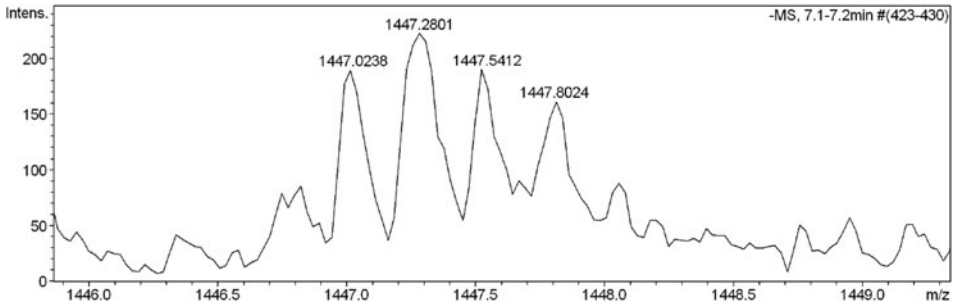


Figure 5. Mass spectrum of the extended mass of primer with *Ap*-TTP.

protected solid-state monomer crystal as a unique *anti*-parallel self-duplex via unique reverse Watson-Crick hydrogen bonding and π–π interactions. Importantly, the enzymatic studies support the single incorporation of *ap*-TTP into DNA primer as like control therminator ddTTP. Hence *ap*-TTP is a different substrate of DNA polymerase to introduce an amine functionality at 3′-position of DNA primer.

Experimental section

Materials. All reagents and solvents were purchased from commercial sources and used as received unless otherwise specified. DNA primers and Template oligonucleotides were purchased from IDT. DNA polymerase enzymes purchased from NEB. DMF and DCM were distilled over CaH_2 and stored on molecular sieves. THF and dioxane were dried over sodium benzophenone and stored over sodium. Pyridine was distilled over potassium hydroxide and stored on CaH_2. Triethyl ammonium bicarbonate (TEAB) prepared by passing CO_2 gas into 2 M aqueous triethylamine solution and stored at 4 °C. HPLC was performed on Waters semipreparative HPLC using Sunfire column(C18; 4.6× 150 mm) using 20 mM TEAB and Acetonitrile (0–10% 30 min).

ESI-Mass experiments: LC-MS experiments were performed on Bruker microTOF-Q II coupled with a Waters Acquity UPLC autosampler. Primer extension reaction products (25 ul) were diluted with 25 µl Milli-Q water and injected about 10 µl vis autosampler by the following program as flow rate 0.2 ml/minute linear gradient.0–10 min80% A, 10–12 min 30% A, 12–13 min 30% A isocratic, 13 and 14min 100% A linear gradient and 14 and 15 min 100% A isocratic. UPLC column (C-18 RP, 2.1 × 5.0 mm, 1.7 µm, Acquity Waters) used for LC-MS experiments. The column temperature maintained at 30 °C during the experiments (buffer A (5 mM Ammonium acetate with pH 7.0) and buffer B (Methanol).

Primer extension reaction Method for mass analyses: Primer extension reactions for LC-MS analysis were carried out with 5 µM of primer and

template, 100 µM *ap*-TTP, dTTP or ddTTP in a total reaction volume of 25 µl with 2.0 units of *Vent exo* DNA polymerase.

Primer extension reaction Method for PAGE gel studies. All primer extension reactions were performed with 1 µM Fam-labeled primer, 1 µM template 100 µM NTP (dTTP, dTTP, and Ap-TTP) in 10.0 µl reaction buffers (provided by the supplier with enzymes). All reactions were performed at 55 °C for 60 min with two units of *Therminator*; *Vent exo or Deep Vent exo* DNA polymerases in 10.0 µl reactions. Though for Bst and Taq DNA polymerases, we used 8.0 and 10.0 units, respectively. Denaturating Gel experiment performed with 20% PAGE (29: 1 Acrylamide, *bis*-acrylamide) and 7.0 M urea. Gels were prepared and run with 1xTBE buffer and visualized under *trans-UV* using the Bio-Rad gel doc system.

Experimental procedures for the synthesis of compounds (2–5)

O-Mesyl-N-Boc Serine methyl ester **(2):** *N*-Boc serine methyl ester (2.0 g, 9.13 mmol) was dissolved in anhydrous pyridine (10 ml) and stirred at 0 °C under nitrogen atmosphere. Mesyl chloride (1.06 ml, 13.7 mmol) was added dropwise with a syringe and stirred at 0 °C for one hour. The reaction mixture was concentrated under vacuum using the rota vapor and then dissolved into water and extracted with organic solvent DCM. The DCM layer was washed with sodium bicarbonate solution followed by brine solution and kept over anhydrous sodium sulfate. The DCM solution was concentrated and purified by silica gel column with Ethyl acetate: Hexanes (1:9) that produced 1.89 g of the oily compound in 70% yield. R_f (0.62) was determined on TLC 40% EtOAc/Hexane.1H-NMR (400 MHz, $CDCl_3$) δ ppm 1.36 (9 H, s), 2.95 (3 H, s), 3.71 (3 H, s), 4.39–4.42(1H, m), 4.47–4.53(2H, m), 5.53-5.55(1H, m) 13C-NMR (100 MHz, $CDCl_3$) δ ppm 28.11, 37.17, 52.89, 53.01, 68.99, 80.43, 155.07, 169.15.HRMS (ESI-TOF) Calculated ($C_{10}H_{17}O_7N\ S + Na$) 320.0774, found 320.0775

N-Bocalanyl Thymine ester **(3)**: Thymine (0.2 g, 1.58 mmol), K_2CO_3 (0.328g, 2.38 mmol) and 18-Crown-6 (0.083 g, 0.31 mmol) was dissolved into anhydrous DMF (5 ml) under a nitrogen atmosphere and stirred for 10 min. The mesyl derivative **2** (0.471 g, 1.58 mmol) was added to DMF reaction mixture and stirred at room temperature for overnight under nitrogen atmosphere. The reaction mixture concentrated under vacuum and purified over silica gel column using solvent system (MeOH and DCM) which gave 0.181 g white solid in 35% yield. R_f (0.46) was determined on TLC using 5% MeOH/DCM. ^{1}H NMR (400 MHz, DMSO-d_6 +CD_3OD) δ ppm 1.27 (9 H, s), 1.72 (3 H, s), 3.54–3.60(1H, m), 3.63 (3 H, s), 4.18–4.23(1H, m), 4.44–4.46(1H, m), 7.18(1 H, m). 13C-NMR (100 MHz, DMSO-d_6 + CD_3OD) δ ppm 11.23, 27.49, 49.19, 51.78, 51.88,

79.40, 109.05, 142.20, 151.43, 156.01, 165.26, 17.42 HRMS (ESI-TOF) Calculated ($C_{14}H_{21}O_6N_3$ +Na) 328.1503, found 328.1509.

N-Boc-aminopropanolyl Thymine **(4)**: Thymine methyl ester (**3**) (0.15 g, 0.45 mmol) was dissolved in anhydrous ethanol under nitrogen atmosphere by stirring for 10 minutes followed by addition of Sodium borohydride (0.052 g, 1.37 mmol) and stirred overnight under nitrogen atmosphere at room temperature. The concentrated reaction mixture had extracted with ammonium chloride and ethyl acetate. The ethyl acetate layer was washed with brine solution and kept over anhydrous sodium sulfate. The organic layers were concentrated with rota-vapor under vacuum and purified by silica gel column chromatography with solvent system MeOH and DCM, which gave 0.109 g of white solid in 80% yield. R_f (0.24) was determined on TLC using 5% MeOH/DCM. ^{1}H-NMR (400 MHz, DMSO-D_6) δ ppm 1.29 (9 H, s), 1.72 (3 H, s), 3.23-3.29 (1H, m), 3.34–3.41 (2H, m), 3.74–3.80 (1H, m), 3.95–4.00 (1H, m), 4.80–4.83 (1H, m), 6.54-6.57 (1H, m), 7.31(1H, s), 11.12 (1H, s) 13C-NMR (100 MHz, DMSO-$D_{6)}$ δ ppm 12.51, 28.52, 49.98, 51.18, 61.90, 78.25, 107.90, 142.58, 151.43, 155.72, 164.81. ESI-HRMS (ESI-TOF) Calculated ($C_{13}H_{21}O_5N_3$) 300.1554, found 300.1556.

Aminopropanolyl thymine triphosphate **(5)**. Triphosphorylationof synthesized nucleoside alcohol was performed by following the Ludwig-Eckstein method.[18] *N*-Boc-aminopropanolyl Thymine alcohol **4** (0.04 g, 0.133 mmol) was dissolved in 3 ml of anhydrous pyridine. Pyridine was evaporated in the Rota vapor under vacuum followed further drying in a vacuum desiccator at ambient temperature for one hour. Desiccator was opened under an argon atmosphere and the round bottom flask was closed with a septum. All the next steps were performed under argon pressure. Salicylchlorophosphidite (0.032 g, 0.160 mmol) dissolved in dry dioxane (1.0 ml) was added and stirred at room temperature for 10 min. Immediately the cocktail of *tri-* butylammonium pyrophosphate (0.146 g, 0.267 mmol) and *tri*-butylamine (0.267 mL, 1.07 mmol) in anhydrous dimethylformamide (DMF) (1 ml) was injected into the flask and stirred for 10 min followed by the addition of iodine (1.0%) in pyridine:water (98:2) solution. After 10 min, 10 ml of 100 mM TEAB buffer (pH 8.0) was added and stirred for one hour, followed by lyophilization. The reaction mixture was dissolved in 5 ml-distilled water and extracted with ethyl acetate. The aqueous layer was lyophilized, and Purified on DEAE Sephadex A-25 anion exchange column with elution buffer (0.1 M-1.0M TEAB). The Fractions containing compounds were concentrated and treated with 1.0 N HCl for removal of Boc. The reaction was quenched with aqueous ammonia and lyophilized before HPLC purification. The purified sample was characterized as *ap*-TTP as a white powder. ^{1}H NMR (400 MHz, D_2O) δ 1.94 (s, 3 H), 3.94 (s, 1 H), 4.04 – 4.20 (m, 2 H), 4.26

(dd, $J = 14.7$, 7.3 Hz, 1 H), 4.41 (dd, $J = 6.7$, 3.1 Hz, 1 H), 7.57 (s, 1 H), 31PNMR (162 MHz, D_2O):(−22.13, t, 1P, P_β), (−11.16, −11.04,d, 1 P, P_γ), (−10.4, −10.16, d, 1P, P_α) ($J_{\alpha\beta}$=19.44 Hz, $J_{\beta\gamma}$=19.44 Hz) HRMS (ESI-TOF) Calculated for $[C_8H_{15}NO_{12}P_3(M\text{-}H)]$ *m/z*437.9863, found *m/z* 437.9969.

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