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Synthesis of α-L-Threofuranosyl Nucleoside 3′-Monophosphates, 3′-Phosphoro(2-Methyl)imidazolides, and 3′-Triphosphates

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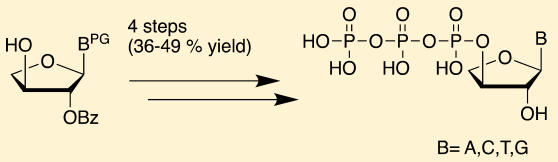
Supporting Information

ABSTRACT: α-L-Threofuranosyl nucleic acid (TNA) is an artificial genetic polymer composed of vicinal 2′,3′-phosphodiester bonds linking adjacent threofuranosyl nucleosides. TNA is one of a small number of genetic polymers that are both highly resistant to nuclease digestion and capable of cross-pairing with DNA and RNA. Although an efficient method for synthesizing TNA nucleosides has been reported, very few advances have been made in the synthesis of phosphorylated TNA compounds. Here, we describe a highly efficient method for synthesizing α-L-threofuranosyl nucleoside 3′-monophosphates (tNMPs), 3′-phosphoro(2-methyl)imidazolides (2-MeImptNs), and 3′-triphosphates (tNTPs) bearing the four genetic bases of adenine (A), cytosine (C), thymine (T), and guanine (G). We suggest that this strategy, which provides access to grams of tNMPs, hundreds of milligrams of 2-MeImptNs, and tens of milligrams of tNTPs, will help advance the use of TNA monomers in exobiology and biotechnology applications.



INTRODUCTION

Nucleoside triphosphates are indispensable reagents for a broad range of molecular biology, biotechnology, and synthetic biology applications.[1−3] In particular, modified nucleoside triphosphates that carry new chemical moieties at the nucleobase, sugar, and phosphate positions are critical to many diagnostic and therapeutic applications,[4−7] as well as fundamental studies aimed at evaluating the specificity of natural enzymes or identifying emergent properties of life either here on Earth or elsewhere in the universe.[8] Unfortunately, despite extensive efforts to develop chemical and/or enzymatic strategies for producing nucleoside triphosphates, no single strategy has been developed that can be applied universally to all nucleosides.[9] This is especially true for chemically modified analogues where a large number of synthetic approaches have been developed to accommodate the idiosyncrasies of different nucleoside chemistries.[10−14] As expected, each strategy comes with its own strengths and weaknesses that must be evaluated on a case-by-case basis.[15] In general, enzymatic strategies suffer from poor substrate specificity, scale, yield, and cost, whereas chemical approaches struggle with functional group compatibility, regiochemistry, yield, and purification.[15]

Synthesizing nucleoside triphosphates of α-L-threofuranosyl nucleic acid (TNA) requires phosphorylating the 3′-hydroxyl position on the furanose ring.[16] However, the 3′-hydroxyl position of α-L-threofuranosyl nucleosides is substantially less reactive than the 5′-hydroxyl position of most DNA and RNA nucleosides because secondary alcohols are less nucleophilic than primary alcohols and the 3′-position is more sterically encumbered than the normal 5′-hydroxyl position (Figure 1). Given the importance of TNA building blocks in many exobiology and synthetic biology projects, a concerted effort has been made to identify synthetic approaches for constructing

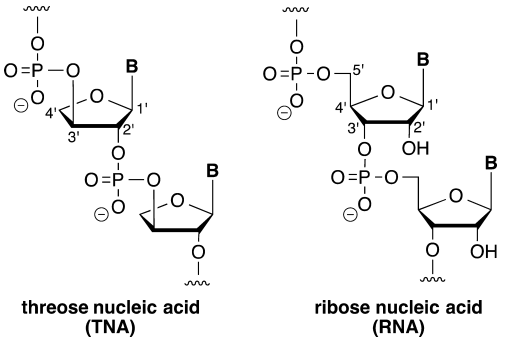


Figure 1. Molecular structure of TNA and RNA.

phosphorylated TNA compounds (Scheme 1) on the scales required to meet the demands of current downstream applications.[17−20] Early efforts by our laboratory and others using the Yoshikawa method with $POCl_3$ proved unsuccessful due to unwanted side reactions. Szostak and co-workers overcame this problem using the classic Ludwig−Eckstein method, which was used to synthesize all four TNA triphosphates from their corresponding 2′-*O*-DMT protected nucleosides.[21] Although a detailed protocol was subsequently established,[22] this method proved cumbersome due to the poor regioselectivity observed when TNA nucleosides are tritylated with DMT.[21] As an alternative strategy, we developed a one-pot four-step synthesis of TNA triphosphates from their 2′-*O*-benzoyl derivatives that is considerably less sensitive to moisture than traditional approaches.[23] Using this method, H-phosphonate intermediates are oxidized in situ with *N*-

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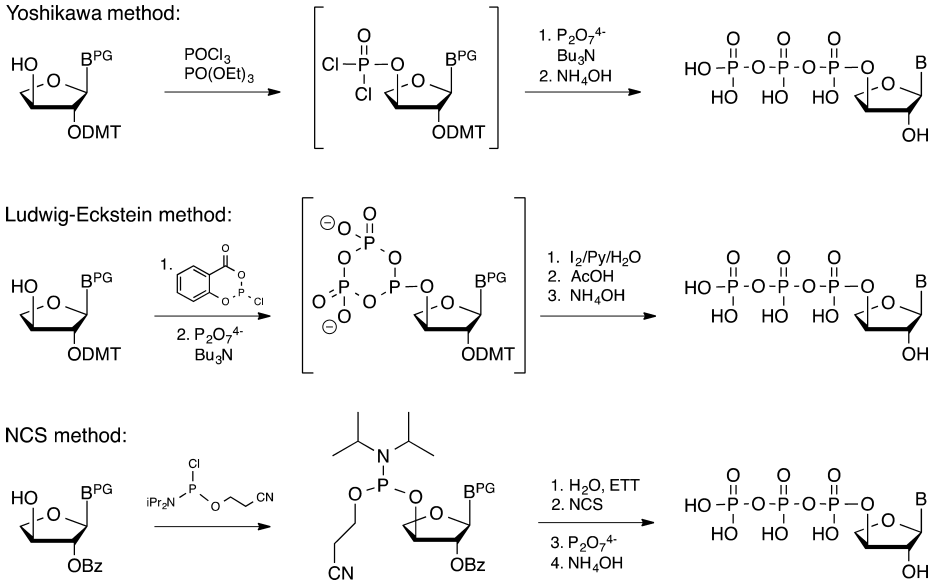
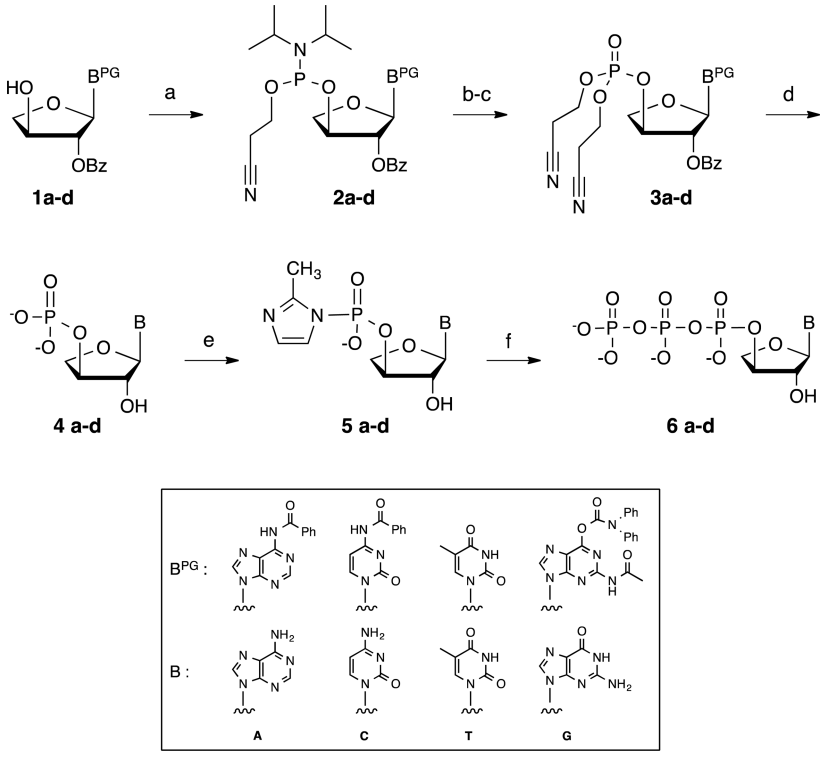
Scheme 1. Previous Strategies for Synthesizing TNA Triphosphates



Scheme 2. Synthesis of TNA Triphosphates Using Activated TNA Monophosphates[a]



[a]Reagents and conditions: (a) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, DMAP, DIPEA, CH_2Cl_2, rt, 30−45 min, 68−75%; (b) 3-hydroxypropionitrile, tetrazole, acetonitrile, rt, 1 h; (c) 30% H_2O_2, rt, 15 min, 71−75% (2 steps); (d) 30−33% NH_4OH, 38 °C, 18 h, 91−98%; (e) 2-methylimidazole, triphenylphosphine, 2,2′-dipyridyldisulfide, DMSO, triethylamine, rt, 6−8 h; (f) tributylammonium pyrophosphate, tributylamine, DMF, 10−12 h, 81−87% (2 steps).

chlorosuccinimide (NCS) to produce chlorophosphate intermediates that are treated with pyrophosphate to generate the desired nucleoside triphosphates.[23] Although TNA nucleoside 3′-triphosphates generated in this way are easy to purify by HPLC, the NCS method suffers from low overall yield (∼10% isolated yield) due to an unwanted side reaction that produces large quantities of the 3′-monophosphate derivative as the major product.

To overcome the shortcomings of previous methods, we developed a robust strategy for generating TNA nucleoside monophosphates, monophosphate derivatives, and triphosphates that is straightforward to perform and could be transferrable to other modified nucleosides. Our approach consists of four chemical transformations to generate TNA monophosphates from a TNA nucleoside intermediate that can be produced in large quantities from L-ascorbic acid.[24] TNA 3′-

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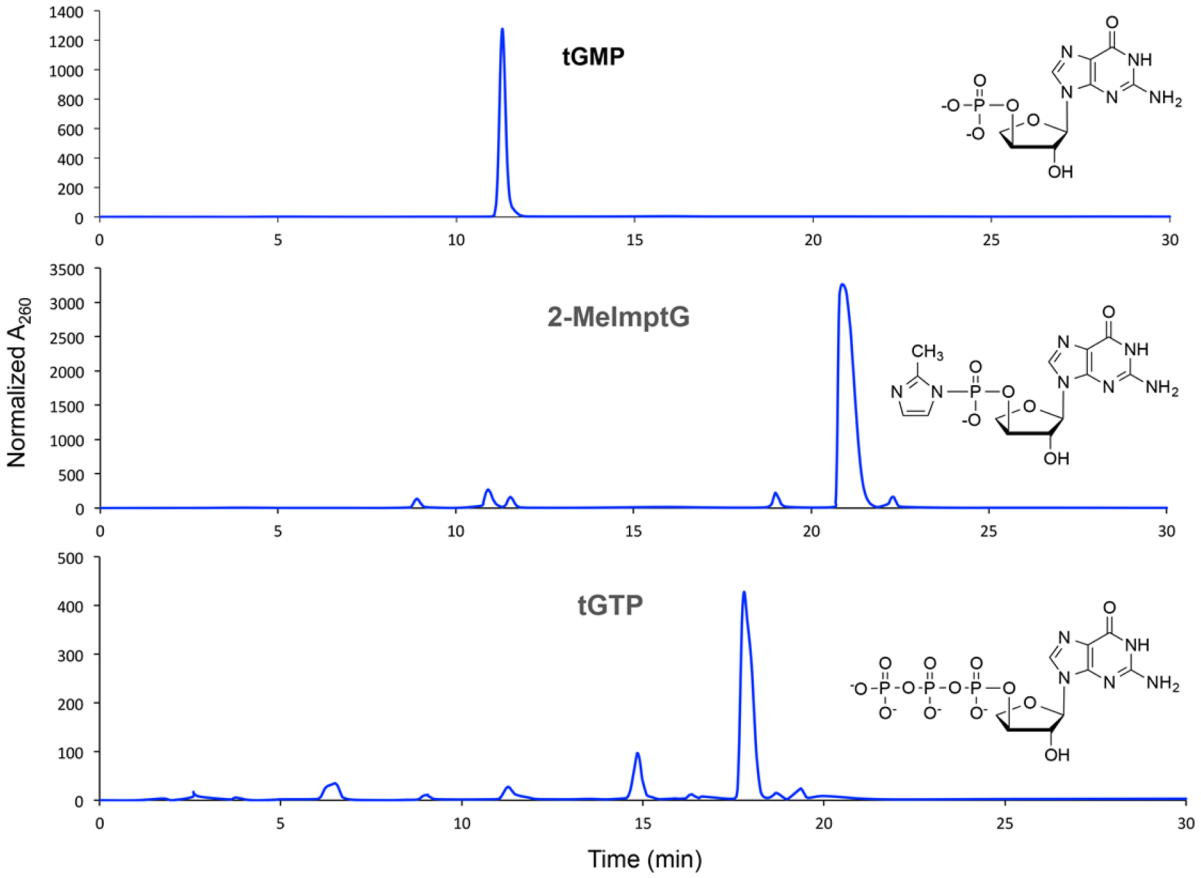

Figure 2. HPLC analysis of the crude reaction mixture for tGMP, 2-MeImptG, and tGTP.

monophosphates are then converted to 3′-phosphoro(2-methyl)imidazolides, which are treated with pyrophosphate to produce nucleoside 3′-triphosphates. HPLC analysis of the crude reaction mixture indicates that the phosphorylation steps proceed cleanly with high yield of the desired compounds. We suggest that the current strategy will be useful for constructing TNA nucleotides for a wide range of exobiology and biotechnology applications, as TNA nucleoside 3′-monophosphates, 3′-monophosphate derivatives, and 3′-triphosphates are obtained as isolated compounds that can be stored for long periods of time without degradation.

RESULTS AND DISCUSSION

We designed a synthetic strategy (Scheme 2) for constructing 3′-phosphorylated TNA compounds from 2′-*O*-benzoyl TNA nucleosides **1a−d**. TNA nucleosides **1a−d** were obtained from L-ascorbic acid using known methodology that involves eight chemical transformations with three crystallization steps.[24] Whereas several phosphorylation reagents were evaluated as possible routes to α-L-threofuranosyl 3′-monophosphates (**4a−d**), a stepwise process that invoked the synthesis of a 3′-phosphoramidite intermediate proved highly efficient for each of the four TNA nucleosides. Accordingly, 2′-*O*-benzoyl nucleosides **1a−d** were converted to their 3′-*O*-phosphoramidite derivatives **2a−d** upon treatment with 2-cyanoethoxy-*N*,*N*-diisopropylchlorophosphine in the presence of Hunig's base and DMAP. The use of DMAP and a slight excess of the chlorophosphoramidite reagent was crucial for obtaining 3′-*O*-phosphoramidites **2a−d** in high yield. Intermediates **2a−d** were subsequently converted to their corresponding trialkyl phosphite by replacing the *N*,*N*-diisopropylamino group with a base-labile cyanoethoxy group. The trialkyl phosphite was then oxidized with H_2O_2 to obtain the phosphate triesters **3a−d**, which were purified by silica gel chromatography. The resulting TNA nucleoside 3′-monophosphates **4a−d** were obtained as an ammonium salt following deprotection with 30% aqueous ammonium hydroxide. Although this strategy is more cumbersome than traditional monophosphate reactions,[25] we found that the higher reactivity of the chlorophosphoramidite reagent, coupled with the ability to purify the protected monophosphate by silica gel chromatography, provided an efficient method for obtaining large quantities (grams) of highly pure TNA 3′-monophosphates.

Next, TNA monophosphates **4a−d** were converted to their 3′-phosphoro(2-methyl)-imidazolide activated derivatives **5a−d** using classic methodology previously developed by Mukaiyama and co-workers.[26] Accordingly, a Mitsunobu-like reaction is performed by incubating **4a−d** with excess 2-methylimidazole and triphenylphosphine in the presence of 2,2′-dipyridyldisulfide. The reactions were monitored by analytical HPLC until >90% conversion of the starting material to the desired product was observed (Figure 2). In some cases, complete conversion of the starting material required the addition of excess 2-methylimidazole and triphenylphosphine to help drive the reaction forward. The desired TNA nucleoside 3′-phosphoro-(2-methyl)imidazolides **5a−d** were isolated as a sodium salt by precipitating the reaction with sodium perchlorate and acetone. Interestingly, we found that the phosphorimidazolide reaction proceeds more efficiently when the monophosphates are precipitated as the ammonium salt rather than the sodium salt.

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TNA nucleoside 3′-triphosphates **6a−d** were generated from the activated nucleoside monophosphates **5a−d** by displacing the activated 2-methylimidazole leaving group with pyrophosphate. In these reactions, TNA nucleosides **5a−d** were incubated at room temperature for 8−12 h in a DMF solution containing tributylammonium pyrophosphate and tributylamine. The reaction was monitored by analytical HPLC, and additional pyrophosphate and tributylamine were added as needed to drive the reaction to completion (Figure 2). In all cases, >90% of the starting nucleoside was consumed within 12 h. The desired TNA nucleoside triphosphates **6a−d** were purified by reverse-phase HPLC, concentrated to dryness, resuspended in methanol, and precipitated as the sodium salt. During the concentration step, we noticed that the pH of the solution becomes acidic due to a buildup of acetic acid from the ammonium acetate running buffer. If left unchecked, this change in pH leads to unwanted acid-catalyzed degradation of the nucleotide triphosphate. Fortunately, this problem is easily overcome by monitoring the pH of the concentrated solution and adjusting as needed with triethylamine.

Next, we evaluated the thermal stability of TNA triphosphates **6a−d**) bearing the four genetic bases of adenine (A), cytosine (C), thymine (T), and guanine (G). In this assay, the stability of each TNA triphosphate was monitored by analytical HPLC using small aliquots of TNA solutions that were stored at temperatures of 4, 24, and 37 °C. A time course analysis performed over 8 days (Table 1) revealed that tNTPs

Table 1. Thermal Stability of Purified TNA Triphosphates[a]

temperature (°C)	days	ATP (%)	GTP (%)	CTP (%)	TTP (%)
4	1	99.7	95.4	96.9	94.4
	5	98.4	92.7	93.6	89.4
	8	95.6	88.7	91.3	84.5
24	1	98.8	94.1	95.6	93.6
	5	90.5	86.5	94.9	83.3
	8	82.8	76.8	91.2	81.8
37	1	97.0	93.1	95.4	93.1
	5	89.6	82.4	93.2	82.4
	8	81.7	73.9	86.9	80.8

[a]Analysis performed in 10 mM Tris, pH 8.0.

are resistant to thermal degradation when stored in a buffered solution containing 10 mM Tris (pH 8.0). Even after 8 days at 37 °C, >70% of tGTP and >80% of tATP, tCTP, and tTTP remained undegraded. This result is consistent with previous thermal stability studies of tNTPs performed under standard PCR conditions.[23]

To confirm that tNTPs synthesized using our new strategy can function as substrates for an engineered TNA polymerase, we performed a standard primer-extension assay in which a library of DNA templates were copied into TNA. To date, the polymerase that most efficiently uses tNTP substrates is Kod-RI, a polymerase that was developed through a combination of directed evolution and scaffold sampling.[20,27] Kod-RI functions with a catalytic rate of ∼1 nucleotide per minute and is able to copy most DNA templates into TNA.[27] As a demonstration, an IR-labeled DNA primer was annealed to a degenerate DNA library and incubated with Kod-RI in the presence of chemically synthesized of tNTP substrates. Analysis of the primer-extension reaction by denaturing polyacrylamide gel electrophoresis (Figure 3) reveals that the primer is extended to full-length product after a 3 h incubation at 55 °C. This result,

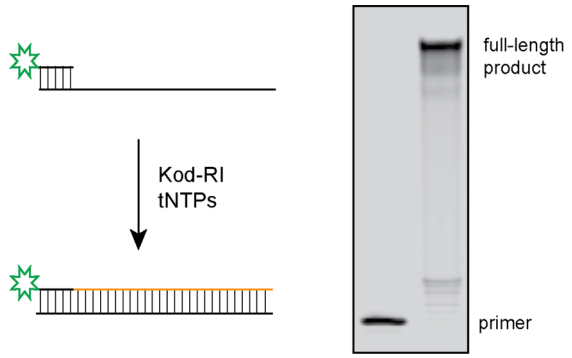

Figure 3. Primer-extension analysis. A 5′-IR-labeled DNA primer annealed to a DNA template was extended with 70 sequential TNA residues. Polymerization reaction was performed at 55 °C for 3 h using Kod-RI TNA polymerase and chemically synthesized tNTPs. The reaction was analyzed by denaturing polyacrylamide gel electrophoresis.

which is consistent with the known activity of Kod-RI,[27] demonstrates that the new synthetic approach produces TNA triphosphates that are recognized by an engineered TNA polymerase.

CONCLUSION

In summary, we report a novel synthetic route for constructing TNA nucleotide 3′-monophosphates, 3′-monophosphate derivatives, and 3′-triphosphates bearing all four nucleobases (A, T, G, C). This strategy overcomes the poor reactivity of the 3′-hydroxyl group on the threose ring by using a more convenient phosphorylating reagent than is commonly employed for nucleoside chemistry. Our approach involves five chemical transformations with one silica gel purification, two precipitation steps, and one HPLC purification. The overall isolated yield of the purified tNTP compounds from their starting nucleosides is 36−49% depending on the identity of nucleoside (A, T, G, C). Finally, we show that the resulting TNA substrates are thermally stable and function as substrates for an engineered TNA polymerase. We suggest that the simplicity of this approach, coupled with the ability to monitor the phosphorylation reaction and isolate intermediate compounds, provides a general approach that could be applied to the phosphorylation of other modified nucleotides.

EXPERIMENTAL SECTION

General Information. All non-aqueous reactions were performed using oven-dried glassware under an atmosphere of argon or nitrogen. All chemicals were commercially available and used as received. Anhydrous solvents were purchased as the highest grade from Sigma-Aldrich. Reactions were monitored by thin layer chromatography using UV-activated TLC plates with silica gel 60 F_{254} and aluminum backing (Sigma-Aldrich, St. Louis, MO). Flash column chromatography was performed using SiliCycle 40−60 mesh silica gel (SiliCycle Inc., Quebec City, Canada). Yields are reported as isolated yields of pure compounds. UV quantification data are analyzed on NanoDrop 2000c using Beer's Law. ^{1}H, ^{13}C, and ^{31}P NMR spectra were analyzed on 400 and 500 MHz NMR spectrometers (Bruker, Billerica, MA). ^{1}H values are reported in parts per million relative to Me_4Si or corresponding deuterium solvents as internal standard. ^{13}C values are reported in parts per million relative to corresponding deuterium solvents as internal standards. ^{31}P NMR values are reported in parts per million relative to an external standard of 85% H_3PO_4. Splitting patterns are designated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet. HPLC analysis was performed on a reverse-phase

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C18 150 × 4.6 mm column with 5 μm particle size, and TNA triphosphate was purified on a preparative reverse-phase C18 250 × 21.2 mm column (Thermo Scientific, USA) using a mobile phase of 100 mM triethylammonium acetate buffer (pH 7.0)/acetonitrile.

1-(2′-*O*-Benzoyl-3′-*O*-[bis(2-cyanoethyoxy)phosphoryl]-α-L-threofuranosyl)thymine (3a). To a stirring solution containing 1 g (3.01 mmol) of 1-(2′-*O*-benzoyl-α-L-threofuranosyl) thymine (**1a**)[24] and 360 mg (3.01 mmol) of DMAP in 12 mL anhydrous CH_2Cl_2 was added 1 mL (7.53 mmol) of *N*,*N*′-diisopropylethylamine followed by the addition of 0.81 mL (3.61 mmol) of 2-cyanoethoxy-*N*,*N*-diisopropylchlorophosphoramidite. After being stirred for 40 min at room temperature, the reaction mixture was diluted with 40 mL of CH_2Cl_2 and extracted twice with 50 mL of saturated aqueous $NaHCO_3$, washed with brine, dried over $MgSO_4$, and evaporated under reduced pressure. The crude 1-[2′-*O*-benzoyl-3′-*O*-(*N*,*N*-diisopropylamino-2-cyanoethoxyphosphinyl)-α-L-threofuranosyl]-thymine (**2a**)[23] was used without further purification.

To a stirring solution containing crude 1-[2′-*O*-benzoyl-3′-*O*-(*N*,*N*-diisopropylamino-2-cyanoethoxyphosphinyl)-α-L-threofuranosyl]-thymine (**2a**)[23] in 10 mL of anhydrous acetonitrile was added 0.62 mL (9.03 mmol) of 3-hydroxypropionitrile followed by the addition of 26.8 mL of tetrazole (0.45 M solution in anhydrous acetonitrile, 12.04 mmol). The reaction mixture was stirred for 1.5 h at room temperature with monitoring by TLC (1:1 hexanes−EtOAc). After complete consumption of the starting material, to the reaction was added 10 mL of 30% H_2O_2 and left to stir for 20 min at room temperature. The crude product was evaporated under reduced pressure and dissolved in 50 mL of ethyl acetate. The organic layer was washed with brine and water, dried over $MgSO_4$, and evaporated under reduced pressure. The crude was purified with silica gel column chromatography affording 1-(2′-*O*-benzoyl-3′-*O*-[bis(2-cyanoethyoxy)phosphoryl]-α-L-threofuranosyl)thymine (**3a**) as a white foam: yield 0.87 g (56%); silica gel TLC (DCM/MeOH, 9:1) R_f = 0.35; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.74 (s, 1H), 8.02 (d, 2H, *J* = 5 Hz), 7.61 (t, 2H, *J* = 7.5 Hz), 7.46 (t, 2H, *J* = 7.5 Hz), 7.30 (s, 1H), 6.11 (d, 1H, *J* = 2 Hz), 5.62 (s, 1H), 5.10 (m, 1H), 4.53 (m, 1H), 4.30−4.39 (m, 5H), 2.82 (t, 4H, *J* = 6 Hz), 1.92 (s, 3H); ^{13}C NMR (125.8 MHz, $CDCl_3$) δ 165.2, 164.1, 150.5, 135.3, 134.1, 130.0, 128.7, 128.3, 116.6, 116.6, 111.0, 89.5, 80.4 (d, $J_{C,P}$ = 6.5 Hz), 79.6 (d, $J_{C,P}$ = 5.2 Hz), 73.5 (d, $J_{C,P}$ = 4.7 Hz), 63.1 (d, $J_{C,P}$ = 5.0 Hz), 63.0 (d, $J_{C,P}$ = 5.0 Hz), 19.7 (d, $J_{C,P}$ = 7.4 Hz), 19.7 (d, $J_{C,P}$ = 7.2 Hz), 12.7; ^{31}P NMR (162 MHz, $CDCl_3$) δ −2.14; HRMS (ESI-TOF) calcd for $C_{22}H_{23}N_4O_9PNa$ $[M + Na]^+$ 541.1100; found 541.1094.

***N*⁴-(2′-*O*-Benzoyl-3′-*O*-[bis(2-cyanoethyoxy)phosphoryl]-α-L-threofuranosyl)cytosine (3b).** To a stirring solution containing 1 g (2.37 mmol) of N^4-benzoyl-1-(2′-*O*-benzoyl-α-L-threofuranosyl) cytosine (**1b**)[24] and 290 mg (2.37 mmol) of DMAP in 12 mL anhydrous CH_2Cl_2 was added 1 mL (5.93 mmol) of *N*,*N*′-diisopropylethylamine followed by the addition of 1.05 mL (4.74 mmol) of 2-cyanoethoxy-*N*,*N*-diisopropylchlorophosphoramidite. After being stirred for 40 min at room temperature, the reaction mixture was diluted with 40 mL of CH_2Cl_2 and extracted twice with 50 mL of saturated aqueous $NaHCO_3$, washed with brine, dried over $MgSO_4$, and evaporated under reduced pressure. The crude N^4-benzoyl-1-[2′-*O*-benzoyl-3′-*O*-(*N*,*N*-diisopropylamino-2-cyanoethoxy-phosphinyl)-α-L-threofuranosyl]cytosine (**2b**)[23] was used without further purification.

To a stirring solution containing crude N^4-benzoyl-1-[2′-*O*-benzoyl-3′-*O*-(*N*,*N*-diisopropylamino-2-cyanoethoxyphosphinyl)-α-L-threofuranosyl]cytosine (**2b**)[23] in 10 mL of anhydrous acetonitrile was added 0.65 mL (7.1 mmol) of 3-hydroxypropionitrile followed by the addition of 21.07 mL of tetrazole (0.45 M solution in anhydrous acetonitrile, 9.48 mmol). The reaction mixture was stirred for 1.5 h at room temperature with monitoring by TLC (1:1 hexanes−EtOAc). After complete consumption of the starting material, to the reaction was added 10 mL of 30% H_2O_2 and left to stir for 20 min at room temperature. The crude product solution was evaporated to dryness and redissolved with 50 mL of ethyl acetate. The organic layer was washed with brine and water, dried over $MgSO_4$, and evaporated under reduced pressure. The crude product was purified with silica gel column chromatography affording N^4-(2′-*O*-benzoyl-3′-*O*-[bis(2-cyanoethyoxy)phosphoryl]-α-L-threofuranosyl)cytosine (**3b**) as a white foam: yield 0.68 g (47.2%); silica gel TLC (DCM/acetone, 10:1) R_f = 0.35; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.30 (s, 1H), 8.21 (d, 1H, *J* = 7.2 Hz), 8.00−8.07 (m, 4H), 7.73 (t, 1H, *J* = 7.6 Hz), 7.57−7.65 (m, 3H), 7.52 (t, 2H, *J* = 7.2 Hz), 7.44 (d, 1H, *J* = 6.4 Hz), 6.07 (d, 1H, *J* = 1.2 Hz), 5.73 (d, 1H, *J* = 1.2 Hz), 5.21 (m, 1H), 4.63 (d, 1H, *J* = 10.4 Hz), 4.47 (dd, 1H, *J* = 3.6, 10.4 Hz), 4.23 (m, 4H), 2.94 (m, 4H); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 167.4, 164.4, 163.7, 154.5, 145.1, 134.1, 133.2, 132.8, 129.7, 128.9, 128.6, 128.5, 118.1, 118.0, 96.3, 90.9, 79.5 (d, $J_{C,P}$ = 6.3 Hz), 78.2 (d, $J_{C,P}$ = 5.0 Hz), 74.1 (d, $J_{C,P}$ = 3.8 Hz), 63.1 (d, $J_{C,P}$ = 4.9 Hz), 63.1 (d, $J_{C,P}$ = 4.9 Hz), 19.1 (d, $J_{C,P}$ = 7.4 Hz), 19.0 (d, $J_{C,P}$ = 7.5 Hz); ^{31}P NMR (162 MHz, DMSO-d_6) δ −2.61; HRMS (ESI-TOF) calcd for $C_{28}H_{26}N_5O_9PNa$ $[M + Na]^+$ 630.1366; found 630.1382.

***N*⁶-(2′-*O*-Benzoyl-3′-*O*-[bis(2-cyanoethyoxy)phosphoryl]-α-L-threofuranosyl)adenine (3c).** To a stirring solution containing 1 g (2.24 mmol) of N^6-benzoyl-9-(2′-*O*-benzoyl-α-L-threofuranosyl) adenine (**1c**)[24] and 273 mg (2.24 mmol) of DMAP in 12 mL anhydrous CH_2Cl_2 was added 0.78 mL (4.48 mmol) of *N*,*N*′-diisopropylethylamine followed by the addition of 0.85 mL (3.81 mmol) of 2-cyanoethoxy-*N*,*N*-diisopropylchlorophosphoramidite. After being stirred for 40 min at room temperature, the reaction mixture was diluted with 40 mL of CH_2Cl_2 and extracted twice with 50 mL of saturated aqueous $NaHCO_3$, washed with brine, dried over $MgSO_4$, and evaporated under reduced pressure. The crude N^6-benzoyl-9-[2′-*O*-benzoyl-3′-*O*-(*N*,*N*-diisopropylamino-2-cyanoethoxyphosphinyl)-α-L-threofuranosyl]adenine (**2c**)[23] was used without further purification.

To a stirring solution containing crude N^6-benzoyl-9-[2′-*O*-benzoyl-3′-*O*-(*N*,*N*-diisopropylamino-2-cyanoethoxy-phosphinyl)-α-L-threofuranosyl] adenine (**2c**)[23] in 10 mL of anhydrous acetonitrile was added 0.6 mL (6.72 mmol) of 3-hydroxypropionitrile followed by the addition of 19.9 mL of tetrazole (0.45 M solution in anhydrous acetonitrile, 8.96 mmol). The reaction mixture was stirred for 1.5 h at room temperature with monitoring by TLC (1:1 hexanes−EtOAc). After complete consumption of the starting material, to the reaction was added 10 mL of 30% H_2O_2 and left to stir for 20 min at room temperature. The crude product solution was evaporated to dryness and redissolved with 50 mL of ethyl acetate. The organic layer was washed with brine and water, dried over $MgSO_4$, and evaporated under reduced pressure. The crude product was purified with silica gel column chromatography affording N^6-(2′-*O*-benzoyl-3′-*O*-[bis(2-cyanoethyoxy)phosphoryl]-α-L-threofuranosyl)adenine (**3c**) as a white foam: yield 0.65 g (46%); silica gel TLC (DCM/MeOH, 9:1) R_f = 0.35; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.80 (s, 1H), 8.62 (s, 1H), 8.06 (m, 4H), 7.72 (m, 1H), 7.66 (m, 1H), 7.57 (m, 4H), 6.52 (d, 1H, *J* = 2.8 Hz), 6.29 (t, 1H, *J* = 2.8 Hz), 5.45 (m, 1H), 4.6 (dd, 1H, *J* = 4, 10.4 Hz), 4.52 (dd, 1H, *J* = 4.8, 10.4 Hz), 4.24 (m, 4H), 2.96 (m, 4H); ^{13}C NMR (125.8 MHz, DMSO-d_6) δ 165.6, 164.7, 152.0, 151.9, 150.5, 143.0, 134.1, 133.3, 132.5, 129.7, 128.9, 128.5, 128.5, 128.4, 125.8, 118.2, 118.1, 87.5, 79.7, 78.6 (d, $J_{C,P}$ = 6.9 Hz), 72.1 (d, $J_{C,P}$ = 4.5 Hz), 63.1 (d, $J_{C,P}$ = 5.0 Hz), 63.0 (d, $J_{C,P}$ = 5.0 Hz), 19.1 (d, $J_{C,P}$ = 7.3 Hz), 19.0 (d, $J_{C,P}$ = 7.2 Hz); ^{31}P NMR (162 MHz, DMSO-d_6) δ −2.25; HRMS (ESI-TOF) calcd for $C_{29}H_{26}N_7O_8PNa$ $[M + Na]^+$ 654.1485; found 654.1478.

***N*²-Acetyl-*O*⁶-diphenylcarbamoyl-9-(2′-*O*-Benzoyl-3′-*O*-[bis-(2-cyanoethyoxy)phosphoryl]-α-L-threofuranosyl)guanine (3d).** To a stirring solution containing 1 g (1.68 mmol) of N^2-acetyl-O^6-diphenylcarbamoyl-9-(2′-*O*-benzoyl-α-L-threofuranosyl) guanine (**1d**)[24] and 210 mg (1.68 mmol) of DMAP in 12 mL anhydrous CH_2Cl_2 was added 0.58 mL (3.36 mmol) of *N*,*N*′-diisopropylethylamine followed by the addition of 0.821 mL (3.67 mmol) of 2-cyanoethoxy-*N*,*N*-diisopropylchlorophosphoramidite. After being stirred for 40 min at room temperature, the reaction mixture was diluted with 40 mL of CH_2Cl_2 and extracted twice with 50 mL of saturated aqueous $NaHCO_3$, washed with brine, dried over $MgSO_4$, and evaporated under reduced pressure. The crude N^2-acetyl-O^6-diphenylcarbamoyl-9-[2′-*O*-benzoyl-3′-*O*-(*N*,*N*-diisopropylamino-2-cyanoethoxyphosphinyl)-α-L-threofuranosyl]guanine (**2d**)[23] was used without further purification.

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To a stirring solution containing crude N^2-acetyl-O^6-diphenylcarbamoyl-9-[2′-*O*-benzoyl-3′-*O*-(*N*,*N*-diisopropylamino-2-cyanoethoxyphosphinyl)-α-L-threofuranosyl]guanine (**2d**)[23] in 10 mL of anhydrous acetonitrile was added 0.36 mL (5.04 mmol) of 3-hydroxypropionitrile followed by the addition of 14.9 mL of tetrazole (0.45 M solution in anhydrous acetonitrile, 6.72 mmol). The reaction mixture was stirred for 1.5 h at room temperature with monitoring by TLC (1:1 hexanes−EtOAc). After complete consumption of the starting material, to the reaction was added 10 mL of 30% H_2O_2 and left to stir for 20 min at room temperature. The crude product solution was evaporated to dryness and redissolved with 50 mL of ethyl acetate. The organic layer was washed with brine and water, dried over $MgSO_4$, and evaporated under reduced pressure. The crude product was purified with silica gel column chromatography affording N^2-acetyl-O^6-diphenylcarbamoyl-9-(2′-*O*-benzoyl-3′-*O*-[bis(2-cyanoethyoxy)-phosphoryl]-α-L-threofuranosyl)guanine (**3d**) as a white foam: yield 0.59 g (45%); silica gel TLC (DCM/acetone, 10:1) R_f = 0.35; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.78 (s, 1H), 8.55 (s, 1H), 8.03 (d, 2H, J = 7.5 Hz), 7.71 (t, 1H, J = 7.5 Hz), 7.44−7.57 (m, 10H), 7.33 (t, 2H, J = 6.5 Hz), 6.39 (s, 1H), 6.21 (s, 1H), 5.41 (s, 1H), 4.75 (m, 1H), 4.47 (m, 1H), 4.24 (m, 4H), 2.94 (m, 4H), 2.20 (s, 3H); ^{13}C NMR (125.8 MHz, DMSO-d_6) δ 168.9, 164.8, 155.3, 154.2, 152.4, 150.1, 144.0, 141.6, 134.1, 129.7, 129.5, 128.9, 128.4, 127.4, 120.3, 118.2, 118.0, 116.7, 87.3, 79.5 (d, $J_{C,P}$ = 7.0 Hz), 78.0 (d, $J_{C,P}$ = 4.3 Hz), 71.6 (d, $J_{C,P}$ = 3.8 Hz), 63.0 (d, $J_{C,P}$ = 5.0 Hz), 63.0 (d, $J_{C,P}$ = 5.0 Hz), 24.7, 19.0 (d, $J_{C,P}$ = 7.9 Hz), 19.0 (d, $J_{C,P}$ = 7.7 Hz); ^{31}P NMR (162 MHz, DMSO-d_6) δ −2.24; HRMS (ESI-TOF) calcd for $C_{37}H_{33}N_8O_{10}PH$ $[M + H]^+$ 780.2281; found 780.2296.

α-L-Threofuranosyl Nucleoside 3′-Monophosphates (4a−d). In a sealed tube, 1-(2′-*O*-benzoyl-3′-*O*-[bis(2-cyanoethyoxy)-phosphoryl]-α-L-threofuranosyl) nucleosides (0.3 mmol) (**3a−d**) were combined with 10 mL of saturated NH_4OH. The reaction was stirred at 37 °C for 16 h. The mixture was cooled to room temperature, and the solvent was evaporated to dryness. The residue was resuspended in 3 mL of methanol at 40 °C with stirring. The 40 mL of acetone was dropwise added into mixture to precipitate the product as ammonium salt. The precipitate was collected by centrifugation at 4400 rpm at room temperature for 15 min, and the resulting pellet was washed twice with 30 mL of acetone and dried under high vacuum. The product α-L-threofuranosyl nucleoside 3′-monophosphates (**4a−d**) were obtained as the ammonium salt (white solid) in near quantitative yield.

α-L-Threofuranosyl Thymine-3′-monophosphate (4a). Product yield: 90.8 mg (98.3%, ε_{267} = 9600); ^{1}H NMR (400 MHz, D_2O) δ 7.61 (d, 1H), 5.82 (s, 1H), 4.62 (s, 1H), 4.46 (m, 2H), 4.34 (m, 1H), 1.90 (s, 3H); ^{13}C NMR (100.6 MHz, D_2O) δ 166.9, 151.8, 138.1, 110.5, 92.0, 79.3 (d, $J_{C,P}$ = 4.4 Hz), 78.2 (d, $J_{C,P}$ = 4.2 Hz), 75.1 (d, $J_{C,P}$ = 4.7 Hz), 11.9; ^{31}P NMR (162 MHz, D_2O) δ 0.21; HRMS (ESI) calcd for $C_9H_{13}N_2O_8PNa$ $[M + Na]^+$ 331.0307; found 331.0311.

α-L-Threofuranosyl Cytosine-3′-monophosphate (4b). Product yield: 84.4 mg (96%, ε_{280} = 13 100); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.65 (d, 1H, J = 7.2 Hz), 5.83 (d, 1H, J = 6.8 Hz), 5.65 (s, 1H), 4.38 (s, 1H), 4.28 (s, 2H), 4.07 (d, 1H, J = 7.6 Hz); ^{13}C NMR (125.8 MHz, DMSO-d_6) δ 163.8, 153.4, 143.1, 93.8, 92.7, 78.8, 78.3, 75.1; ^{31}P NMR (162 MHz, DMSO-d_6) δ −0.65; HRMS (ESI-TOF) calcd for $C_8H_{11}N_3O_7P$ $[M - H]^-$ 292.0326; found 292.0335.

α-L-Threofuranosyl Adenine-3′-monophosphate (4c). Product yield: 88.5 mg (93%, ε_{259} = 15 200); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.30 (s, 1H), 8.16 (s, 1H), 5.91 (d, 1H, J = 1.6 Hz), 4.57 (s, 2H), 4.23 (d, 1H, J = 8 Hz), 4.12 (dd, 1H, J = 4, 5.2 Hz); ^{13}C NMR (125.8 MHz, DMSO-d_6) δ 156.0, 153.0, 149.5, 140.2, 118.6, 89.3, 79.3 (d, $J_{C,P}$ = 5.2 Hz), 78.2 (d, $J_{C,P}$ = 4.7 Hz), 73.5 (d, $J_{C,P}$ = 4.8 Hz), 75.1; ^{31}P NMR (162 MHz, DMSO-d_6) δ 1.49; HRMS (ESI-TOF) calcd for $C_9H_{11}N_5O_6P$ $[M - H]^-$ 316.0479; found 316.0447.

α-L-Threofuranosyl Guanine-3′-monophosphate (4d). Product yield: 90.9 mg (91%, ε_{253} = 13 700); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.88 (s, 1H), 5.68 (s, 1H), 4.45 (d, 2H, J = 26.4 Hz); ^{13}C NMR (125.8 MHz, DMSO-d_6) δ 157.0, 154.1, 151.2, 136.3, 116.0, 88.6, 79.2 (d, $J_{C,P}$ = 4.8 Hz), 78.0 (d, $J_{C,P}$ = 3.6 Hz), 73.1 (d, $J_{C,P}$ = 4.0 Hz); ^{31}P NMR (162 MHz, DMSO-d_6) δ −0.17; HRMS (ESI-TOF) calcd for $C_9H_{11}N_5O_7P$ $[M - H]^-$ 332.0407; found 332.0396.

α-L-Threofuranosyl Nucleosides 3′-Phosphor-2-methylimidazolides (5a−d). To a solution containing α-L-threofuranosyl thymidine-3′-monophosphate (**4a−d**) (0.27 mmol) and 2-methylimidazole (2.7 mmol) in 5 mL of anhydrous DMSO were added triethylamine (2.7 mmol), triphenylphosphine (1.08 mmol), and 2,2′-dipyridyldisulfide (1.08 mmol). The reaction was stirred under a nitrogen atmosphere for 6−8 h at room temperature with monitoring by analytical HPLC. After consumption of the starting material, the product was precipitated by the dropwise addition of the reaction mixture to a stirring solution containing 80 mL of acetone, 60 mL of diethyl ether, 5 mL of triethylamine, and 5 mL of saturated $NaClO_4$ in acetone. The precipitate was collected by centrifugation at 4400 rpm for 15 min at room temperature. The pellet was washed twice with 30 mL of washing solution (acetone/diethyl ether 1:1) and dried under high vacuum to afford the α-L-threofuranosyl nucleoside 3′-phosphor-2-methylimidazolides (**5a−d**) as the sodium salt (white solid).

α-L-Threofuranosyl Thymine-3′-phosphor-2-methylimidazolide (5a). Product yield: 97.9 mg (97.4%, ε_{267} = 9600); ^{31}P NMR (162 MHz, D_2O) δ −7.88; HRMS (ESI-TOF) calcd for $C_{13}H_{17}N_4O_7PNa$ $[M + Na]^+$ 395.0732; found 395.0717.

α-L-Threofuranosyl Cytosine-3′-phosphor-2-methylimidazolide (5b). Product yield: 93.1 mg (96.6%, ε_{280} = 13 100); ^{31}P NMR (162 MHz, D_2O) δ −8.07; HRMS (ESI-TOF) calcd for $C_{12}H_{16}N_5O_6PNa$ $[M + Na]^+$ 380.0736; found 380.0735.

α-L-Threofuranosyl Adenine-3′-phosphor-2-methylimidazolide (5c). Product yield: 100.5 mg (97.7%, ε_{259} = 15 200); ^{31}P NMR (162 MHz, D_2O) δ −8.37; HRMS (ESI-TOF) calcd for $C_{13}H_{16}N_7O_5PNa$ $[M + Na]^+$ 404.0848; found 404.0847.

α-L-Threofuranosyl Guanine-3′-phosphor-2-methylimidazolide (5d). Product yield: 104 mg (97%, ε_{253} = 13 700); ^{31}P NMR (162 MHz, D_2O) δ −8.40; HRMS (ESI-TOF) calcd for $C_{13}H_{16}N_7O_6PNa$ $[M + Na]^+$ 420.0797, found 420.0807.

α-L-Threofuranosyl Nucleosides 3′-Triphosphates (6a−d).[21−23] To a solution containing 0.1 mmol of α-L-threofuranosyl nucleosides 3′-phosphor-2-methylimidazolides (**5a−d**) and 2 mL of in anhydrous DMF were added tributylamine (0.2 mmol) and tributylammonium pyrophosphate (0.2 mmol). The reaction mixture was then stirred under nitrogen atmosphere for 8−12 h at room temperature with monitoring by analytical HPLC. After the reaction was finished, the reaction mixture was added dropwise to a stirring solution containing 30 mL of acetone and 5 mL of saturated $NaClO_4$ in acetone. The precipitate was collected by centrifugation at 4400 rpm for 15 min at room temperature and dried under vacuum for 1 h. The crude precipitate was dissolved in 3 mL of 0.1 M triethylammonium acetate buffer and purified by a semipreparative HPLC. Fractions containing triphosphates were collected and concentrated, pH adjusted by triethylamine to 7.0, and lyophilized to afford the product as a triethylammonium salt. The solid product was resuspended in 3 mL of methanol and was added dropwise to a solution containing 40 mL of acetone and 2 mL of saturated $NaClO_4$ in acetone. The solution was centrifuged at 4400 rpm for 15 min at room temperature. The supernatant was discarded, and the pellet was washed with 30 mL of acetone and dried under vacuum for 1 h. The resulting white solid was dissolved in RNase-free water containing 10 mM of Tris pH 8.0 to afford the α-L-threofuranosyl nucleotide 3′-triphosphate (**6a−d**) solution.

Threofuranosyl Thymine-3′-triphosphate (6a). Product yield after HPLC purification: 42.6 mg (91.1%, ε_{267} = 9600).

Threofuranosyl Cytosine-3′-triphosphate (6b). Product yield after HPLC purification: 40 mg (88.3%, ε_{280} = 13 100).

Threofuranosyl Adenine-3′-triphosphate (6c). Product yield after HPLC purification: 43 mg (90.1%, ε_{259} = 15 200).

Threofuranosyl Guanine-3′-triphosphate (6d). Product yield after HPLC purification: 44.2 mg (89.7%, ε_{253} = 13 700).

Thermal Stability Analysis of TNA Triphosphates. A small volume (30 μL) of 4 mM of **6a−d** in 10 mM of Tris buffer (pH 8) was maintained at different temperatures (4, 24, and 37 °C) for a period of 8 days. At specific time periods (1, 5, and 8 days), 5 μL of sample was

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transferred to 150 μL of HPLC running buffer [0.1 M triethylammoniumacetate (TEAA)], and the samples (50 μL) were analyzed by analytical reverse-phase HPLC with a gradient of 0 to 7% acetonitrile in 0.1 M TEAA buffer over 40 min. Analytical HPLC traces were analyzed, and relative peak areas are reported in Table 1.

Polymerase-Mediated Primer Extension. The primer extension experiment was done in a single PCR tube with 100 μL of reaction volume containing a DNA primer−template complex (50 pmol). The complex was labeled with an IR800 dye at the 5′-end of the DNA primer. The primer−template complex was annealed in 1× thermoPol buffer (20 mM Tris·HCl, 0 mM $(NH_4)_2SO_4$), 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8) by heating for 5 min at 95 °C and cooling for 10 min at 4 °C. An engineered polymerase, KOD-RI (10 μL), was pretreated with $MnCl_2$ (1 mM) and added to the reaction mixture. Newly formed **6a−d** (100 μM) were then added to the reaction mixture, and the solution was incubated for 3 h at 55 °C. The reaction was analyzed by denaturing polyacrylamide gel electrophoresis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.7b00892.

^1H, ^{13}C, and ^{31}P NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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