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Substrate/Inhibition Studies of Bacteriophage T7 RNA Polymerase with the 5′-Triphosphate Derivative of a Ring-Expanded ('Fat') Nucleoside Possessing Potent Antiviral and Anticancer Activities

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Abstract—As part of an effort to explore the mechanism of potent, broad spectrum antiviral and anticancer activities of a number of ring-expanded ('fat') nucleosides that we recently reported, a representative 'fat' nucleoside 4,6-diamino-8-imino-8*H*-1-β-D-ribofuranosylimidazo[4,5-*e*][1,3]diazepine (**1**) was converted to its 5′-triphosphate derivative (**2**), and biochemically screened for possible inhibition of nucleic acid polymerase activity, employing synthetic DNA templates and the bacteriophage T7 RNA polymerase as a representative polymerase. Our results suggest that **2** is a moderate inhibitor of T7 RNA polymerase, and that the 5′-triphosphate moiety of **2** appears to be essential for inhibition as nucleoside **1** alone failed to inhibit the polymerase reaction.

Introduction

Ring-expanded nucleosides and nucleotides are potentially useful probes for nucleic acid metabolism, structure, and function. With their structural resemblance to natural purines, they are a rich source of substrates or inhibitors of enzymes of nucleic acid metabolism as well as of those requiring energy cofactors such as ATP or GTP. As ring-expansion is anticipated to considerably affect the electronic, spatial, and geometric characteristics, they are also excellent probes for steric and conformational constraints of nucleic acid double-helices.

We have recently discovered that a number of ring-expanded ('fat') nucleosides that we have synthesized[1–6] possess potent, broad spectrum in vitro antiviral and anticancer activities with minimum toxicity.[7–11] Included in this list is the diamino-imino-substituted 'fat' nucleoside (**1**), whose synthesis we reported in 1994 (Scheme 1).[6] This nucleoside, although not the most potent of all the 'fat' nucleosides that exhibited the antiviral and/or anticancer activity, was nevertheless our initial compound of choice as a prototype for further explorations into the mechanism of action of biological activity of ring-expanded nucleosides in general containing analogous heterocyclic ring systems. This choice was based upon several considerations: (1) the synthesis of **1** is simple, short (only three steps), and efficient; (2) compound is reasonably stable to normal physiological and biochemical experimental conditions; (3) nucleoside **1** is planar as suggested by molecular modeling[12] and is potentially aromatic;[13] (4) a planar, larger surface area of **1** as compared to that of purines may, in addition, result in enhanced hydrophobic interactions with nucleic acid bases if **1** gets incorporated into nucleic acid double-helices; and (5) the compound is theoretically capable of existing in several tautomeric forms in solution as shown above, and depending upon what particular tautomeric form it assumes, **1** can mimic adenine, guanine, and/or isoguanine, in addition

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to 2,6-diaminopurine to which it has the best structural resemblance.

The last three features mentioned under items (3), (4) and (5) above allow one to investigate several intriguing possibilities for the mechanism of biological activity of the compound, including that: (a) **1** could be phosphorylated in vivo by kinases to its 5′-triphosphate derivative, and subsequently incorporated into viral or tumor nucleic acids during transcription of a DNA/RNA template by a polymerase, and cause double-helical distortion, ultimately leading to chain termination; (b) **1** could bind to an active or allosteric site of the polymerase, either as a nucleoside or nucleotide, and cause competitive or non-competitive inhibition; or (c) **1** could be a substrate or inhibitor of any or several of the enzymes of purine metabolism, mimicking one of the natural purines, and thus act as an effective antimetabolite or inhibitor. The work presented here is aimed at exploring the first two (a and b) of these possibilities.

In view of the fact that nucleoside **1** is a riboside, an RNA polymerase was the logical choice for initial biochemical explorations employing the 5′-triphosphate derivative of **1**. The most commonly employed template-dependent RNA-polymerase for biochemical investigations and preparations of RNA transcripts is T7 RNA polymerase from bacteriophage T7.[14] Studies of modified nucleosides with this enzyme have been reported, for example, it has been shown to accept base-modified nucleotides as substrates.[15] Thus, base pairs between isoguanosine and isocytidine, and also between xanthosine and diaminopyrimidine nucleosides can be formed using the appropriate template.[15,16] With templates containing the 2′-deoxy-1-methylpseudouridine ($d^m\Psi$), T7 RNA polymerase catalyzes the incorporation of either adenosine triphosphate or formycin triphosphate into a growing chain of RNA with the same efficiency as with templates containing thymidine.[17] In each case, the overall rate of synthesis of full-length products containing formycin is about one-tenth the rate of synthesis of analogous products containing adenosine.[17] The sugar-modified nucleotide analogues, 2′-fluoro-2′-deoxynucleoside-5′-triphosphate and 2′-amino-2′deoxyuridine-5′-triphosphate, are also substrates for T7 RNA polymerase. Transcription of two tRNA genes indicated that such templates can be transcribed to full-length products essentially without premature termination using any of the analogues as substrates.[18] On the other hand, there are also known nucleotide analogues which can inhibit the synthesis of RNA without being incorporated into RNA. For example, 5-formyl-l-(α-D-ribofuranosyl)uracil-5′-triphosphate is a noncompetitive inhibitor of *Escherichia coli* DNA dependent RNA polymerase.[19] Similarly, the regulatory nucleotide ppGpp has been shown to slow down the transcriptional elongation process of *E. coli* RNA polymerase by binding and altering the enzyme structure in a manner that impedes the passage of the protein through certain DNA sequences.[20]

This report describes the effects of 4,6-diamino-8-imino-8*H*-1-β-D-ribofuranosylimidazo[4,5-*e*][1,3]diazepine (**1**) and its 5′-triphosphate derivative **2** on the synthesis of RNA transcripts catalyzed by bacteriophage T7 RNA polymerase.

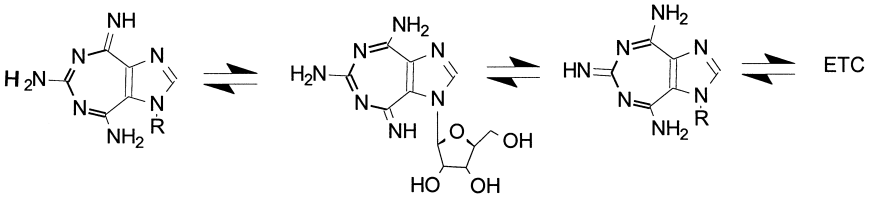
Chemistry

Nucleoside **1** was synthesized using the procedure that we reported earlier,[6] except that four equivalents of guanidine instead of equimolar amounts of guanidine and 4,5-dicyanoimidazole were employed for condensation to prepare the required heterocyclic base 4,6,8-triaminoimidazo[4,5-*e*][1,3]diazepine in a consistently high yield of 80% or more. The Vorbrüggen ribosylation,[21,22] followed by deprotection of the sugar hydroxyls, as reported in the published procedure,[6] afforded **1** in ≥77% overall yield for the two steps. The method of Vrang et al.[23] was employed for phosphorylation of **1** (Scheme 2), which included: (a) treatment with phosphorus oxychloride to phosphorylate the 5′-hydroxyl function; and (b) condensation with bis(tributylammonium) pyrophosphate at 0°C to form the triphosphate. The yield of the reaction was ~50%. However, during purification on DEAE -Sephadex A-25 column using TEAB buffer, partial decomposition of the 5′-triphosphate was observed. The compound was isolated in a pure form by preparative TLC on cellulose plates, using methanol: water (7:3) as the developing solvent. The compound was precipitated as a sodium salt with NaI in acetone, giving the target 5′-triphosphate derivative **2**. The latter was characterized by ^1H and ^{31}P NMR, as well as by high resolution mass spectral analysis.

Results and Discussion

Assessment of incorporation of 'fat' nucleoside-5′-triphosphate (FNTP, 2) during transcription catalyzed by T7 RNA polymerase

In order to determine if the 'fat' nucleotide **2** (FNTP) is incorporated into RNA during transcription, experiments



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Scheme 1.

were performed using bacteriophage T7 RNA polymerase (RNAP) and a synthetic 42-mer DNA template oligonucleotide shown below that is annealed to a 17-nucleotide oligomer that encodes the promoter which is specific for T7 RNAP. This promoter-template enables the synthesis of 25-mer RNA in which the purine analogue could potentially be incorporated at positions +1, +11, +13, +15, +21, +22, and +23.

5′T AAT ACG ACT CAC TAT A

3′A TTA TGC TGA GTG ATA TCG GAA GGA AGC

ACG TGG GAG CTT AA 5′

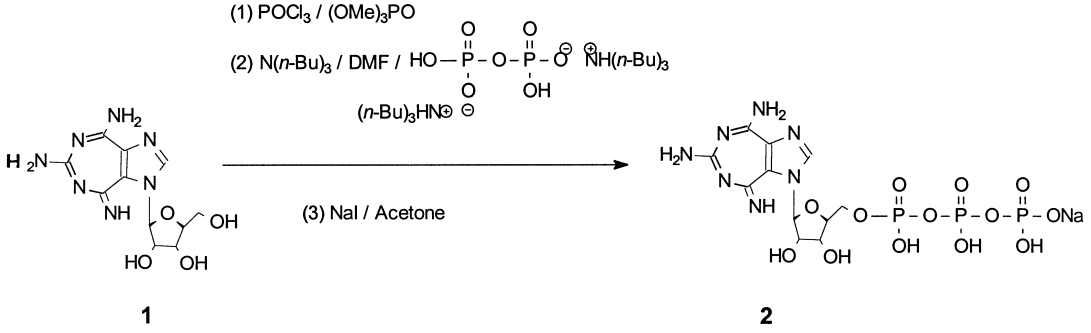
Transcription reaction conditions were optimized with the use of α-^{32}P CTP for labeling RNA transcripts. Transcription products obtained from the 42-mer template, using 1 mM each of four natural NTPs, contained a 25-mer RNA as the major product, together with shorter and longer labeled transcripts (Fig. 1, lane 1). The additional bands observed are consistent with the known ability of T7 RNA polymerase to add non-coded residues at the 3′-end of transcripts.[24] With the use of 1 mM nucleotide **2** (FNTP), the yield of transcription was reduced (lane 2).

The relative ability of nucleotide **2** to act as a purine in T7 RNA polymerase-catalyzed polymerization was assessed by comparison of reactions from which ATP or GTP was omitted. In the absence of ATP (lane 3), when only CTP, GTP and UTP were used, a 14-mer RNA was synthesized as the major product. When nucleotide **2** was used (lane 4), the 14-mer RNA was once again observed as the major product, and none of the longer transcripts were seen. These results indicate that nucleotide **2** is not incorporated into the nascent chain.

In the absence of GTP (1 mM GMP was used to initiate the synthesis of RNA) (Fig. 1, lane 5), a 10-mer was seen as the major product. When nucleotide **2** was used (lane 6), a 10-mer RNA was again seen as the major product, and the extent of synthesis was reduced as well. These results suggest that nucleotide **2** is not a substrate for T7 polymerase.

Assessment of inhibition of the T7 RNA polymerase-catalyzed transcription by 'fat' nucleoside-5′-triphosphate (FNTP, 2)

With the use of a synthetic 37-mer DNA template, annealed to a 17-nucleotide promoter strand for T7 RNAP as shown below, a 20 nucleotide-long transcript



Scheme 2.

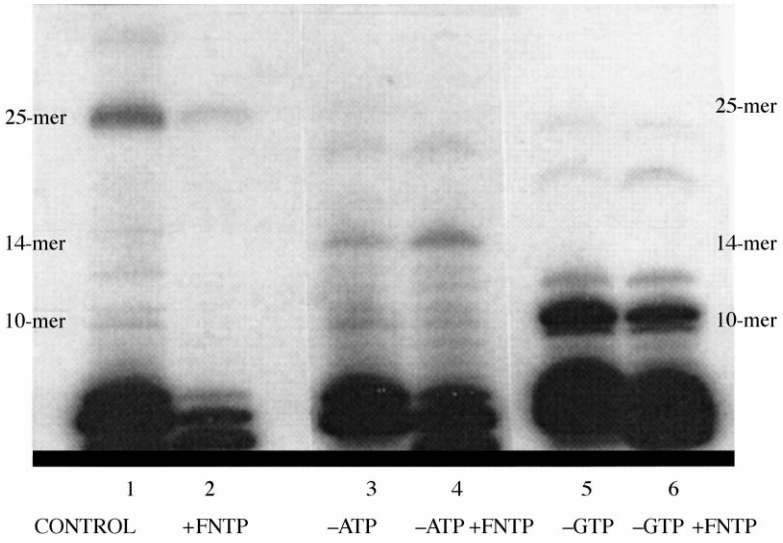


Figure 1. Autoradiograph of gels obtained from electrophoretic separation of transcription products using the 42-mer template: 1 mM each of NTP (lane 1); with the addition of 1 mM FNTP (lane 2); with 1 mM each of GTP, CTP, and UTP (lane 3); with 1 mM each of GTP, CTP, UTP and FNTP (lane 4); with 1 mM each of ATP, CTP, and UTP plus 1 mM of GMP to initiate RNA synthesis (lane 5); with 1 mM each of ATP, CTP, UTP, and FNTP, plus 1 mM of GMP to initiate RNA synthesis (lane 6).

RNA was obtained when the reaction mixture contained all four NTPs in appropriate concentrations, along with many shorter 'abortive' fragments.[24] For the reaction with modified nucleotide,

5′T AAT ACG ACT CAC TAT A
3′A TTA TGC TGA GTG ATA TCC TGA
+1
TCG CCT CCG ATC AGG 5′
+10 +20

typically the concentration of the appropriate natural nucleotide was reduced.[18] This strategy is however less effective if the analogue is the first nucleotide in the transcript because of the large number of 'abortive' initiation products.[25] In our experiments, we compared the synthesis of RNA under standard conditions (using 4 NTPs in 0.2 mM concentration each) with the synthesis of RNA using an additional 'fat' nucleoside triphosphate **2** (FNTP) in varying (0.05–10 mM) concentrations. The reaction mixtures were incubated at 37°C for 2 h, and the products were denatured in a buffer containing urea. The products were separated by electrophoresis on 20% polyacrylamide-urea gel and visualized by autoradiography.

Figure 2 is an autoradiograph of a typical gel in which the products of the four reactions were analyzed. With autoradiography, the bands of the long 20-mer RNA along with those of abortive shorter fragments could be seen (Fig. 2, lane 1). In the reactions containing 'fat' nucleotide **2**, the bands of long RNA decreased in intensity with increasing concentrations of nucleotide **2** (lanes 2–4).

The quantitation of the products formed was carried out either by (laser) scanning densitometry of autoradiographs of the [α-^{32}P]GTP–labeled transcripts that were analyzed by gel electrophoresis, or with the use of a PhosphorImager. The assayed yields of transcription at varying concentrations of nucleotide **2** are presented as percentages of the values obtained with the absence of nucleotide **2** (Fig. 3).

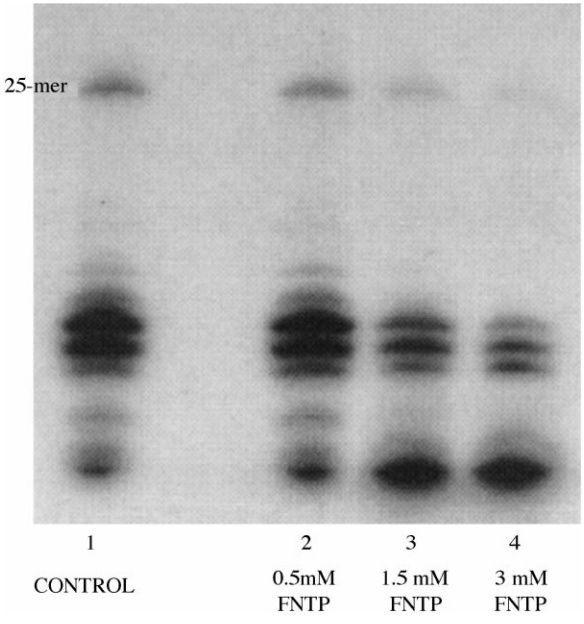


Figure 2. Autoradiograph of the products of transcription experiments using a 37-mer template: 0.2 mM each of NTP (lane 1); with addition of 0.5 mM FNTP (lane 2), with addition of 1.5 mM FNTP (lane 3); with addition of 3 mM FNTP (lane 4).

Effect of nucleoside 1 on transcription catalyzed by T7 RNA polymerase

Since, as delineated above, there was no evidence that the nucleotide **2** is incorporated into RNA during the transcription of a DNA template by T7 RNA polymerase, we wondered if the 5′-triphosphate derivative would at all be necessary for the inhibition. Consequently, we repeated the above transcription experiments using nucleoside **1** in place of nucleotide **2**. The autoradiographs of the polyacrylamide gels clearly indicate that there is neither incorporation nor inhibition by **1** in the transcription reaction.

Conclusions

In an effort to explore the mechanism of broad spectrum antiviral and anticancer activities of ring-expanded ('fat') nucleosides in general, a representative 'fat' nucleoside **1** was converted into its 5′-triphosphate derivative **2**. The transcriptions of two DNA templates (a 42-mer and a 37mer) were investigated in the presence and absence of nucleotide **2**, using the bacteriophage T7 RNA polymerase. Our results suggest that nucleotide **2** is a moderate inhibitor of T7 polymerase, but there is no indication that **2** is being incorporated into RNA during transcription. The observed low inhibition of an RNA polymerase may indeed have some beneficial implications and correlations in terms of the observed low or little toxicity of **1** since HBV is known to employ the host RNA polymerase to synthesize pregenomic HBV RNA and other smaller RNA transcripts from the HBV DNA template in the host nucleus.[26,27] Only after leaving the cell nucleus and entrance into the cytoplasm, these RNA molecules are further reverse transcribed by HBV DNA polyrnerase to produce ±DNA strands and/or directly used as mRNA for further translation and packaging into viral particles as

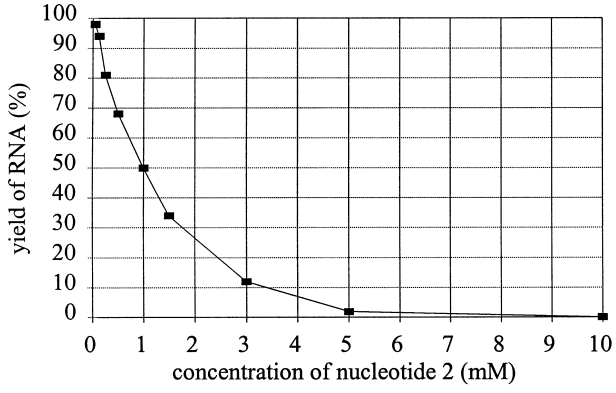


Figure 3. Relative yields (determined by scanning densitometry of autoradiographs, analyzed by gel electrophoresis of the [α^{32}P]GTP-labeled transcript) of full-length RNA transcript (20-mer) in reactions using T7 RNA polymerase, and varying concentrations of nucleotide **2**, as described under Experimental.

well as viral surface and core proteins.[26,27] Since nucleoside **1** itself failed to cause any inhibition of transcription, the 5′-triphosphate moiety appears to be critical for the observed inhibition of the polymerase. These results lead us to believe that the nucleotide **2** may simply be binding to the polymerase at an active or allosteric site to cause inhibition without actually being incorporated into the developing nucleic acid during the transcription process. While no direct mechanistic extrapolations or correlations can be made between the observed T7 RNA polymerase inhibition by nucleotide **2** and the observed potent, broad spectrum antiviral and anticancer activities of nucleoside **1**, the results are nevertheless interesting and encouraging for further explorations of polymerases as one of the possible mechanistic targets. In view of the potent anti-HBV activity of **1**, HBV DNA polymerase is a logical target for further studies. Nevertheless, due to the fact that other alternate mechanistic possibilities, such as the ones mentioned as item (c) in the Introduction, are yet to be explored, it is not possible to pinpoint polymerase inhibition as the principal mode of action of these intriguing nucleoside analogues. In any event, should the polymerase inhibition be a viable mechanism of the observed biological activity, then nucleoside **1** will necessarily have to be transformed in vivo by the appropriate kinases into the corresponding 5′-triphosphate derivative **2**.

Experimental

Chemistry

^{1}H NMR spectra were recorded on a General Electric QE-300 (300 MHz) instrument. ^{31}P NMR spectra were run on a Bruker Avance 600 MHz instrument operating at 242.93 MHz for phosphorus nucleus. The chemical shift data are reported with reference to Me_4Si for ^{1}H NMR and to H_3PO_4 for ^{31}P NMR, used as internal and external standards, respectively, and are shown in the following format: chemical shift, multiplicity (s = singlet, d = doublet, and m = multiplet), integration, coupling constants, and assignment of resonance. The mass spectral data were obtained from the Mass Spectral Facility, Department of Biochemistry, Michigan State University, East Lansing, MI. Thin layer chromatography was performed on Merck Kieselgel 60 GF_{254} plates (0.2 mm thickness). Melting points were determined on a Thomas — Hoover capillary melting point apparatus, and are uncorrected.

Synthesis of 4,6-diamino-8-imino-8*H*-1-β-D-ribofuranosylimidazo[4,5-*e*][1,3]diazepine-5′-phosphate (2). To an ice-cold suspension of nucleoside **1**[6] (77 mg, 0.2 mmol) in 0.7 mL of trimethyl phosphate, 80 μL (0.85 mmol) of phosphorus oxychloride was added. The mixture was stirred at 0°C for 5 h, until all precipitate had dissolved. Then a solution of bis(tributylammonium) pyrophosphate (782 mg, 1.95 mmol) in 1.5 mL of dry DMF and 0.4 mL (1.95 mmol) of dry tributylamine was added, and the mixture was stirred in an ice-bath for 30 min. The reaction mixture was quenched by addition of triethylammonium hydrogen carbonate (TEAB) buffer (1 M, pH 7.5) so as to adjust the pH to about 7. The solution was extracted with EtOAc and the aqueous layer (≈20 mL) was loaded onto the DEAE Sephadex A-25 column (40×2.5 cm) which was pre-equilibrated with 0.01 M TEAB buffer. The column was subsequently eluted with a linear gradient of TEAB buffer (0.01–1 M, 1200 mL). The fractions were monitored by TLC (2-propanol-concd NH_4OH–water, 7:1:2). Appropriate UV-absorbing fractions were pooled and evaporated. The residue was purified by preparative TLC on Cellulose F plates, using a mixture of methanol:water (7:3) as the developing solvent. The product 5′-triphosphate obtained from the preparative TLC was dissolved in 3 mL of water and reprecipitated with 100 mg of NaI in 10 mL of acetone. The precipitated sodium salt was collected by centrifugation, washed with acetone, and dried over P_2O_5 to yield **2** (20 mg, 17.5%), and was stored in a −20°C freezer: R_f (silica gel, 2-propanol–concd NH_4OH–water, 7:1:2) 0.06; ^{1}H NMR (D_2O) δ 8.5 (s, 1H, H-2), 6.1 (d, J = 7.2 Hz, 1H, sugar H-1′), 4.48 (m, 1H, sugar H), 4.13 (m, 1H, sugar H), 3.74 (m, 1H, sugar H), 3.53–3.21 (m, 2H, sugar 5′-H); ^{31}P NMR (D_2O) δ −6.41 (γ-P), −7.89 (α-P), −20.16 (β-P); UV (pH 7) λ_{max} 246 nm (ε 32,000); mass spectrum (FAB) m/z 572 (MH^+), HRMS calcd for $C_{11}H_{18}O_{13}N_7P_3Na$: 572.0073. Found: 572.0079.

Transcription reactions. General. Nucleoside triphosphates were purchased from Sigma, dissolved in water to concentration of 50 mM adjusted to pH 8 with 1 M NaOH, and then stored at −20°C. [α^{32}P]-CTP and [α^{32}P]-GTP (specific activity 3000 Ci/mmol) were purchased from Amersham. Synthetic DNAs were from Gibco BRL, Life Technologies; Inc., Gaithersburg MD. DNAs were PAGE purified, and their concentrations were determined spectrophotometrically.

The oligonucleotide templates were prepared by combining the two complementary oligonucleotides, each at a final concentration of 2.5 μM, in 10 mM Tris–HCl pH 8, 0.1 mM EDTA, heating to 80°C for 10 min and slow cooling to room temperature. The bacteriophage T7 polymerase (20 units/μL) was purchased from Promega-P2075. Kodak XAR-5 films were employed for autoradiography.

Transcription reaction conditions and procedures. Transcription samples (total volume 10 μL) contained: 40 mM Tris–HCl, pH 8.1, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 1 mM spermidine, 0.01% Triton-X 100, 50 μg/mL bovine serum albumin, 250 nM synthetic DNA template with a promotor for T7 RNAP, four units of T7 RNA polymerase, and either 0.2 mM (for 37-mer template) or 1.0 mM (for 42-mer template) ribonucleoside triphosphates. A single α ^{32}P-labeled NTP was added for labeling (typically 1 μL of 3000 Ci/mmol). Inhibition assays were monitored with varying concentrations (0.05–10 mM) of nucleotide **2**. The reaction mixtures were incubated at 37°C for 2 h at which time 1 μL of 50% glycerol and 3 μL of formamide containing 0.02% bromophenol blue and 0.02% xylene cyanol were added to each reaction. The mixtures were heated at 80°C for 3 min, and loaded onto a pre-electrophoresed 20% denaturing gel (19:1 acrylamide:bisacrylamide, 8 M urea). Electrophoresis was performed

on slab gels (18×16 cm, or 35×44 cm).[28] Gels were pre-electrophoresed (400 V for 2 h) and samples were run at 400 V for 2–3 h or at 1000 V overnight. Bands were visualized by autoradiography or by phosphorimaging. The latter method involved exposing the gel to a Phosphor screen for 12–15 h, followed by scanning using a Molecular Dynamics Storm instrument. The intensities of RNA bands were quantitated using the Image Quant™ software.

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