

Enzymatic Incorporation of Emissive Pyrimidine Ribonucleotides

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In memory of Thomas L. Netzel

Abstract: The enzymatic incorporation of a series of emissive pyrimidine analogues into RNA oligonucleotides is explored. T7 RNA polymerase is challenged with accepting three non-natural, yet related, triphosphates as substrates and incorporating them into diverse RNA transcripts. The three ribonucleoside triphosphates differ only in the modification of their uracil nucleus and include a thieno[3,2-d]pyrimidine nucleoside, a thieno[3,4-d]pyrimidine derivative, and a uridine containing a thiophene ring conjugated at its 5-position. All thiophene-containing uridine triphosphates (UTPs) get incorporated into RNA oligonucleotides at positions

that are remote to the promoter, although the yields of the transcripts vary compared with the transcript obtained with only native triphosphates. Among the three derivatives, the 5-modified UTP is found to be the most “polymerase-friendly” and is well accommodated by T7 RNA polymerase. Although the fused thiophene analogues cannot be incorporated next to the promoter region, the 5-modified non-natural UTP gets incorporated

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near the promoter (albeit in relatively low yields) and even in multiple copies. Labeling experiments shed light on the mediocre incorporation of the fused analogues, suggesting the enzyme frequently pauses at the incorporation position. When incorporation does take place, the enzyme fails to elongate the modified oligonucleotide and yields aborted transcripts. Taken together, these results highlight the versatility and robustness, as well as the scope and limitation, of T7 RNA polymerase in accepting and incorporating reporter nucleotides into modified RNA transcripts.

Introduction

RNA is no longer viewed as a passive carrier of genetic information. Rather, this complex biopolymer is now known to be involved in key cellular events, far beyond its classical role as a mediator between DNA and proteins.^[1] Recent discoveries, demonstrating new regulatory roles for RNA, such as the functions seen in RNA interference and riboswitches,^[2,3] further fueled interest in RNA as a central regulatory molecule. RNA is therefore now viewed as a potential target for the control of cell function by exogenous ligands and for therapeutic intervention by drug-like small molecules.^[4,5] Much like the development of ligands as modulators of protein function, one could envisage the discovery of

small molecules as specific effectors of RNA function and hence of cellular response.^[6]

To explore RNA recognition events and to fabricate discovery assays for biologically active ligands, effective biophysical tools need to be advanced.^[7] Fluorescence spectroscopy is among the most effective and sensitive techniques and researchers have long relied on such tools to decipher the fundamental structural, folding, and recognition features of biomolecules. Many proteins contain fluorescent aromatic amino acids (e.g., tryptophan), or interact with fluorescent cofactors (e.g., NADH), thus providing researchers with inherently emissive, “built-in” probes.^[8] RNA molecules, in contrast, present challenges as the native nucleobases are practically nonemissive.^[9] To overcome these limitations, fluorescent nucleoside analogues can be synthesized and incorporated into reporter oligonucleotides.^[10] A key criterion for the potential utility of modified nucleosides is their ability to replace the native nucleosides while preserving the folding and recognition properties of the unmodified oligonucleotides.^[11] Importantly, to serve as reporter probes, such nucleobases have to display favorable photophysical characteristics and, in particular, be responsive to changes in their mi-

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croenvironment.^[12] Several successful motifs of isosteric nucleobase analogues have been reported and implemented.^[13,14]

Two major protocols are available for the modification of RNA oligonucleotides including solid-phase and enzymatic syntheses. Although solid-phase synthesis can be applied to almost any nucleoside analogue (provided it can be converted into a suitable building block),^[15] enzymatic synthesis can be employed only if RNA polymerase accepts the nucleoside triphosphate as a building block and incorporates it effectively.^[16,17] Despite this potential obstacle, transcription reactions are, in many cases, highly advantageous, owing to the following practical considerations: a) the necessary triphosphate can typically be synthesized in one step from unprotected nucleosides circumventing multistep synthetic protocols; b) reasonably large quantities of RNA can be synthesized by high turnover transcription reactions with relatively small amounts of the modified triphosphates.^[18] These factors make this method within reach to almost all laboratories that are not necessarily equipped for complex organic synthesis and solid-phase RNA synthesis.^[19,20]

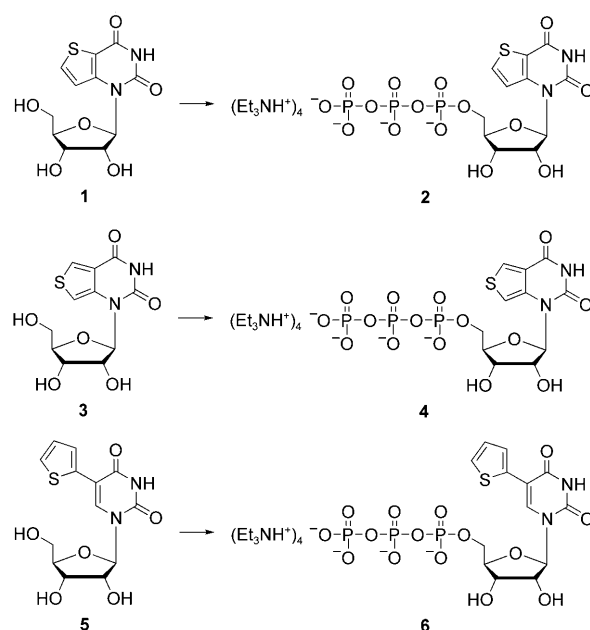
To explore the utility and limitations of *in vitro* transcription reactions for the incorporation of fluorescent non-natural pyrimidine nucleotides, we comprehensively compare the behavior of three related triphosphates in the synthesis of emissive RNA oligonucleotides. Two isomeric thiophene-fused pyrimidine analogues (**1** and **3**) and one pyrimidine conjugated to thiophene at the 5-position (**5**) are phosphorylated and subjected to *in vitro* transcription reactions with nine different DNA templates.^[21] Our results shed light on the structural features most accommodated by RNA polymerase in accepting modified analogues as substrates and the impact that proximity to the promoter has on the success of transcription reactions that generate emissive RNA oligonucleotides.

Results and Discussion

Synthesis and Basic Photophysical Properties

The fused-thiophene-modified ribonucleosides **1** and **3** were synthesized by employing our previously reported procedures.^[11f,18c] The new 5-thieno-conjugated uridine **5** was prepared by a Stille cross-coupling reaction by using 5-iodouridine and 2-(tributylstannyl)thiophene in the presence of a catalytic amount of palladium(0), using protocols used for related analogues.^[13h] Ribonucleosides **1**, **3**, and **5** were then converted to the 5'-triphosphates **2**, **4**, and **6**, respectively, by using freshly distilled POCl₃ and tributyl ammonium pyrophosphate (Scheme 1).^[22] The analytically pure triphosphates, obtained by anion-exchange chromatography followed by HPLC purification, were thoroughly characterized by using mass spectrometry as well as, ¹H, ¹³C, and ³¹P NMR spectroscopy.

All nucleosides investigated are emissive and responsive (Table 1).^[11f,18c] However, their absorption and emission maxima differ significantly. Nucleoside **1**, built around the



Scheme 1. Syntheses of thiophene-modified nucleoside triphosphates **2**, **4**, and **6**. Reagents and conditions: i) POCl₃, (MeO)₃PO, 0 °C; ii) tributylammonium pyrophosphate, Bu₃N, 0–4 °C. See the Experimental Section for details.

Table 1. Basic photophysical characteristics of nucleosides **1**, **3**, and **5** in different solvents.

Nucleoside	Solvent	$\lambda_{\text{max}}^{\text{[a]}}$ [nm]	$\lambda_{\text{em}}^{\text{[a]}}$ [nm]	$I_{\text{rel}}^{\text{[b]}}$	$\Phi_{\text{r}}^{\text{[c]}}$
1	water	292	351	1.5	0.058 ± 0.001
	methanol	294	350	1.1	
	acetonitrile	294	344	1.0	
3	water	304	412	3.3	0.48 ± 0.05
	methanol	304	404	2.9	
	acetonitrile	304	386	1.0	
5	water	315	439	1.3	0.024 ± 0.001
	methanol	319	428	1.3	
	acetonitrile	319	421	1.0	

[a] Lowest energy maximum is given. [b] Relative emission intensity with respect to intensity in acetonitrile. [c] Relative quantum yield in water. For details, see the Supporting Information.

thieno[3,2-d]pyrimidine core, displays the highest energy of absorption and emission in water, both of which are in the ultraviolet region. Nucleoside **3**, containing the thieno[3,4-d]pyrimidine, and nucleoside **5**, containing a conjugated thiophene at position 5, both display absorption maxima above 300 nm and emission in the visible range when in a polar environment. As solvent polarity is decreased from water to methanol, and finally acetonitrile, a hypsochromic shift and hypochromic effects are observed for the emission maxima of all nucleosides (Table 1). The most responsive nucleoside is **3**, which exhibits a strong emission in water ($\Phi_{\text{r}}=0.48$) with a more than threefold higher intensity relative to its emission in acetonitrile.^[18c] Notably, a good correlation between the emission energy and $E_{\text{T}}(30)$, a micro-

scopic solvent polarity parameter, is seen for all nucleosides (Figure S1 in the Supporting Information), which suggests utility as nucleobase probes.^[14g]

Enzymatic Incorporation of Modified Triphosphates

To investigate the enzymatic incorporation of the modified nucleotides into RNA oligonucleotides, transcription reactions with T7 RNA polymerase and triphosphates **2**, **4**, and **6** were carried out. Short DNA templates were designed to evaluate the incorporation efficiency of a single or several modified nucleotides into the RNA transcript and the impact of the modification position on the yield of the modified RNA oligonucleotides (Figure 1). All promoter-template duplexes were constructed by annealing an 18-mer oligodeoxyribonucleotide, containing the T7 RNA polymerase consensus sequence, and synthetic single-stranded DNA templates **7a–i** (Figure 1). The templates possess dA residues to direct the incorporation of the modified U analogues and a single T residue at the 5' end to guide the incorporation of a unique A residue at the 3' end of the transcript. When performed in the presence of α -³²P adenosine triphosphate (ATP), a successful runoff transcription reaction would result in the formation of a labeled RNA transcript. Early-terminated transcripts would remain undetected following gel electrophoretic separation.

To first compare the incorporation efficiency of the three unnatural triphosphates, a promoter–template duplex was constructed with DNA template **7a** (Figure 1).^[17d] This template was designed to possess a unique dA residue away from the promoter region at position +7. A phosphorimage of the PAGE-resolved transcription reaction with template **7a** in the presence of the modified triphosphates revealed the formation of the corresponding 10-mer full-length RNA product (Figure 2, lanes 3–5). The incorporation efficiency of triphosphates **2**, **4**, and **6** as compared with uridine tri-

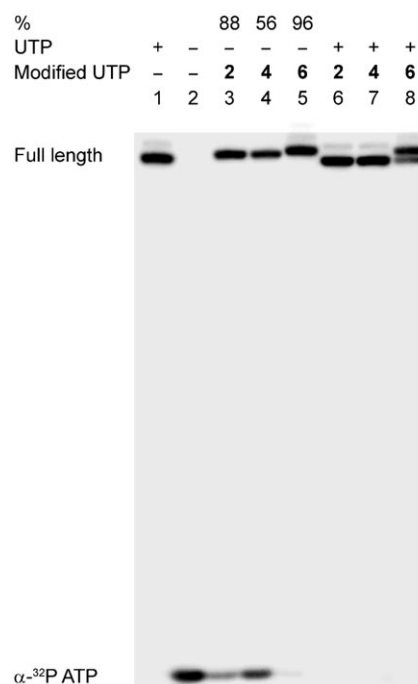


Figure 2. Transcription reactions with template **7a** in the presence of thiophene-modified UTPs **2**, **4**, and **6**. Lane 1: control transcription reaction in the presence of natural NTPs. Lane 2: control reaction in the absence of UTP and modified UTPs. Lanes 3–5: reaction in the presence of **2**, **4**, and **6**, respectively. Lanes 6–8: reaction in the presence of equimolar concentration of **2/4/6** and UTP, respectively. Incorporation efficiencies of **2**, **4**, and **6** are reported with respect to transcription in the presence of UTP. All reactions were performed in triplicate and the standard deviations were $\pm 4\%$.

phosphate (UTP) was found to be $88 \pm 3\%$, $56 \pm 4\%$, and $96 \pm 3\%$, respectively. The successful incorporation of higher-molecular-weight modified nucleosides into the RNA

transcripts is evident from the slower migration of the transcripts **8**, **9**, and **10a** as compared with the transcript formed in the presence of only natural nucleoside triphosphates (NTPs; Figure 2, compare lane 1 and lanes 3–5). Importantly, control experiments in the absence of UTP and modified UTPs yielded no full-length transcripts (Figure 2, lane 2). This indicates that the full-length transcription products seen in Figure 2, lanes 3–5 are formed as a result of incorporation of the modified UTPs and not owing to adventitious misincorporation.

The apparent difference in migration of the unmodified

T7 promoter	5' TAA TAC GAC TCA CTA TAG 3'	
Template 7a	3' ATT ATG CTG AGT GAT ATC GCG GCA CGT 5'	
	T7 RNA polymerase natural NTPs/2/4/6 ↓ α-³²P ATP	
	5' _{ppp} GCG CCG <u>X</u> GC A 3' X = 1 (8)	
		X = 3 (9)
		X = 5 (10a)
DNA templates	Transcripts	
7a 3' ATT ATG CTG AGT GAT ATC GCG GCA CGT 5'	10a 5' _{ppp} GCG CCG <u>5</u> GC A 3'	
7b 3' ATT ATG CTG AGT GAT ATC ACG GCG CGT 5'	10b 5' _{ppp} G <u>5</u> G CCG CGC A 3'	
7c 3' ATT ATG CTG AGT GAT ATC GAG GCC CGT 5'	10c 5' _{ppp} GCG C <u>5</u> CCG GGC A 3'	
7d 3' ATT ATG CTG AGT GAT ATC GCA GCG CGT 5'	10d 5' _{ppp} GCG C <u>5</u> G CGC A 3'	
7e 3' ATT ATG CTG AGT GAT ATC GCA GCA CGT 5'	10e 5' _{ppp} GCG C <u>5</u> G <u>5</u> GC A 3'	
7f 3' ATT ATG CTG AGT GAT ATC GCG GAA CGT 5'	10f 5' _{ppp} GCG CC <u>5</u> <u>5</u> GC A 3'	
7g 3' ATT ATG CTG AGT GAT ATC GCG GCA CAT 5'	10g 5' _{ppp} GCG CCG <u>5</u> <u>5</u> GC A 3'	
7h 3' ATT ATG CTG AGT GAT ATC GCG GCA AGT 5'	10h 5' _{ppp} GCG CCG <u>55</u> C A 3'	
7i 3' ATT ATG CTG AGT GAT ATC GCG GCA AAT 5'	10i 5' _{ppp} GCG CCG <u>555</u> A 3'	

Figure 1. Enzymatic incorporation of thiophene-modified ribonucleotide triphosphates **2**, **4**, and **6**. DNA templates (**7a–7i**) were annealed to 18-mer consensus T7 promoter DNA. Transcripts **8**, **9**, and **10a** contain nucleosides **1**, **3**, and **5** respectively, whereas transcripts **10b–10i** contains nucleoside **5**. Modified bases in the RNA transcripts are underlined.

and modified transcripts allowed us to investigate the preference of T7 RNA polymerase toward natural UTP and modified UTPs in a competition experiment. Transcription reactions, when performed with template **7a** in the presence of equimolar concentrations of either **2** or **4** and UTP, exhibited complete preference for UTP (Figure 2, lanes 6 and 7). In contrast, T7 RNA polymerase displayed little preference for the modified UTP **6** over the parent UTP when transcription reactions were performed in the presence of equimolar concentrations of the two (Figure 2, lane 8). Similar behavior has been previously observed for transcription reactions with the analogous furan-conjugated UTP.^[18a]

Characterization of RNA Transcripts

MALDI-TOF mass spectrometry analysis of the isolated modified 10-mers obtained with template **7a** in large-scale transcription reactions showed the expected mass for the full-length modified oligonucleotides (see Figure S2–S4 in the Supporting Information). To unequivocally confirm the incorporation of the modified analogues into the RNA transcripts, they were digested in the presence of alkaline phosphatase, phosphodiesterase, RNase A, and RNase T1. The

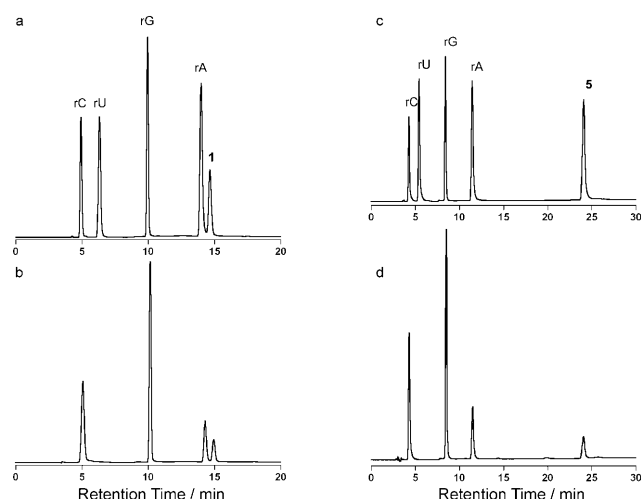


Figure 3. HPLC profile of enzymatic digestion of transcript **8** and **10a** at 260 nm. a) and c) Authentic nucleoside samples and modified nucleoside **1/5**, respectively. b) and d) Digested RNA transcript **8** and **10a**, respectively. See reference [18c] for the enzymatic digestion of **9**.

resulting nucleoside mixture was resolved and quantified by reverse-phase HPLC. Figure 3 shows selected HPLC profiles for the digestion of RNA **8** and **10a**.^[23] Importantly, the chromatograms show both the presence of the modified nucleosides and the correct stoichiometry (G:C:A:U:5). Furthermore, fractions corresponding to each nucleoside were collected and subsequently analyzed by mass spectrometry, confirming the authenticity of the native and modified nucleosides. Taken together, these results unambiguously demonstrate the ability of T7 RNA polymerase to incorporate

fluorescent thiophene-modified U analogues **2**, **4**, and **6** into RNA oligonucleotides in transcription reactions in vitro.^[24]

Effect of Sequence Variations on the Incorporation Efficiency

Transcription efficiency is known to be greatly impacted by the sequence of the DNA template, particularly near the promoter region (positions +1 to +6, see Figure 1).^[25] It has previously been established that replacing the +1 or +2 guanosine nucleotides with other nucleotides dramatically reduces the transcription efficiency, whereas changes at positions +3 to +6 show more-subtle effects on the transcription yield.^[25,26] Other sequence variations, such as the placement of contiguous AU base pairs can also result in poor transcription. To evaluate the incorporation efficiency of the unnatural UTP analogues, we designed several templates that would lead to a single, as well as multiple incorporations of the modified UTPs in positions +2 to +9 (Figure 1). We envisioned that these templates would provide valuable information regarding the suitability of in vitro transcription to generate non-natural RNA strands with single or multiple modifications.

Transcription in the presence of triphosphate **6** and template **7b**, which would place the incoming modified nucleotide at position +2 resulted in negligible amounts of product (Figure 4, lane 4). A slight improvement in the transcription yield (~22%) was observed for template **7c** and **7d** result-

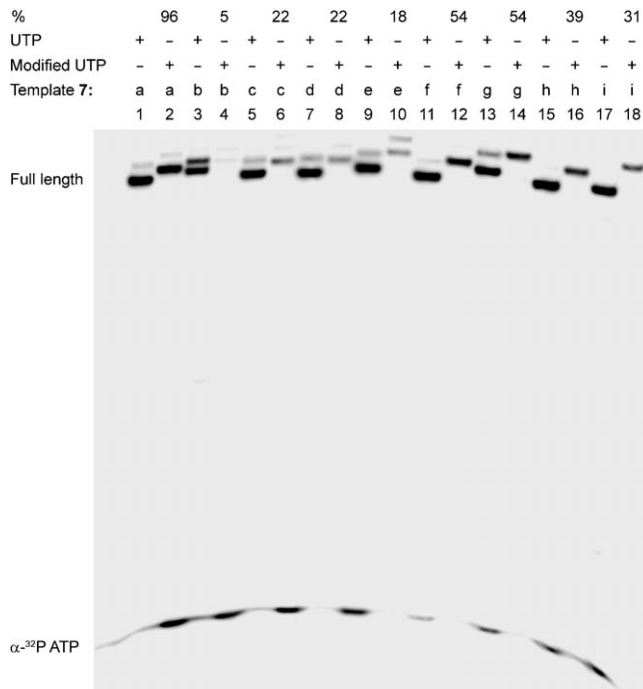


Figure 4. Transcription reactions with templates **7a–i** in the presence of UTP and modified UTP **6**. Incorporation efficiency of **6** is reported with respect to transcription yield in the presence of natural NTPs only. All reactions were performed in triplicate and the standard deviations were $\pm 8\%$.

ing in single substitution at positions +3 and +4, respectively (Figure 4, lanes 6 and 8). Template **7e**, which would lead to the incorporation of the modified nucleotide at positions +4 and +7, gave a low overall yield of approximately 18% (Figure 4, lane 10). Interestingly, reactions performed with templates **7f–i**, which direct double and triple modifications at positions +6 and beyond, exhibited reasonable transcription efficiency yielding full-length products in approximately 30–55% yield (Figure 4, lanes 12, 14, 16, and 18). Notably, attempts to incorporate non-natural UTPs **2** and **4** near the promoter region and in multiple positions by using templates **7b–i**, did not result in any detectable full-length RNA product (Figure S8 and S9 in the Supporting Information).

The negligible formation of full-length transcripts with analogues **2** and **4** could, in principle, be caused by several possible scenarios, depending on the nucleotide and the incorporation position. In one extreme case, the modified nucleoside does not get incorporated at all, and transcription ceases as the enzyme approaches the incorporation position. Alternatively, the modified nucleoside could get incorporated, but elongation of the modified nascent transcript fails past the modification position containing the non-natural nucleotide. To investigate the progression of the transcription reaction in the presence of non-natural nucleotides, we envisioned that 5' labeling of the nascent transcript by using γ - 32 P-GTP (the initiator nucleotide; GTP=guanosine triphosphate) would provide valuable information regarding the actual events during this process. This approach would allow us to determine the point of premature termination in the presence of the modified UTPs and provide insight regarding the observed variations in the incorporation efficiencies between the nucleotides.

Template **7a**, which gives moderate to excellent yields for the reactions in the presence of modified UTPs, has been chosen as the first template to assess the path of the enzymatic incorporation by using γ - 32 P-GTP. Importantly, 5' labeling of the growing transcripts illustrates that T7 RNA polymerase tends to abort transcription rather frequently, even in the presence of natural triphosphates (Figure 5, lane 1), a phenomenon previously observed.^[25] Control reactions in the absence of UTP or modified UTP with template **7a**, which directs the addition of uridine at position +7, results in premature termination to afford a 6-mer RNA product, as expected (Figure 5, lane 2). Although reactions performed in the presence of modified UTPs **2** and **4** exhibit the formation of full-length RNA products, it appears the transcription stalls to a considerable extent, producing the RNA products that correspond to the unmodified 6-mer, and to a lesser extent, the modified 7-mer (Figure 5, lanes 3 and 4). In the presence of **6**, T7 RNA polymerase incorporates the modified nucleotide with an efficiency similar to that of natural UTP with no apparent truncation before and after modification (Figure 5, compare lanes 1 and 5). This is consistent with the results reported above, which show that T7 RNA polymerase accommodates the modified nucleotide **6** as well as the native UTP.

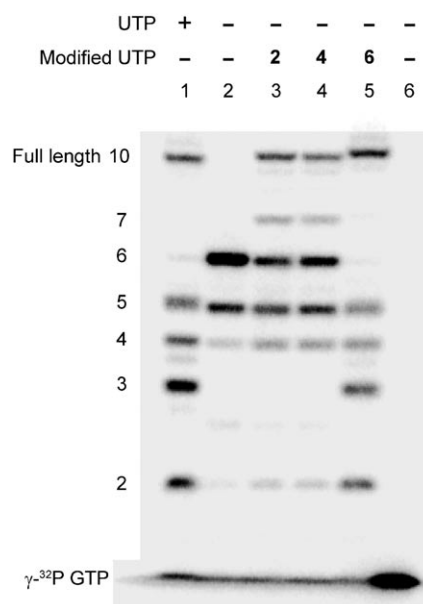


Figure 5. 5' Labeling of RNA transcripts by using γ - 32 P-GTP by a transcription reaction with template **7a** in the presence of UTP and modified UTPs **2**, **4**, and **6**.

As noted above, the transcription efficiency tends to significantly drop for templates that incorporate modified nucleotide analogues near the promoter region (+1 to +6).^[18a,25,26] To explore these events more closely, template **7d**, which directs a U residue into position +4, has been subjected to transcription reactions with γ - 32 P-GTP. Reactions in the presence of **2**, **4**, and **6** reveal a progressive abortion of the transcription reaction before and after modification, as compared to the reaction with UTP with little formation of full-length products (Figure 6, lanes 3–5). These observations corroborate the results reported above wherein the reaction leading to the incorporation of modified nucleotides produced either no or small amounts of full-length RNA product. The course of a transcription reaction with template **7g**, which directs the incorporation of modified UTP analogues **2**, **4**, and **6** into positions +7 and +9, showed similar behavior. As seen in Figure 6, transcription reactions involving **2** and **4** were markedly terminated before the first modification step (position 7) and were also completely abrogated before the second modification (Figure 6, lanes 8 and 9). With **6**, the enzyme successfully incorporated the modified UTP at both positions with no apparent loss in transcription efficiency during the pre- and post-modification steps (Figure 6, lane 10). Taken together with previous observations, we note that T7 RNA polymerase tolerates 5-modified pyrimidines extremely well. As these analogues are known to have useful fluorescence properties, in vitro transcription reactions with **6** and related analogues provide a useful entry to fluorescently tagged RNA oligonucleotides.

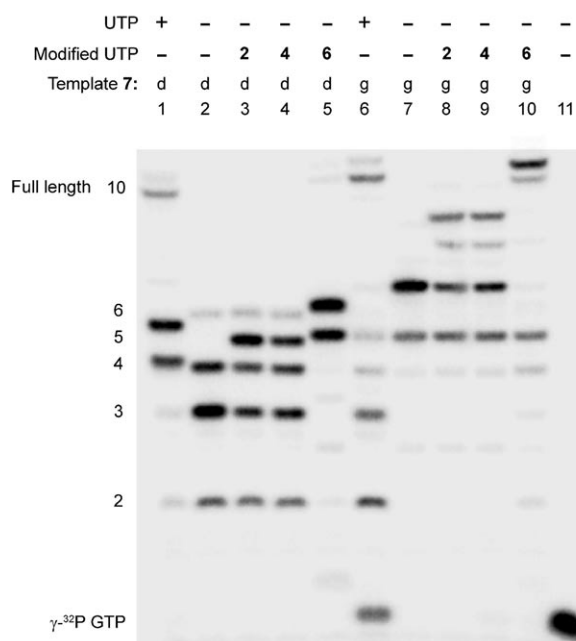


Figure 6. 5' Labeling of RNA transcripts by using γ - ^{32}P -GTP by a transcription reaction with templates **7d** and **g** in the presence of UTP and modified UTPs **2**, **4**, and **6**. Template **7d** directs the incorporation of rU at position 4 near the promoter region. Template **7g** directs the incorporation of rU at positions 7 and 9.

Conclusions

The modification of RNA oligonucleotides with fluorescent reporter nucleotides can be of significant utility for biophysical evaluation of RNA recognition events.^[7,18,27] To explore the potential of *in vitro* transcription reactions for the synthesis of emissive RNA oligonucleotides, T7 RNA polymerase was challenged with accepting three related modified triphosphates as substrates and incorporating them into diverse RNA transcripts. The three ribonucleoside triphosphates differ only in the modification of their uracil nucleus and included a thieno[3,2-d]pyrimidine nucleoside (**1**), a thieno[3,4-d]pyrimidine derivative (**3**) and a related pyrimidine containing a thiophene ring conjugated at position 5 (**5**). All non-natural UTPs were incorporated into RNA oligonucleotides at positions that are remote to the promoter, although the yields of the transcripts, compared with that obtained with only native triphosphates, varied substantially. Among the three derivatives, the 5-modified UTP was found to be the most "polymerase friendly" and was well accommodated by T7 RNA polymerase. While the fused thiophene analogues cannot be incorporated next to the promoter region, suggesting interference by the fused ring at the 5,6-positions, the 5-modified unnatural UTP gets incorporated near the transcript's 5'-end and even in multiple copies closer to the 3'-end. Labeling experiments shed light on the problematic incorporation of the fused analogues, suggesting that the enzyme frequently pauses at the incorporation position, and, when incorporation does take place, T7 RNA polymerase fails to elongate the modified oligonu-

cleotides and yields aborted transcripts. Taken together, these results highlight the versatility and robustness, as well as the scope and limitation, of T7 RNA polymerase in accepting and incorporating reporter nucleotides.

Experimental Section

Materials

Unless otherwise specified, materials obtained from commercial suppliers were used without further purification. 5-Iodouridine was purchased from MP Biomedicals, Inc. 2-(Tributylstannyl)thiophene and bis-(triphenylphosphine)-palladium(II) chloride were obtained from Aldrich and Acros Chemicals, respectively. Trimethyl phosphate was obtained from Aldrich. Bis-tributylammonium pyrophosphate (0.5 M in dimethyl formamide; DMF) was prepared according to a literature report.^[28] DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. Oligonucleotides were purified by gel electrophoresis and desalted on Sep-Pak (Waters Corporation). NTPs, T7 RNA polymerase, ribonuclease inhibitor (RiboLock) were obtained from Fermentas Life Science. Radio-labeled α - ^{32}P ATP and γ - ^{32}P GTP (800 Ci mmol⁻¹) were obtained from MP Biomedicals, Inc. Alkaline phosphatase, phosphodiesterase, RNase A, and RNase T1 were purchased from Boehringer Mannheim, Germany. Autoclaved water was used in all biochemical reactions. For the synthesis of **1**, **3**, and **4** see references [11 f] and [18 c].

Instrumentation

NMR spectra were recorded on a Varian Mercury 400 MHz spectrometer. Mass spectra were recorded at the UCSD Chemistry and Biochemistry Mass Spectrometry Facility by utilizing a LCQDECA (Finnigan) ESI with a quadrupole ion trap. All MALDI-TOF spectra were collected on a PE Biosystems Voyager-DE STR MALDI-TOF spectrometer in positive-ion, delayed-extraction mode. UV/Vis spectra were recorded on a Hewlett Packard 8452 A diode array spectrometer. Reverse-phase HPLC (Vydac C18 column) purification and analysis were carried out by using the Hewlett Packard 1050 Series chromatographs. Steady-state fluorescence experiments were carried out in a micro fluorescence cell with a path length of 1.0 cm (Hellma GmbH & Co. KG, Müllheim, Germany) on a Horiba Jobin Yvon (FluoroMax-3) spectrometer. Polyacrylamide gels containing radiolabeled RNA were analyzed by using a BioRad phosphorimager.

Synthesis

Triphosphate 2. To an ice-cold suspension of nucleoside **1** (0.075 g, 0.25 mmol, 1.0 equivalent) in trimethyl phosphate (1 mL) was added freshly distilled POCl₃ (57 μL , 0.62 mmol, 2.5 equiv). The solution was stirred for 32 h at 4–6°C. A solution of bis-tributylammonium pyrophosphate (0.5 M in DMF, 3.0 mL, 1.5 mmol, 6.0 equiv) and tri-*n*-butyl amine (0.60 mL, 2.5 mmol, 10 equiv) was then rapidly added under ice-cold conditions. The reaction was quenched after 30 min with 1 M triethylammonium bicarbonate buffer solution (TEAB, pH 7.5, 15 mL) and was extracted with ethyl acetate (20 mL). The aqueous layer was evaporated under vacuum. The residue was purified first on a diethylaminoethyl (DEAE) sephadex-A25 anion-exchange column (0.01–1.0 M, TEAB buffer solution, pH 7.5) followed by reverse-phase HPLC (Vydac C18 column, 1.0 $\times$ 25 cm, 5 μm TP silica, 0–15 % acetonitrile in 100 mM triethyl ammonium acetate buffer solution, pH 7.0, 30 min). Appropriate fractions were lyophilized to afford the desired triphosphate (0.114 g, 48 %). ¹H NMR (400 MHz, D₂O): δ = 8.20 (d, J = 3.2 Hz, 1H), 7.30 (d, J = 3.2 Hz, 1H), 6.08 (d, J = 6.8 Hz, 1H), 4.61 (t, J = 6.8 Hz, 1H), 4.35 (dd, J_1 = 6.8 Hz, J_2 = 4.8 Hz, 1H), 4.16–4.03 ppm (m, 3H); ¹³C NMR (100 MHz, D₂O): δ = 160.0, 152.0, 145.1, 137.5, 118.8, 114.3, 89.4, 83.2 (d, J = 9.1 Hz), 70.4, 68.6, 65.2 ppm; ³¹P NMR (162 MHz, D₂O): δ = -9.86 (br, P_γ), -10.68 (d, J = 19.8 Hz, P_α), -22.48 ppm (br, P_β); ESIMS (negative mode): Calculated for C₁₁H₁₅N₂O₁₅P₃S [M] 539.94, found [M-H]⁻ = 538.91.

Thiophene-modified ribonucleoside 5. 2-(Tributylstannyl)thiophene (1.81 g, 4.85 mmol, 7.0 equiv) was added to a suspension of 5-iodouridine

(0.257 g, 0.69 mmol, 1.0 equiv) and bis-(triphenylphosphine)-palladium(II) chloride (0.010 g, 0.014 mmol, 0.02 equiv) in anhydrous dioxane (6 mL). The reaction mixture was heated at 95°C for 5 h, cooled, and then filtered through a celite bed. The solvent was evaporated and the resulting residue was triturated with hexanes (2 × 25 mL). The residue was purified by silica gel chromatography to afford the nucleoside as a white solid (0.141 g, 62%). TLC (CH₂Cl₂/MeOH = 8:2) *R*_f = 0.63; $\epsilon_{260}(\text{H}_2\text{O}) = 10140 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{316}(\text{H}_2\text{O}) = 8700 \text{ M}^{-1} \text{ cm}^{-1}$; ¹H NMR (400 MHz, [D₆]DMSO): δ = 11.72 (s, 1H), 8.67 (s, 1H), 7.46 (d, *J* = 5.2 Hz, 1H), 7.40 (d, *J* = 3.6 Hz, 1H), 7.06–7.04 (m, 1H), 5.83 (d, *J* = 4.0 Hz, 1H), 5.49 (d, *J* = 5.2 Hz, 1H), 5.45 (t, *J* = 4.4 Hz, 1H), 5.12 (d, *J* = 4.8 Hz, 1H), 4.15–4.06 (m, 2H), 3.92–3.91 (m, 1H), 3.78–3.73 (m, 1H), 3.66–3.62 (m, 1H); ¹³C NMR (100 MHz, [D₆]DMSO): δ = 161.3, 149.5, 135.7, 133.9, 126.4, 125.7, 122.4, 108.2, 88.6, 84.5, 74.3, 69.3, 60.1 ppm; ESIMS negative mode (*m/z*): Calculated for C₁₃H₁₄N₂O₆S [M] 326.06, found [*M*–H][–] = 325.03.

Triphosphate 6. To an ice-cold solution of nucleoside **5** (0.060 g, 0.18 mmol, 1.0 equiv) in trimethyl phosphate (1 mL) was added freshly distilled POCl₃ (50 μ L, 0.55 mmol, 3.1 equiv). The solution was stirred for 27 h at 4–6°C. TLC revealed nearly 50% conversion and hence, another 1 equivalent of POCl₃ was added. The solution was further stirred at 4–6°C for 21 h. However, there was no significant consumption of the starting material after this period. A solution of bis-tributylammonium pyrophosphate (0.5 M in DMF, 1.8 mL, 0.90 mmol, 5.0 equiv) and tri-*n*-butylamine (0.43 mL, 1.80 mmol, 10 equiv) was then rapidly added under ice-cold conditions. The reaction was quenched after 30 min with 1 M TEAB buffer solution (pH 7.5, 15 mL) and was extracted with ethyl acetate (20 mL). The aqueous layer was evaporated under vacuum. The residue was purified first on a DEAE sephadex-A25 anion-exchange column (0.010–1.0 M, TEAB buffer solution, pH 7.5) followed by reverse-phase HPLC (Vydac C18 column, 1.0 × 25 cm, 5 μ m TP silica, 0–15% acetonitrile in 100 mM triethyl ammonium acetate buffer solution, pH 7.0, 30 min). Appropriate fractions were lyophilized to afford the desired triphosphate **6** (0.046 g, 26%). ¹H NMR (400 MHz, D₂O): δ = 8.12 (s, 1H), 7.50 (d, *J* = 3.6 Hz, 1H), 7.44 (d, *J* = 5.2 Hz, 1H), 7.13–7.11 (m, 1H), 6.01 (d, *J* = 5.2 Hz, 1H), 4.47–4.42 (m, 2H), 4.28–4.19 ppm (m, 3H); ¹³C NMR (100 MHz, D₂O): δ = 163.5, 151.1, 136.3, 132.4, 127.5, 126.8, 125.2, 110.5, 88.5, 84.0, 73.8, 70.0, 65.5 ppm; ³¹P NMR (162 MHz, D₂O): δ = –9.01 (br, P_γ), –10.65 (br, P_α), –22.11 ppm (br, P_β); ESIMS (negative mode): Calculated for C₁₃H₁₇N₂O₁₅P₃S [M] 565.96, found [*M*–H][–] = 564.99.

In vitro Transcription Reactions

Transcription reactions with α -³²P-ATP. Single-strand DNA templates were annealed to an 18-mer T7 RNA polymerase consensus promoter sequence in TE buffer solution (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 7.8; EDTA = ethylenediaminetetraacetate, Tris = tris(hydroxymethyl)aminomethane) by heating a 1:1 mixture (5 μ M) at 90°C for 3 min and cooling the solution slowly to room temperature. Transcription reactions were performed in 40 mM Tris-HCl buffer solution (pH 7.9) containing 250 nM annealed template, 10 mM MgCl₂, 10 mM dithiothreitol (DTT), 10 mM NaCl, 2 mM spermidine, 1 U μ L^{–1} RNase inhibitor (RiboLock), 1 mM GTP, 1 mM CTP (CTP = cytosine triphosphate), 1 mM modified UTP **2/4/6**, 20 μ M ATP, 5 μ Ci α -³²P-ATP (800 Ci mmole^{–1} stock) and 2.5 U μ L^{–1} T7 RNA polymerase (Fermentas) in a total volume of 20 μ L. In the control reaction 1 mM UTP was used instead of modified UTPs. After 3 h at 37°C, reactions were quenched by adding 55 μ L of loading buffer solution (7 M urea in 10 mM Tris-HCl, 100 mM EDTA, pH 8 and 0.05% bromophenol blue), heated to 75°C for 3 min, and samples (4 μ L) were loaded onto an analytical 20% denaturing polyacrylamide gel. The products on the gel were analyzed by using a phosphorimager.

Large-scale transcription reactions and transcript analysis. Large-scale transcription reactions with template **7a** was performed in a 250- μ L reaction volume under similar conditions to isolate RNA for enzymatic digestion. The reaction contained 2 mM NTPs, 2 mM modified UTP **2/4/6**, 20 mM MgCl₂ and 500–600 units T7 RNA polymerase. After incubation for 12 h at 37°C, the precipitated magnesium pyrophosphate was removed by centrifugation. The reaction volume was reduced to half by Speed Vac and 25 μ L of loading buffer solution was added. The mixture

was heated at 75°C for 3 min, and loaded onto a preparative 20% denaturing polyacrylamide gel. The gel was UV shadowed; the appropriate band was excised, extracted with 0.5 M ammonium acetate, and desalted on a Sep-Pak. By using the above-described condition, 14–20 nmoles of full length products containing the fluorescent label were obtained.

For composition analysis, the transcripts obtained were enzymatically digested and analyzed. The fluorescently labeled transcripts (2–5 nmol of **8**, **9**, or **10a**) from large-scale transcription reactions were digested with snake-venom phosphodiesterase I, calf-intestine alkaline phosphatase, and RNase A in 50 mM Tris-HCl buffer solution (pH 8.5, 50 mM MgCl₂, 0.1 mM EDTA) for 15 h at 37°C. The mixture was further treated with RNase T1 for 4 h at 37°C. The ribonucleoside mixture obtained was analyzed by reverse-phase analytical HPLC by using a Vydac C18 column (0.46 × 25 cm, 5 μ m TP silica) at 260 nm. Mobile phase A: 100 mM triethyl ammonium acetate buffer solution (pH 7.0); mobile phase B: acetonitrile. Flow rate: 1 mL min^{–1}.

For transcript **8**: Gradient: 0–7.5% mobile phase B in 20 min and 7.5–100% mobile phase B in 10 min.

For transcript **9**:^[18c] Gradient: 0–7.5% mobile phase B in 20 min and 7.5–100% mobile phase B in 10 min.

For transcript **10a**: Gradient: 0–15% mobile phase B in 30 min and 15–100% mobile phase B in 10 min.

Transcription reactions with γ -³²P-GTP. Single-strand DNA templates were annealed to an 18-mer T7 RNA polymerase consensus promoter sequence in TE buffer solution (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 7.8) by heating a 1:1 mixture (5 μ M) at 90°C for 3 min and cooling the solution slowly to room temperature. Transcription reactions were performed in 40 mM Tris-HCl buffer solution (pH 7.9) containing 250 nM annealed template, 10 mM MgCl₂, 10 mM DTT, 10 mM NaCl, 2 mM spermidine, 1 U μ L^{–1} RNase inhibitor (RiboLock), 1 mM ATP, 1 mM CTP, 1 mM modified UTP **2/4/6**, 500 μ M GTP, 5 μ Ci γ -³²P-GTP (800 Ci mmole^{–1} stock) and 2.5 U μ L^{–1} T7 RNA polymerase (Fermentas) in a total volume of 20 μ L. In the control reaction 1 mM UTP was used instead of modified UTPs. After 3 h at 37°C, reactions were quenched by adding 55 μ L of loading buffer solution (7 M urea in 10 mM Tris-HCl, 100 mM EDTA, pH 8 and 0.05% bromophenol blue), heated to 75°C for 3 min, and samples (4 μ L) were loaded onto an analytical 20% denaturing polyacrylamide gel. The products on the gel were analyzed by using a phosphorimager.

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