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Synthesis, Photophysical Characterization, and Enzymatic Incorporation of a Microenvironment-Sensitive Fluorescent Uridine Analog

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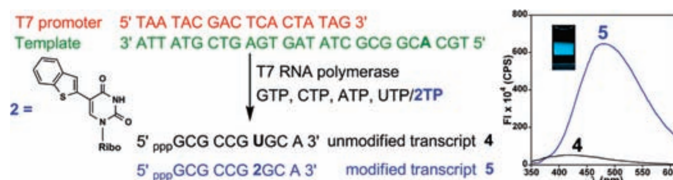
ABSTRACT



The synthesis of a microenvironment-sensitive base-modified fluorescent ribonucleoside analog based on a 5-(benzo[*b*]thiophen-2-yl)pyrimidine core, enzymatic incorporation of its corresponding triphosphate into RNA oligonucleotides, and photophysical characterization of fluorescently modified oligoribonucleotides are described.

Microenvironment-sensitive fluorescent ribonucleoside analogs that report changes in RNA conformation during folding or the recognition process in the form of changes in basic photophysical properties have become very important analytical tools in investigating RNA structure and function.[1,2] An appealing feature of many of these analogs is that they closely resemble natural ribonucleosides and form stable Watson–Crick (WC) base pairs. Therefore, these emissive ribonucleoside reporters can be site-specifically placed in a target oligoribonucleotide in a nonperturbing fashion and utilized in monitoring subtle conformational changes taking place around the point of investigation. In particular, the minimally perturbing nature and exquisite sensitivity of 2-aminopurine (2AP) to conformational changes have been widely used in designing several fluorescence-based assays to probe the structure, dynamics, and function of RNA molecules.[1,2] However, the emission maximum (~370 nm) in the near-UV region and low quantum yields when incorporated into nucleic acids substantially limit the utility of 2AP to in vitro systems only.[3] Consequently, several microenvironment-sensitive ribonucleoside analogs with improved photophysical properties such as emission in the visible region and a high quantum yield have been developed.[1] However, only a few have been effectively applied in the RNA-based biophysical assays.[4] Therefore, we sought to develop new fluorescent ribonucleoside analogs that (a) are structurally noninvasive, (b) exhibit emission in the visible region, (c) display a reasonable quantum yield when incorporated into oligoribonucleotides, and

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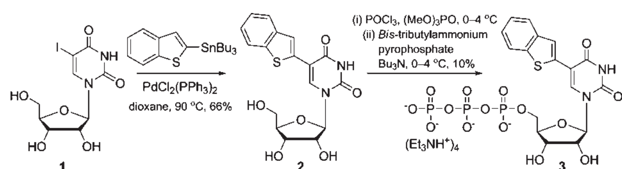
Scheme 1. Synthesis of Ribonucleoside **2** and Triphosphate **3**[7]



(d) report changes in their surrounding environment via changes in their photophysical properties. Here, we report the synthesis, photophysical characterization, and enzymatic incorporation of a new microenvironment-sensitive fluorescent benzo[*b*]thiophene-conjugated uridine analog **2** into RNA oligonucleotides. Interestingly, unlike 2AP,[5] the fluorescence quantum yield of **2** is not significantly compromised when placed within an ssRNA or a perfect complementary duplex. Furthermore, using steady-state and time-resolved fluorescence spectroscopy techniques, we illustrate the ability of the fluorescently modified oligoribonucleotide in distinguishing between pyrimidines and purines upon hybridization to complementary and mismatched oligonucleotides.

Benzo[*b*]thiophene-conjugated uridine **2** has been synthesized by performing a palladium catalyzed cross-coupling reaction between the commercially available 5-iodoridine **1** and 2-tributylstannylbenzo[*b*]thiophene (Scheme 1). The modified triphosphate substrate **3** required for in vitro transcription reactions has been synthesized by reacting ribonucleoside **2** with $POCl_3$, followed by reaction with *bis*-tributylammonium pyrophosphate[6] in a one-pot two-step reaction.

In order to access the responsiveness of the emissive ribonucleoside **2** to solvent polarity, we performed UV-absorption, steady-state, and time-resolved fluorescence spectroscopic measurements in solvents of different polarity (Table 1, Figure 1). In water, **2** shows two distinct absorption maxima at 274 and 318 nm corresponding to the base and conjugated benzothiophene, respectively. When the solvent polarity is sequentially decreased from water to dioxane, the highest and the lowest absorption energy maxima are marginally affected (Figure 1). However, solvent polarity has a significant influence on both the emission maximum and intensity. When a solution of **2** in dioxane is excited at 322 nm, a strong fluorescence band is observed in the visible region (435 nm). As the solvent

Table 1. Photophysical Properties of Fluorescent Ribonucleoside **2** in Various Solvents[7]

solvent	$\lambda_{max}{}^{a}$ (nm)	λ_{em} (nm)	$I_{rel}{}^{b}$	Φ^{c}	$\tau_{ave}{}^{c}$ (ns)	k_r/k_{nr}
water	318	458	1.0	0.035	1.04	0.037
methanol	321	446	1.7	0.047	0.60	0.049
acetonitrile	321	443	1.3	0.034	0.49	0.036
dioxane	322	435	2.1	0.060	0.45	0.064

[a] The lowest energy maximum is given. [b] Emission intensity relative to intensity in water. [c] Standard deviations for Φ and τ_{ave} are $\leq$0.003 and 0.02 ns, respectively. k_r and k_{nr} are radiative and nonradiative decay rate constants, respectively.

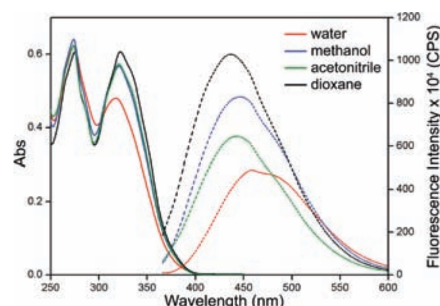


Figure 1. Absorption (50 μM, solid) and emission (5.0 μM, dash) spectra of ribonucleoside **2** in various solvents.[7]

polarity is increased from dioxane to water, the ribonucleoside displays a marked bathochromic shift (435 to 458 nm) and a nearly 2-fold fluorescence quenching (Table 1, Figure 1). The relative quantum yield determined in various solvents is also in consensus with the observed emission intensity in respective solvents (Table 1). A plot of the Stokes shift determined in different solvents vs Reichardts' microscopic solvent polarity parameter, $E_T(30)$,[8] shows a very good linear correlation, which further ascertains the responsiveness of the fluorescent ribonucleoside **2** to its microenvironment (Figure S1).

Time-resolved fluorescence measurements of **2** as a function of solvent polarity reveal distinct excited state decay profiles (Table 1, Figure S2). An aqueous solution of **2** shows the highest lifetime, and as the solvent polarity is decreased from water to dioxane a progressive decrease in lifetime is observed (Table 1). We then used the lifetimes and quantum yields to calculate the radiative (k_r) and nonradiative (k_{nr}) decay rate constants in different solvents (Table 1).[7] The nearly 2-fold higher k_r/k_{nr} ratio in dioxane indicates that the radiative pathway is significantly favored in dioxane as compared to water. Taken together, emission in the visible region, a reasonable quantum yield, and sensitivity to changes in solvent polarity as exemplified by photophysical measurements adequately bestow probe-like character to the emissive ribonucleoside analog **2**.

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(5) Up to a 100-fold reduction in quantum yield is observed when 2AP is incorporated into oligonucleotides. See ref 3a.

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(7) See Supporting Information (SI) for details.

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Figure 2. Enzymatic incorporation of ribonucleoside triphosphate **3**.[7] Synthetic oligonucleotides (**6**−**10**) used in this study.

Figure 3. In vitro transcription reactions with templates **T1**−**T5** in the presence of UTP and modified UTP **3**.[7] % incorporation of **3** is reported with respect to reaction in the presence of NTPs. Unreacted α-^{32}P ATP is not shown here (see SI, Figure S3).

In many instances, modified RNA oligonucleotides have been synthesized using an in vitro transcription reaction as it does not involve elaborate protection−deprotection steps.[9,10] The efficacy of T7 RNA polymerase in incorporating the modified ribonucleoside triphosphate **3** into RNA transcripts was investigated by performing in vitro transcription reactions as illustrated in Figure 2. A series of duplexes were assembled by annealing an 18-mer T7 RNA polymerase consensus deoxyoligonucleotide promoter sequence to deoxyoligonucleotide templates (**T1**−**T5**, Figure 2). The templates were designed to possess one or two dA residues at different positions (e.g., near the promoter region, away from the promoter region, and at multiple sites) to direct single or multiple incorporations of **3**. In addition, all templates contained a unique dT residue at the 5′-end to direct the incorporation of a single adenosine at the 3′-end of each transcript. Therefore, successful transcription reactions performed in the presence of GTP, CTP, UTP/**3**, and α-^{32}P ATP would result in the formation of 3′-end ^{32}P-labeled RNA transcripts. The labeled oligoribonucleotide products could then be resolved by analytical denaturing polyacrylamide gel electrophoresis and imaged. Unsuccessful transcription reactions resulting in transcripts shorter than the full-length oligoribonucleotide products would stay undetected. We surmise that reactions with such templates can provide valuable information on the efficiency and scope of the transcription reaction in generating fluorescent oligoribonucleotides.

The transcription reaction in the presence of template **T1** containing a dA residue away from the promoter at the +7 position results in the formation of the 10-mer modified full-length oligoribonucleotide transcript **5** (Figure 3, lane 2).[11] Relative to natural UTP, **3** is incorporated into the transcript with an excellent efficiency of 85 ± 1%. The slower migration of transcript **5** clearly indicates the incorporation of a higher molecular weight modified ribonucleoside during the transcription reaction (Figure 3, compare lanes 1 and 2). Importantly, a control transcription in the absence of UTP and **3** did not yield any full-length oligoribonucleotide product, which clearly rules out the formation of full-length transcripts due to adventitious misincorporation (Figure 3, lane 3).

Interestingly, reaction in the presence of equimolar concentrations of UTP and **3** demonstrates that T7 RNA polymerase has an almost equal preference for UTP and unnatural ribonucleotide **3** (1:1.1, Figure 3, lane 4). When reactions are performed in the presence of templates **T2** and **T3** containing the dA residue near the promoter at +3 and +4 positions, the incorporation efficiency drops significantly (Figure 3, lanes 6 and 8). This is understandable as the efficiencies of transcription reactions are known to be significantly reduced by template sequences that lead to modifications near the promoter region.[12] In the presence of template **T4**, the RNA polymerase incorporates **3** in two successive positions (+6 and +7) with reasonable efficiency (62 ± 1%, Figure 3, lane 10). However, for a reaction in the presence of template **T5**, which would direct the addition of a modified ribonucleoside in alternating positions (+7 and +9), the incorporation efficiency was found to be low (12 ± 3%, Figure 3, lane 12). To further confirm the presence of benzo[*b*]thiophene-conjugated uridine **2** in the transcript, a large-scale transcription reaction was performed with template **T1**. MALDI-TOF mass measurement revealed the presence of a modified ribonucleoside in the full-length transcript **5** (Figure S4).

Emissive nucleoside analogs when incorporated into oligonucleotides experience diverse interactions with neighboring bases such as stacking, hydrogen bonding, and collisional interactions, which can affect the photophysical properties of nucleosides.[3] In order to evaluate the effect of neighboring bases on the fluorescence of ribonucleoside **2**, we have photophysically characterized the RNA transcript **5** and duplexes of **5**. These duplexes have been

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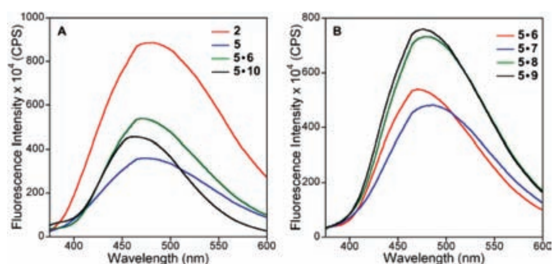

Figure 4. (A) Emission spectra of **2**, **5**, and duplexes **5•6** and **5•10**. (B) Emission spectra of duplexes containing ribonucleoside **2** opposite to purines and pyrimidines.[7]

constructed by annealing transcript **5** with various cDNA and RNA oligonucleotides in which the ribonucleoside **2** has been placed opposite to its complementary and mismatch bases (Figure 2). Upon excitation at 320 nm, oligoribonucleotide **5** in cacodylate buffer shows a slightly red-shifted emission (482 nm) corresponding to a quantum yield of 0.010 ± 0.002, which is 2.7-fold lower than that for the free ribonucleoside (Figure 4A, Table S2).[7] This fluorescence quenching accompanied by a small spectral shift is presumably due to the solvation effect and partial stacking of the fluorophore with neighboring bases.[3,13,14] Surprisingly, perfect RNA−DNA (**5•6**) and RNA−RNA (**5•10**) duplexes display a small enhancement in fluorescence intensity (Figure 4A, Table S2). This observation is particularly interesting because most fluorescent nucleoside analogs (eg., 2AP, pyrroloC) exhibit progressive fluorescence quenching upon incorporation into ss- and ds-oligonucleotides due to stacking interactions with adjacent bases.[3,15,16] Furthermore, similar quantum yields, excited state decay profiles with average lifetimes of 1−1.2 ns, and k_r/k_{nr} ratios for the emissive ribonucleoside in **5** and complementary duplexes (**5•6** and **5•10**) strongly suggest that in all these constructs the fluorophore experiences similar interactions with neighboring bases (Figure S6, Table S2).

Interestingly, duplexes **5•8** and **5•9** in which the emissive ribonucleoside **2** is placed opposite to pyrimidines show an ~40% increase in fluorescence intensity as compared to duplexes **5•6** and **5•7** containing **2** opposite to purines (Figure 4B). It is more likely that in duplexes **5•6** and **5•7** the modified uridine analog is localized due to the formation of a stable WC and wobble base pair with dA and dG, respectively, which results in a better stacking of the fluorophore with adjacent bases. Hence, the observed fluorescence enhancement can be attributed to a flexible and less stacked benzothiophene moiety in **5•8** and **5•9** as compared to **5•6** and **5•7**. In addition, duplexes **5•8** and **5•9** have longer excited state lifetimes than **5•6** and **5•7,** which further indicates a distinct microenvironment around the emissive ribonucleoside (Figure S7 and Table S2). Ribonucleoside **2** therefore photophysically distinguishes between pyrimidine and purine bases, albeit with moderate fluorescence enhancement.

The incorporation of **2** can potentially perturb the native structure of the oligoribonucleotide **5** and, hence, can result in ineffective hybridization. Thermal denaturation studies with a **2**-containing duplex and corresponding unmodified duplex show only marginal destabilization due to benzo[*b*]thiophene modification (Figure S8). Additionally, native gel retardation experiments performed with ^{32}P-labeled transcript **5** and its corresponding duplexes under the conditions utilized for the fluorescence experiments clearly reveal complete hybridization (Figure S9). Together, these results clearly indicate that the duplexes are completely intact and the observed changes in photophysical properties of the duplexes are solely due to the differences in the microenvironment of the emissive ribonucleoside.

In summary, we have developed a new fluorescent uridine analog based on a (benzo[*b*]thiophen-2-yl)pyrimidine core that has an emission maximum in the visible region and displays excellent solvatochromism. The corresponding triphosphate substrate is amenable to incorporation into oligoribonucleotides by transcription reactions. Interestingly, T7 RNA polymerase equally incorporates both natural UTP and modified UTP **3** into RNA oligonucleotides, a trait that can be utilized in the fluorescent labeling of RNA by a ribonucleoside salvage pathway.[17] The results reported here also demonstrate that polarity-sensitive fluorescent ribonucleoside analog **2** can be used as a probe in investigating nucleic acid dynamics and the recognition process by monitoring changes in fluorescence properties such as fluorescence intensity, lifetime, and anisotropy.[18] Efforts in these directions are currently in progress and will be reported in due course.

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Supporting Information Available. Experimental procedures and spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

(13) Steady-state Stern−Volmer titrations with NMPs reveal very small fluorescence quenching due to collisional interaction (Figure S5).

(14) The solvation effect can potentially change the relative contribution of two different emissive states of the ribonucleoside within an oligonucleotide, which can lead to a spectral shift and/or fluorescence quenching (see Figure 1, emission spectrum of **2** in water).

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