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Fluorescent Ribonucleoside as a FRET Acceptor for Tryptophan in Native Proteins

Yun Xie, Tucker Maxson, and Yitzhak Tor*

Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, California 92093

Received June 15, 2010; E-mail: ytor@ucsd.edu

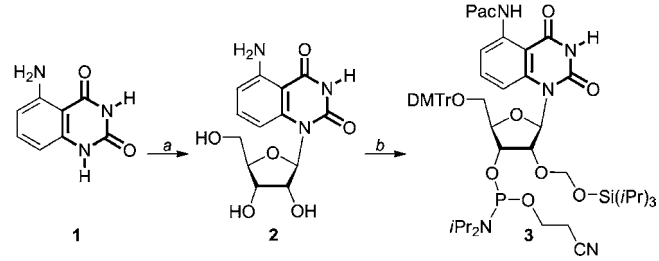
Abstract: A new fluorescent ribonucleoside analogue, containing 5-aminoquinazoline-2,4(1*H*,3*H*)-dione, acts as a Förster resonance energy transfer acceptor for tryptophan ($R_0 = 22$ Å) and displays visible emission (440 nm). As tryptophan is frequently found at or near the recognition domains of RNA binding proteins, this FRET pair facilitates the study of RNA binding to native proteins and peptides, which is demonstrated here for the HIV-1 Rev association with the Rev Response Element (RRE).

While tryptophan is one of the most infrequently occurring amino acids in proteins,[1] it is recurrently found at, or near, the recognition domains of RNA-binding proteins.[2,3] As such recognition phenomena are crucial for transcriptional regulation, mRNA maturation, RNA interference, translation, and more, the development of biophysical tools for evaluating these fundamental processes is of key importance. Although the indole chromophore imparts useful emissive properties,[4] tryptophan's emission is highly sensitive to diverse environmental perturbations,[5] a feature that, while useful, can significantly complicate the readout and interpretation of binding events. Förster resonance energy transfer (FRET) pairs can overcome many of such difficulties, but the incorporation of large "classical FRET" chromophores into the binding partners can perturb these delicate recognition events. We have therefore sought to develop a new isomorphic ribonucleoside analogue that could seamlessly be incorporated into RNA and serve as a FRET acceptor to tryptophan residues residing on RNA binding proteins. Here we report the design, synthesis, photophysical characteristics, and implementation of an emissive uridine mimic that fulfills such stringent requirements and facilitates the study of RNA−protein interactions.

A fundamental challenge in developing minimally perturbing nucleoside analogues is the tendency of small aromatic chromophores to display high emission energies, which frequently overlap with the emission bands of native amino acids.[4] A molecular feature that can help alleviate such constraints and facilitate FRET pairing with Trp is the implementation of a charge-transfer character upon the chromophore, a mechanism frequently found in naturally occurring visibly emissive fluorophores (e.g., oxyluciferin and GFP).[4] Heterocycle **1** and the corresponding ribonucleoside **2** (Scheme 1), containing an electron-rich ring fused into the electron-deficient pyrimidine, fulfill this requirement and display red-shifted absorption and emission bands. The extinction coefficient of **2** at 280 nm, the absorption maximum of tryptophan, is minimal, while the emission of tryptophan, centered around 350 nm ($\Phi_F = 0.12$), overlaps well with the absorption band of **2**, which emits at 440 nm ($\Phi_F = 0.42 \pm 0.04$), suggesting excellent FRET pairing (Figure 1). The critical Förster radius (R_0) was experimentally determined to be 22 Å, a suitable value for monitoring RNA−protein recognition events.

To unequivocally demonstrate the utility of this ribonucleoside and its FRET pairing with natively occurring Trp residues we have investigated the Rev peptide and the Rev Responsive Element (RRE), its cognate RNA target. Rev, a key HIV-1 regulatory protein,

Scheme 1. Heterocycle **1**, Ribonucleoside **2**, and the Corresponding Phosphoramidite **3**[a]



[a] Reagents: (a) (i) *N*,*O*-bis(trimethylsilyl)acetamide, $CF_3SO_3Si(CH_3)_3$, β-D-ribofuranose 1-acetate 2,3,5-tribenzoate, CH_3CN; (ii) conc. NH_4OH, 81%. (b) (i) $(CH_3)_3SiCl$, phenoxyacetic anhydride, H_2O, conc. NH_4OH, pyridine, 75%; (ii) DMTrCl, Et_3N, pyridine, 82%; (iii) iPr_2NEt, nBu_2SnCl_2, iPr_3SiOCH_2Cl, $ClCH_2CH_2Cl$, 30%; (iv) iPr_2NEt, $(iPr_2N)P(Cl)O-CH_2CH_2CN$, $ClCH_2CH_2Cl$, 60%.[8]

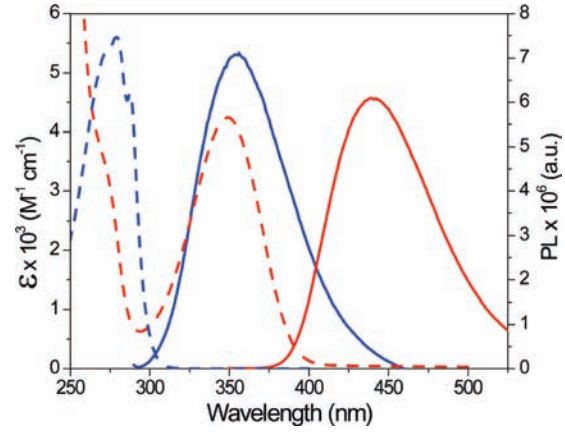


Figure 1. Absorption (---) and emission (—) spectra of tryptophan (blue) and **2** (red) in water.[8]

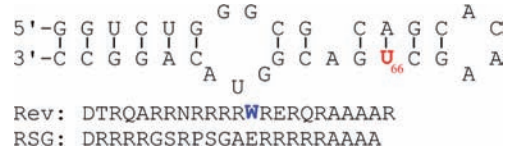


Figure 2. Model RRE construct and peptides used for FRET binding and competition experiments.

is involved in the transport of immature viral mRNAs from the nucleus to the cytoplasm of the host cell.[9−13] The specific and high-affinity binding between the protein and RNA has been attributed to the arginine-rich domain of Rev and Stem-loop IIB of the RRE (Figure 2).[11,12,14] While the Rev protein contains a single Trp residue (Trp_{45}), it is strategically embedded within the RNA binding domain.[12] Residue U66 of the RRE, neighboring the binding site, was therefore identified as the RNA modification position.[6,15,18]

To incorporate the modified nucleoside into RNA oligonucleotides, phosphoramidite **3** was prepared (Scheme 1). 5-Amino-

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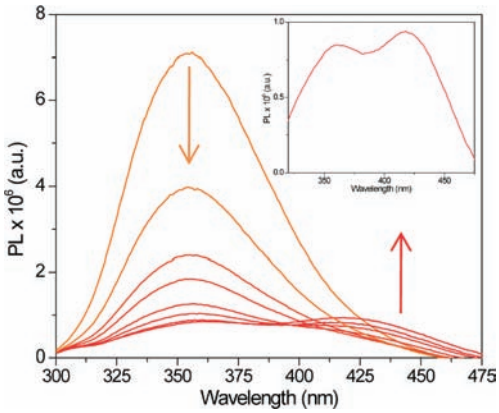

Figure 3. Fluorescence response as labeled RRE is titrated into Rev. Inset shows the emission spectrum at saturation. Conditions: Rev (1.0×10^{-5} M), cacodylate buffer pH 7.0 (2.0×10^{-2} M), NaCl (1.0×10^{-1} M).

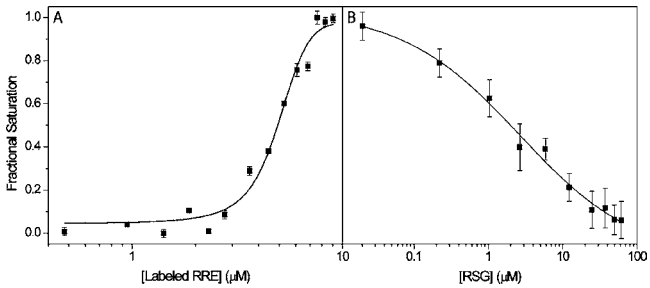

Figure 4. Normalized response of the fluorescent acceptor (■) in the labeled RRE in the following experiments: (A) titration of the labeled RRE into Rev; (B) displacement of Rev from the labeled RRE by RSG. Conditions same as those for Figure 3.

quinazoline-2,4(1*H*,3*H*)-dione **1** was glycosylated to provide the modified nucleoside **2** after saponification of all esters. Protection of the 5′-hydroxyl as the 4,4′-dimethoxytrityl (DMTr) derivative and the 2′-hydroxyl as the (trisisopropylsiloxy)methyl (TOM) derivative, followed by phosphitylation of the 3′-hydroxyl, provided phosphoramidite **3** (Scheme 1). Standard solid-phase oligonucleotide synthesis was utilized to prepare the 34-mer RRE model construct, where U66 is replaced by **2** (Figure 2). The oligonucleotide was purified by PAGE, and MALDI-TOF mass spectrometry confirmed its full length and the presence of the intact emissive nucleoside **2** (Figure S1). The folded RNA construct was as stable as the unmodified RRE construct ($T_m = 66$ and 68 ± 1 °C, respectively, cacodylate buffer, pH 7.0), suggesting minimal perturbation by the unnatural nucleosides. The emission profile of the emissive RRE construct, excited at 350 nm, resembled that of the parent nucleoside in water, albeit with a lower quantum yield.[8]

Titration of the Rev peptide into the emissive RRE construct, excited at 280 nm (Trp's absorption maximum), showed a continuous decrease of tryptophan emission at 350 nm and an increase of the acceptor emission at 440 nm (Figure 3). At equimolar concentrations (and saturation), the emission intensities of acceptor **2** and Trp are comparable (Figure 3 inset). Based on FRET efficiency, the calculated distance between nucleoside **2** and Trp is 18 (±3) Å, which is in good agreement with our structure-based, estimated distance between U66 and Trp.[18]

Titration curves, generated by plotting the normalized sensitized emission of acceptor **2**, yield a K_D value of $(7 \pm 5) \times 10^{-8}$ M, in agreement with literature values (Figure 4A).[12,16b,19] To ensure that the modification of U66 with **2** is nonperturbing, fluorescence anisotropy measurments[20] were used to independently determine the affinity of Rev to both the modified ($K_D = (2 \pm 1) \times 10^{-8}$) and unmodified RRE ($K_D = (3 \pm 2) \times 10^{-8}$) (Figure S2).[8] The placement of **2** at U66 of the RRE is therefore nonperturbing and enables a faithful monitoring of peptide−RNA binding.

Importantly, this FRET system can also report the displacement of the Rev peptide by competing ligands that do not contain tryptophan residues. The RSG peptide (Figure 2) is known to have a higher affinity to the RRE compared to Rev.[11] As RSG is titrated into a solution containing the Rev-bound labeled RRE, the emission of fluorescent nucleoside **2**, acting as the acceptor, decreases (Figure 4B). Fitting the decreased FRET efficiency yields an IC_{50} value of $(2 \pm 1) \times 10^{-6}$ M, confirming that it is indeed a tighter binder than Rev.[8]

In conclusion, we have identified a fluorescent nucleoside analogue **2** that is suitable for monitoring protein−RNA interactions via Förster resonance energy transfer with native Trp residues. To the best of our knowledge, **2** is the first isomorphic and visibly emitting nucleoside that can efficiently pair with tryptophan. While illustrated here for the HIV-1 Rev and RRE, the Trp−**2** FRET pair is likely to find utility in exploring other systems due to the high abundance of Trp residues within the RNA recognition domains of proteins.[21]

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Supporting Information Available: Synthetic details, thermal denaturation measurements, titration spectra, photophysical data, and MALDI-TOF MS spectrum. This information is available free of charge via the Internet at http://pubs.acs.org.

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