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## Emissive Nucleosides as Molecular Rotors

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### Keywords

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Naturally occurring nucleosides, the building blocks of nucleic acids, are characterized by two distinct molecular elements: a rigid aromatic nucleobase and a more flexible (2'-deoxy)- $\beta$ -ribose moiety. The structural features of nucleosides and their corresponding nucleotides are context-dependent and are largely dictated by two major and distinct “degrees” of conformational freedom: (a) the sugar pucker, and (b) the syn/anti orientation of the nucleobase with respect to the ribose ring (Figure 1A).<sup>[1]</sup>

Numerous modified pyrimidines, extensively explored and utilized in recent years, contain a conjugated aromatic residue at position 5.<sup>[2]</sup> Although typically benign with respect to the impact on higher structures (e.g., duplexes), the introduction of a rotatable bond separating two aromatic entities adds a new level of interplay between structure and properties, as the conformation around the aryl–aryl bond directly impacts the conjugation of the extended nucleobase and hence its electronic nature (Figure 1B). As a result, many of these modified nucleosides are fluorescent, a feature essentially nonexistent in naturally occurring nucleobases.<sup>[2]</sup> We hypothesized that this unique motif, not present in natural nucleobases, endows such modified nucleosides with molecular rotor behavior, a feature that typically manifests itself in remarkable sensitivity of the photophysical properties toward viscosity and molecular crowding effects. Here we demonstrate that emissive and highly responsive pyrimidines containing five-membered aromatic heterocycles (Figure 2), indeed possess a molecular rotor element. This feature ultimately results in dual probing capabilities where environmental polarity impacts their Stokes shift ( $\nu_{\text{abs}}-\nu_{\text{em}}$ ), while structure rigidifying factors impact the emission quantum yields (and hence the chromophore's brightness). This property has far reaching implications on the application of these and related chromophores for probing nucleic acids and other confined biomolecular cavities, but has so far been overlooked.

Although the relationship between molecular structure and fluorescence properties is neither straightforward nor predictable, it was understood early on that increased structural rigidification leads to enhanced emission quantum yield.<sup>[3]</sup> Experiments with diphenyl- and triphenylmethane dyes, while practically non-emissive in common solvents, revealed strong fluorescence in highly viscous media, illustrating that structural rigidity does not necessarily need to be intrinsic.<sup>[4]</sup> These early observations have triggered the recent development of viscosity sensitive fluorescent molecular probes, also referred to as molecular rotors, a term

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likely related to “Kreiselkörper”, introduced by Kramers in 1923.<sup>[5]</sup> These chromophores can undergo a rotational motion leading to twisted excited states.<sup>[6, 7]</sup> In contrast to media of low viscosity, viscous media impede free rotation resulting in structural rigidification. This diminishes the contribution of non-radiative decay pathways with consecutive enhancement of the fluorescence intensity.<sup>[6, 7]</sup>

Exploring the hypothesis delineated above, we have selected several related emissive nucleoside analogs with demonstrated utility in probing nucleic acid structures and lesions.<sup>[8–11]</sup> A furan modified dU (**1**), serves as a prototypical example, and is compared to the corresponding thiophene modified dU (**2**), furan modified dC (**3**), an extended ethynyl furan dU derivative (**4**) and, as a control, to a fused analogs (**5**) (Figure 2).<sup>[8–11]</sup> To establish a molecular rotor character, their photophysical features were examined in solvents with distinct viscosity and polarity.

The responsiveness of **1** to changes in viscosity is revealed by its temperature-dependent absorption and emission characteristics measured in solvents of distinct viscosity but nearly identical polarity: ethylene glycol ( $E_T(30) = 56.3$  kcal/mol,  $\eta_{20^\circ\text{C}} = 21.26$  cP), and glycerol ( $E_T(30) = 57.0$  kcal/mol,  $\eta_{20^\circ\text{C}} = 1317$  cP).<sup>[12]</sup> In contrast to the minimal effect on the absorption spectra of **1**, the emission signatures reveal a dramatic response to changes in solvent viscosity as introduced by temperature variations (Figures 3 and 4A). We stress that, although solvent viscosity drops dramatically upon increasing temperature, the polarity is minimally impacted.<sup>[13, 14]</sup> Using the logarithmic form of the Loutfy and Arnold equation [eq. 1, where  $\Phi$ ,  $B$ ,  $\eta$ ,  $T$ , and  $x$  represent fluorescence quantum yield, a constant, viscosity, temperature (in K), and a free volume parameter, respectively]<sup>[15]</sup> and plotting the data as log fluorescence intensity vs.  $\log(\eta/T)$ ,<sup>[16]</sup> a linear behavior, characteristic of molecular rotors, is obtained (Figure 4B).<sup>[14]</sup> The slope, representing the ‘free volume parameter’, is 0.78 and 0.51 for ethylene glycol and glycerol, respectively.

$$\Phi = B \left( \frac{\eta}{T} \right)^x \quad (1)$$

To corroborate the molecular rotor characteristics of **1**, 9-(dicyanovinyl)-julolidine (DCVJ), an established molecular rotor and viscosity probe,<sup>[7]</sup> was interrogated under identical conditions. The trend observed is very similar (Figure 4B), although the ‘free volume parameter’ obtained for DCVJ in glycerol is higher than the value determined for **1**, likely reflecting the differences in rotor size, which are especially prevalent in highly viscous milieus like glycerol.

The photophysics of **1** was evaluated in binary solvent mixtures at a constant temperature to further substantiate the observations reported above for temperature-mediated viscosity changes. To cover a wide viscosity window, solutions of **1** in binary mixtures of methanol ( $\eta_{20^\circ\text{C}} = 0.583$  cP) and glycerol were prepared and studied at 20 °C.<sup>[17]</sup> As before, the ground state absorption spectrum is minimally affected by changes in viscosity, but the fluorescence signal shows substantial enhancement upon increasing viscosity (Figure 5A).<sup>[22]</sup> A double log plot of emission intensity as a function of viscosity according the Förster-Hoffmann relation (eq. 2, where  $\Phi$ ,  $C$ ,  $x$ , and  $\eta$  represent fluorescence quantum yield, a constant, a dye dependent constant, and viscosity, respectively) gives a linear behavior, typical of molecular rotors.<sup>[24]</sup>

$$\log \Phi = C + x \log \eta \quad (2)$$

The observations described above indicate that **1** responds to viscosity changes regardless of the method of their introduction (different solvents, solvent mixtures, or temperature alterations). To support the generality of the observed phenomena and to gain insight into the relationship between molecular structure and sensitivity to viscosity, the photophysical characteristics of nucleosides **2–5** were examined in binary mixtures of methanol and glycerol.<sup>[14]</sup> Table 1 lists the slopes of the double log plots for **2–5**, obtained using the Förster-Hoffmann correlation as was done for **1** (Figures 5B and 6).

As expected, the emission spectra of the analogous thiophene-containing nucleoside **2** display the same sensitivity toward viscosity changes, when compared to **1**. Similarly, the dC analog **3** exhibits very similar behavior (Figure 6). These observations suggest that the electronic nature of the pyrimidine and the ring conjugated to its 5 position have a limited effect on the photophysical sensitivity to environmental viscosity, as long as the molecular footprint of the rotor element is comparable. Examining **4**, where the pyrimidine and the aromatic five membered ring are separated by a conjugating ethynyl linkage, further supports this conclusion. This modified nucleoside displays an attenuated responsiveness to viscosity changes, compared to the analogous **1**, where a single rotatable bond separates the pyrimidine and the furan. Finally, the highly emissive nucleoside **5**, where the thiophene heterocycle is fused to the pyrimidine 5,6 positions, lacks any sensitivity to changes in viscosity, illustrating that a related rigid emissive nucleoside missing a rotatable linkage is completely devoid of a molecular rotor behaviour (Table 1, Figure 6). As the response to viscosity of **1**, **2**, and **3** is comparable to the behavior of DCVJ, an established molecular rotor and viscosity probe, it is likely that torsional relaxation, by internal conversion, is the main channel for non-radiative decay for these fluorescent nucleosides.<sup>[25]</sup>

In order to complete the analysis of these emissive nucleoside analogs, their dual sensitivity to both solvent polarity and viscosity needs to be put into perspective. The fluorescent T-mimic **1** was previously employed as a microenvironmental probe relying on its high sensitivity to changes in polarity.<sup>[11]</sup> Because alteration of viscosity and polarity, from a spectroscopic viewpoint, translates predominantly to changes in fluorescence intensity and Stokes shift ( $\nu_{\text{abs}} - \nu_{\text{em}}$ ), respectively, nucleoside **1** and analogous derivatives can be viewed as dual probes. Sensitivity to polarity is fundamentally governed by the electronic nature of the chromophore and responsiveness to viscosity, as demonstrated, requires certain structural requirements to be fulfilled. In addition to the viscosity sensitivity of nucleosides **1–5**, Figure 6 also overlays the responsiveness of these fluorophores to changes in polarity as determined by monitoring their absorption and emission maxima in dioxane–water mixtures of distinct polarity.<sup>[14]</sup> Although **1** displays high sensitivity to both, nucleoside **5** responds only to changes in environmental polarity. Intermediate behavior is seen for nucleosides **2–4**. Evidently, there is no immediate parallel between the sensitivity for both parameters, showing that sensitivity toward polarity and viscosity stems from different molecular features, which are not, however, necessarily decoupled. Although the former relies on distinct electronic features (e.g. donor-acceptor interactions leading to greater charge separation in the excited state), the latter predominantly originates from geometrical properties (the presence of rotatable bonds separating  $\pi$  systems of distinct electronic nature).

Finally, to illustrate the significance and potential application of our findings, we discuss the detection of abasic sites, known cytotoxic DNA lesions, by nucleoside **1**. When **1**, in a complementary probe oligonucleotide, was hybridized and placed opposite an abasic site in a defective oligonucleotide, a significant emission enhancement, when compared to the perfect duplex, was observed.<sup>[8]</sup> It was hypothesized that the modified nucleobase undergoes an *anti* to *syn* conformational change and helix internalization (Figure 7).<sup>[8]</sup> Although the exact molecular morphology of an abasic site is unknown, the intrahelical vacant but

confined space between two WC base pairs likely limits the free rotation of the furan-uracil single bond, effectively mimicking a medium of elevated viscosity. The results reported here, revealing the molecular rotor entity in **1**, thus corroborate our previously postulated intrahelical model, suggesting restricted mobility within this biomolecular cavity.

In summary, we have shown that the fluorescence intensity of **1** (as well as of **2–4**) drastically responds to viscosity changes, demonstrating the presence of a molecular rotor element in such nucleosides. This observation, in addition to their established responsiveness to polarity, endows these nucleosides with dual probing characteristics, which have so far been overlooked. Importantly, the findings described are not necessarily limited to these nucleosides alone, but are likely applicable to the numerous modified nucleosides which contain a rotatable bond separating a pyrimidine or purine and a conjugated aromatic ring.<sup>[2, 10]</sup> In addition to the fundamental significance of this discovery, our observations expand the utility landscape of emissive nucleosides to include information concerning microenvironmental polarity as well as molecular crowding effects.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

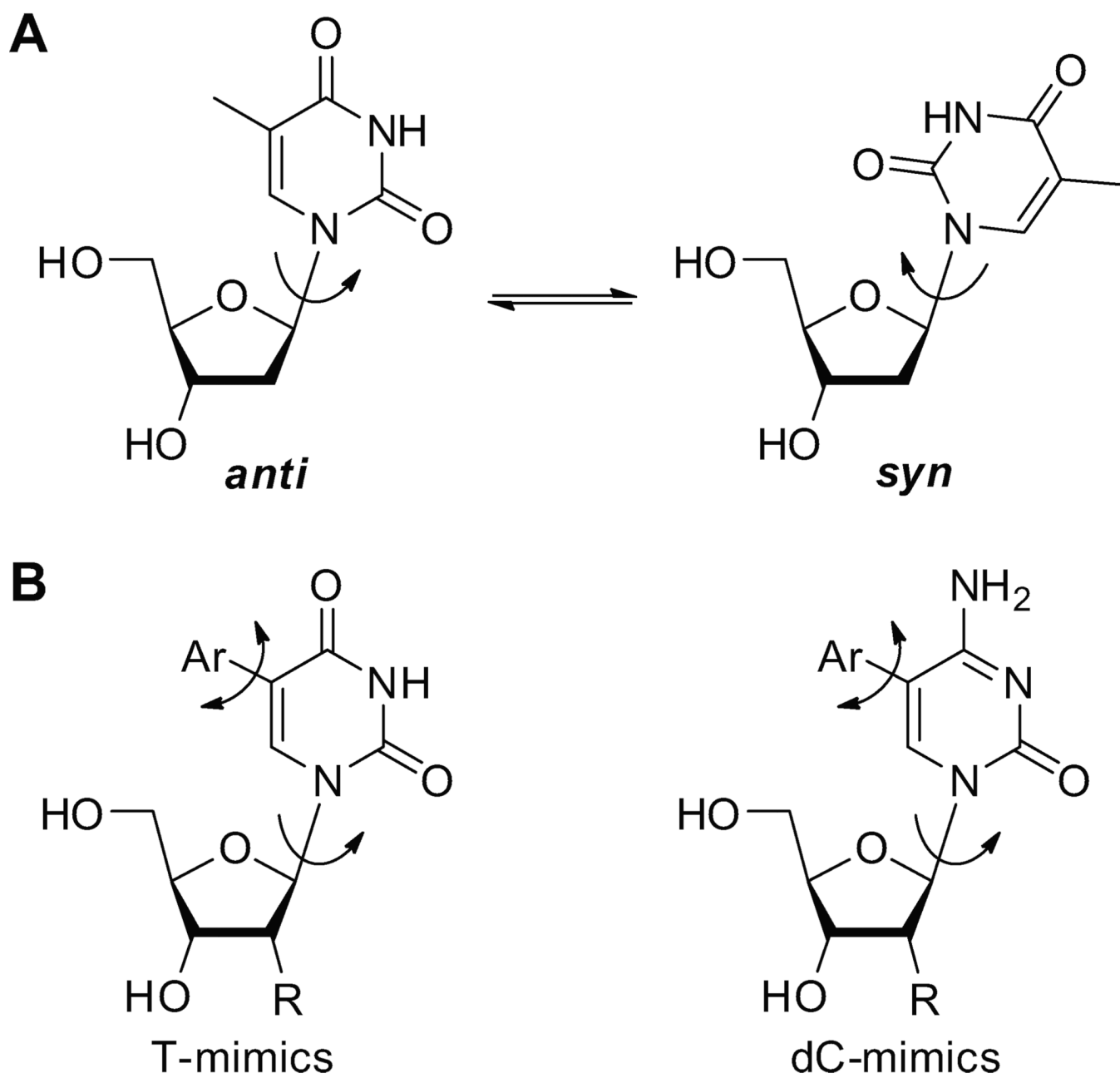
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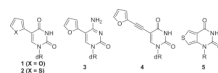
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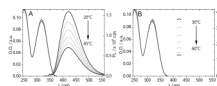


**Figure 1.**

(A) *Syn* and *anti* orientation of the nucleobase in T, a native pyrimidine. (B) The conformation around the aryl-aryl bond in 5-modified pyrimidines is likely to impact the electronic nature of the chromophore.

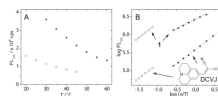


**Figure 2.**  
Emissive nucleosides used in this study where R and dR stand for D-ribose and 2'-deoxy-D-ribose, respectively.

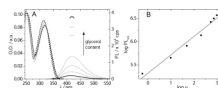


**Figure 3.** Absorption (dashed lines) and emission spectra (solid lines) of **1** in A) ethylene glycol, and B) glycerol. Note that the maximum fluorescence intensity in graph 3B is ~6-fold higher than the minimum in graph 3A.

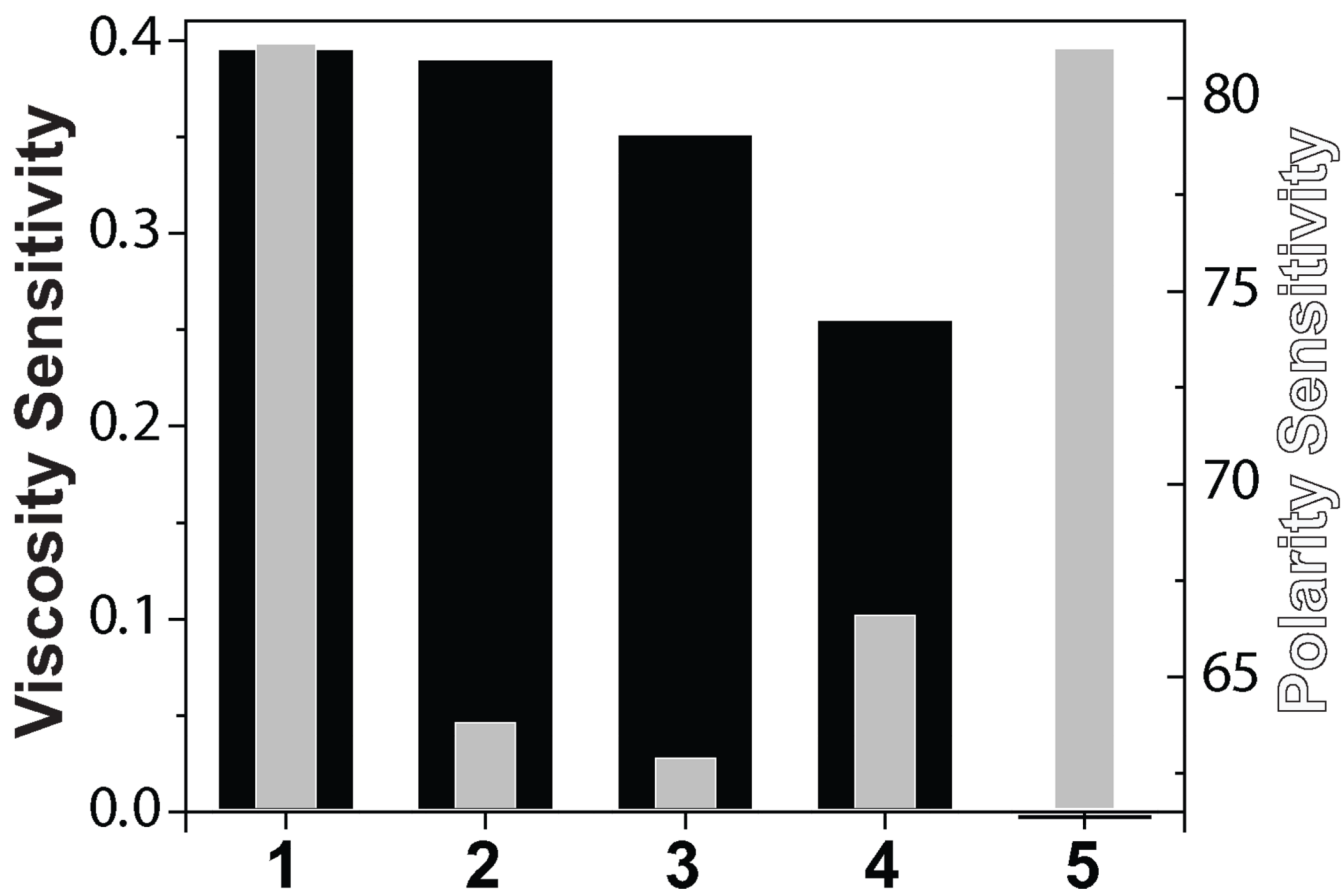




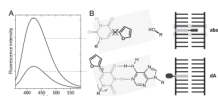
**Figure 4.** Plots showing A) Fluorescence intensity (PL<sub>int.</sub>) vs. T, and B) log PL<sub>int.</sub> vs. log (η/T) for **1** (solid grey lines) and DCVJ (dashed grey lines) in ethylene glycol (open circles) and glycerol (solid stars).



**Figure 5.** A) Absorption (dashed lines) and emission (solid lines) spectra of 1 in methanol, glycerol (black lines), and mixtures thereof (grey lines). B)  $\log PL_{\text{int}}$  vs.  $\log \eta$  (solid circles) and a linearization (grey line). The curves represent an average of three independent measurements.



**Figure 6.** Viscosity (black) and polarity (grey) sensitivity for compounds 1–5. The viscosity sensitivity is expressed by the slopes of the double log plot (Table 1). The polarity sensitivity is expressed by the slope of the relationship between Stokes shift and the sample's  $E_T(30)$  value.



**Figure 7.**

A) The fluorescence intensity of **1** incorporated in a duplex across an abasic site (top) and opposite dA (bottom). B) Schematic representation of a DNA duplex containing **1** opposite of dA and opposite of an abasic site. In the latter, the modified nucleobase is sandwiched between two WC base pairs, which restricts its conformational freedom.

**Table 1**

Viscosity and polarity sensitivity parameters.

Nucleoside	Viscosity Sensitivity <sup>[a]</sup>		Polarity Sensitivity <sup>[b]</sup>	
	Y-intercept	Slope	Y-intercept	Slope
<b>1</b>	5.3	0.40	4317	81.9
<b>2</b>	5.2	0.39	5188	63.8
<b>3</b>	5.1	0.35	6817	62.9
<b>4</b>	5.6	0.26	6135	66.6
<b>5</b>	5.8	0.01	3794	81.3

<sup>[a]</sup>Viscosity sensitivity is determined in MeOH-glycerol mixtures @ 20 °C.

<sup>[b]</sup>Polarity sensitivity is expressed by the slope of the Stokes shift vs. sample ET(30) values relation in water-dioxane mixtures @ 20 °C. The error is typically <7%.