Published in final edited form as:

Chemphyschem. 2012 October 8; 13(14): 3350–3356. doi:10.1002/cphc.201200375.

Modified 6-Aza Uridines: Highly Emissive pH Sensitive Fluorescent Nucleosides

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Abstract

Optimized facile syntheses and highly desirable spectroscopic properties of two isomorphous fluorescent pyrimidines, comprising a 1,2,4-triazine motif conjugated to a thiophene (**1a**) or a furan (**1b**), are described. Although structurally related to their 5-modified uridine counterparts, these modified 6-aza-uridines reveal dramatically improved fluorescence properties and a remarkable sensitivity to polarity and pH changes. The thiophene derivative **1a** has an absorption maximum around 335 nm, which upon excitation yields visible emission with a polarity sensitive maximum and fluorescence quantum yield ranging from 415 nm ($\Phi = 0.8$) to 455 nm ($\Phi = 0.2$) in dioxane and water, respectively. Nucleoside **1a** also displays susceptibility to acidity. Correlating emission intensity and solution pH yields a pK_a value of 6.7–6.9, reasonably close to physiological pH values. The results illustrate that highly sought-after fluorescence features (brightness and responsiveness) are not necessarily the trait of large fluorophores alone, but can be observed with probes that meet stringent isomorphous design criteria.

Keywords

Fluorescent Probes; Nucleosides; Photophysics Donor-acceptor Systems; Acidity

1. Introduction

The sensitivity and simplicity of fluorescence spectroscopy has made it one of the most useful tools for the study of biomolecules. Despite numerous technological and instrumental advances, the quality of fluorescence-based analyses ultimately relies on the fluorescent probes themselves. Countless fluorescent tags, labels and probes have been designed over the years, but only a limited fraction meets the stringent design criteria of isomorphism.^[1] Useful isomorphous probes are characterized by a high structural resemblance to the molecular building blocks they mimic, while maintaining distinct spectroscopic properties. This imposes structural and application-specific constraints, where probes capable of innocently replacing membranes, proteins, or nucleic acid components have to be fabricated.^[1] The design of such probes is further challenged by the fact that the relationship between structure and photophysical properties is hard to deduce, and can typically only be elucidated experimentally.

To explore nucleic acids, the non-emissive canonical nucleosides need to be structurally modified to endow them with useful fluorescent properties. Such alterations cannot impede

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native W-C base-pairing and subsequent helix formation.^[2,3] Successful nucleoside probes reconcile these structural and photophysical demands with an exclusively excitable wavelength, red-shifted from the ~260 nm absorption band of the native nucleobases. Responsive emissive nucleoside probes, unlike fluorescent tags or labels, are characterized by spectroscopic properties, which are sensitive to changes in the local environment. Several groups, including ours, have reported a rich variety of isomorphous fluorescent nucleoside analogs.^[1,4,5] The basic design principle of our first generation emissive nucleobase analogs has relied on conjugating five membered aromatic heterocycles to the pyrimidine core at the 5-position.^[2] The modification at this position appears to be structurally benign, with no impact on the anti orientation or sugar pucker of the nucleosides.^[6] The resulting nucleosides emit in the visible range (390–443 nm), have rather large Stokes shifts (8400–9700 cm⁻¹), but suffer from relatively low emission quantum efficiency ($F=0.01-0.035$).^[6] These nucleosides behave like molecular rotors, with the 5-membered heterocycles and the pyrimidine core acting as the “donor and acceptor”, respectively.^[7] Rotation around the biaryl linkage provides an effective channel for non-radiative torsional relaxation, thus leading to low emission quantum yield in non-viscous media.^[7,8] We hypothesized that altering the electronics by replacing the pyrimidine with the corresponding 1,2,4-triazine core (i.e., 6-aza-pyrimidine)^[9] would enhance the charge transfer character, ideally yielding a bathochromic shift as well as a hyperchromic effect and higher brightness. Herein we report a simplified synthetic pathway to thiophene and furan-modified 6-azauridines, discuss their most relevant photophysical properties, and reveal their responsiveness to environmental polarity and pH.

2. Chromophore Design

As articulated above, replacing the uridine core with 6-azauracil, a more electron deficient heterocycle, while keeping the electron rich thiophene and furan moieties in the 5-position, was expected to maintain an extended π -system while augmenting the donor-acceptor nature of this motif (Scheme 1). This enhanced “push-pull” interaction is expected to affect a red-shift of the absorption maxima and likely a concomitant red-shifted emission maxima. Such a donor-acceptor interplay is also frequently associated with enhanced sensitivity toward environmental polarity.^[10–13] The single bond linkage between the 5-membered heterocycle and the 6-membered nucleobase introduces a molecular rotor element likely to render **1a** and **1b** sensitive toward viscosity (molecular crowding) as demonstrated for other 5-aryl modified pyrimidines.^[7,8] Chromophores possessing such a feature typically reveal much higher emission intensity in viscous compared to non-viscous media. Viscosity (molecular crowding) hampers formation of a twisted excited state thereby limiting the rotational relaxation, a non-emissive decay pathway, to the ground state.^[14,15]

Only a few fluorescent nucleosides have been shown to be pH sensitive.^[8,16,17] 8-Aza-guanosine, an isomorphous fluorescent purine analog,^[18] shows its pH-dependent emission characteristics as a nucleoside^[19] and after enzymatic incorporation into RNA.^[20] This feature was exploited in the investigation of the catalytic function of RNA.^[21] An application reveals interesting opportunities for fluorescent nucleosides that display spectral sensitivity toward (de)protonation. No emissive pyrimidines with pK_a values near physiological pHs have, however, been documented. Importantly, the presence of the nitrogen atom at position 6 has a profound influence on the acidity of the NH, lowering its pK_a to 6.8 vs. a pK_a value of 9.3 for the parent uridine.^[22] Like uridine, 6-aza-uridine has no appreciable fluorescence properties hampering visualization of (de)protonation events with fluorescence spectroscopy. Assuming the emissive features discovered for the parent 5-modified uridines translate to (or are enhanced in) the 6-aza motif, deprotonation and protonation events are likely to be revealed by changes in the emission profile. This new

motif would therefore constitute the first example of a pH responsive emissive pyrimidine with a pK_a value near physiological pHs.

3. Results

3.1. Synthesis

Condensation of commercially available thiophene glyoxylic acid (**2a**) with semicarbazide (**3**) gave imine **4a** which, *in situ*, cyclized upon heating under basic conditions to furnish **5a** in good yields. The same approach, starting from **2b**, provided **5b**. Glycosylation of **5a** and **5b** was performed in a two-step one-pot procedure. First, the nucleobase was activated by reaction with bis(trimethylsilyl)acetamide (BSA) in acetonitrile for ~30 minutes at rt, turning the initial suspension into a clear solution. Secondly, the reaction mixture was brought to 85°C after which β -D-ribofuranose 1-acetate 2,4,5-tribenzoate (**6**) was added, immediately followed by addition of trimethylsilyltriflate (TMSOTf). The reaction was allowed to run for 30 minutes after which TLC analysis indicated full conversion of the nucleobase to the desired glycosylated product. An aqueous work-up followed by purification with column chromatography yielded **7a** and **7b** in good yields. Deprotection with methanolic ammonia at 60°C, followed by column chromatographic purification gave desired **1a** and **1b**.

3.2. Photophysics

3.2.1. Basic photophysics in water—In unbuffered deionized water, **1a** is characterized by a red-shifted absorption maximum at 332 nm, which upon excitation gives a visible fluorescence signal, peaking at 455 nm, with a respectable quantum yield ($\Phi = 0.20$) and a fluorescence lifetime of 4.9 ns (Table 1). In contrast, a solution in dioxane yields an absorption maximum at 335 nm, an emission maximum at 415 nm ($\Phi = 0.80$) and a slightly longer fluorescence lifetime of 5.4 ns. The small change in absorption maximum, but 40 nm difference between the emission maxima in the two solvents prompted us to study the sensitivity to polarity in greater detail (see below).

3.2.2. Sensitivity to polarity—Influence of polarity on spectroscopic parameters of fluorescent probes was studied using samples in dioxane ($E_T(30) = 36.0$ kcal/mol), water ($E_T(30) = 63.1$ kcal/mol) and mixtures thereof.^[23] Water–dioxane samples of **1a** and **1b** were prepared from a concentrated DMSO stock solution and subjected to an absorption and steady state emission spectroscopy study (Figure 1 and Table 1). The small amount of DMSO has been established to have negligible impact on polarity.^[24]

The Stokes shifts ($\nu_{\text{abs}} - \nu_{\text{em}}$) were calculated for the samples in dioxane, water, and mixtures thereof and plotted, after conversion from cm^{-1} to kcal/mol, as a function of the experimentally-determined samples' $E_T(30)$ values (Figure 3). The data points clearly line-up linearly as was established by a good linear fit with a slope value of 0.268, quantifying the sensitivity to polarity (Table 2). In addition to a poorer linear fit, the sensitivity to polarity for **1b** was lower (Table 2 and SI).

3.2.3. Sensitivity to pH—The sensitivity of the emissive 6-aza-U chromophores to pH was studied in aqueous phosphate buffers with pH values ranging from 0.5 to 12. Absorption spectra only showed small differences in maxima ($\lambda_{\text{max}} = 326$ nm), while the corresponding emission spectra revealed a strong influence of pH on the position as well as intensity of the emission maximum (Figure 2a, Table 1). The emission maximum shifted from ~426 nm ($\Phi = 0.39$) to ~462 nm ($\Phi = 0.13$), going from pH 10.13 to 2.55, respectively. Curve-fitting the normalized fluorescence intensity as a function of pH gave a sigmoidal curve from which a pK_a value of 6.7 was calculated (Figure 2b). Using the same approach a

pK_a value of 6.6 for probe **1b** was determined.^[25] The difference between the quantum yield under acidic and basic conditions was also reflected by the difference in fluorescence lifetimes, 3.0 and 6.6 ns, respectively (Table 1).

3.2.4. Sensitivity to viscosity—Absorption and emission spectra of **1a** in samples of methanol, glycerol and mixtures thereof reveal virtually no responsiveness toward viscosity for **1a** (Figure 3), but indicate a significant sensitivity to viscosity for **1b**.^[25] A double log plot of the fluorescence intensity as a function of sample viscosity for **1b**, however, reveals poor linearity.^[25] The high fluorescence quantum yields for **1a** in methanol (0.50) and glycerol (0.66), are accompanied by the longest lifetimes observed in this study, 6.6 and 7.9 ns, respectively.

4. Discussion

4.1. Synthesis

The earliest scheme described for the synthesis of **5b**, is a two-pot two-step procedure starting from furanglyoxylic acid (**2b**), which was reacted with semicarbazide hydrochloride (**3**) to give the corresponding formic acid semicarbazone (**4b**). This, in turn, yielded **5b** upon refluxing in a mixture of propylene glycol–ethanol in the presence of sodium ethoxide.^[26] Later, Walker *et al.* reported a procedure for the synthesis of **2a**, but used thio-semicarbazide instead of semicarbazide (**3**), necessitating a third step to convert the thiocarbonyl into a carbonyl moiety.^[27] To shorten the synthesis of **5a** and **5b** we started from semicarbazide and convert the original two-pot two-step synthesis^[26] to a simplified one-pot two-step procedure.^[28] Importantly, this streamlined approach works well for the synthesis of **5a** and, albeit with lower yield, also for **5b**, while the three-step three-pot synthesis by Walker *et al.* works for the synthesis of **5a** it but proved very challenging for the synthesis of **5b**.^[27,29] Glycosylation of 5-substituted 6-aza uracils has been reported.^[27,30,31] We used a slightly modified procedure involving activation of the nucleobase with BSA in CH_3CN instead of $TMSCl$ and Et_3N in benzene. We performed the glycosylation directly *in situ* after BSA treatment, activating the ribose (**6**) with $TMSOTf$, instead of changing the solvent from benzene to dichloroethane and using stannic chloride to activate the ribose (**6**). Our procedure proved facile, quick, and high yielding for the synthesis of **7a**, and **7b**. Deprotection of the benzoyl groups to give **1a** and **1b** was performed using methanolic ammonia for 24 hours at 60°C in a pressure vessel. Deprotection also succeeded with aqueous ammonia in methanol in 17 hours at 40°C, albeit in lower yield. Both **1a** and **1b** were synthesized in 3 steps from commercially available starting materials in 41% and 20% overall yield, respectively.

4.2. Fluorescence Characteristics and their Sensitivity to Polarity

Diverse events can impact the structure of nucleic acids (e.g., (un)folding, binding of low MW ligand, lesions), which often result in changes in the local polarity. Nucleic acid probes able to detect such changes in environmental polarity have proven to be useful tools.^[1,24,32–39] We therefore thoroughly evaluate the influence of polarity on a probe's photophysical properties. We regularly use water–dioxane mixtures, as they not only vary in polarity, but also present changes in hydrogen bonding donor and acceptor composition prone to exert specific solvent–solute interactions.^[23] Moreover, the polarity window obtainable with such water–dioxane mixtures likely reflects most biological relevant environmental polarities. Using a microenvironmental polarity parameter such as the $E_T(30)$ scale with values ranging from 36.0 to 63.1 kcal/mol for pure dioxane to water, respectively,^[11] typically provides good correlation with spectral features like Stokes shifts.^[23]

While the quantum yield for **1a** in water is modest ($\Phi = 0.20$) and low for **1b** ($\Phi = 0.05$), the quantum yield displayed by **1a** in dioxane ($\Phi = 0.80$) is among the highest values reported for an isomorphous nucleoside (Table 1).^[1] Both the quantum yield in water and dioxane represent a huge improvement compared to the values reported for the corresponding 2'-deoxyuridine modified with either a thiophene (**8a**) ($\Phi_{\text{water}} = 0.01$) or furan (**8b**) ($\Phi_{\text{water}} = 0.03$) in the five position (Scheme 1).^[2,6] Interestingly, uridine analogs **8a** and **8b** display the opposite behavior with even lower quantum yield in apolar organic media.^[2,6,24] Incidentally, this remarkable difference in fluorescence quantum yield between these isoelectronic nucleoside analogs shows that photophysical properties are difficult to predict from the molecular structure alone.

It is worth noting that we previously reported the sensitivity to polarity for **8a** and **8b** in $\text{cm}^{-1}/\text{kcal mol}^{-1}$ (Table 2). To obtain unitless values, the values for the Stokes shift in cm^{-1} were converted to kcal/mol, and replotted as a function of the samples' $E_T(30)$ value. The slope for the linear relationship, the polarity sensitivity, for modified uridines **8a** and **8b** was determined to be 0.182 and 0.227,^[25] respectively, both significantly less sensitive than **1a** (Table 2). These substantial differences illustrate how the enhanced push-pull motif present in **1a** and **1b**, as compared to **8a** and **8b** indeed results in the expected enhanced susceptibility to polarity.

4.3. Sensitivity to pH

Sensitivity to pH as a characteristic of fluorescent nucleosides is relatively uncommon, although a couple of examples have been reported.^[8,17,19] In these cases the absorption spectra show a two-state transition with an isosbestic point. The influence of pH on the emission properties is reflected by a strong increase or decrease of the fluorescence intensity not necessarily accompanied by significant shifts of the emission maxima.

Nucleoside **1a** reveals its pH sensitivity by small changes in absorption, but large changes in emission intensity and maxima (Figure 2a). Changing from basic to acidic conditions the fluorescence curve of **1a** shifts from a maximum located at 426 nm to 462 nm with a concurrent drop in the fluorescence quantum yield from 0.39 to 0.13, respectively (Figure 2a, Table 1). Fluorescence decay analysis reveals that the fluorescence lifetime in basic conditions is more than doubled compared to the value in acidic conditions. The drop in normalized fluorescence intensity as a function of pH gives, after a Boltzmann fit of the data points, an S-curve indicative of a two-state (de)protonation process with a pK_a of 6.7 and 6.6 for **1a**, and **1b** respectively (Figure 2b and SI). These values are in agreement with a reported value for 6-aza-uridine ($pK_a = 6.8$).^[22] It further shows that the nature of the modification in the five position of the six membered heterocycle has little effect on the pK_a of the NH as its electronic influence is limited. The observed pK_a values significantly deviate from the reported values for the parent uridine (9.3 – 9.5),^[22,40,41] demonstrating that the additional nitrogen renders the NH ~1000 fold more acidic. This finding is corroborated by others who have reported that substitution of H-6 in native uracil with an electron withdrawing chloride has a spectacular influence on the pK_a value causing a drop from 9.5 to 5.8.^[42] Closer inspection of Figure 2a shows a downward trend of the fluorescent intensity at very low pHs. Although speculative at this stage, this might suggest that the 6-aza nitrogen gets protonated at pHs far below 2. A normalized plot excluding these low pH data points ($\text{pH} < 2.5$) gives a slightly higher pK_a value of 6.85 for the heterocycle's NH (Figure S4.1).^[25] Regardless of this putative second transition, **1a** shows fluorescence intensity as well as emission maximum sensitivity with a pK_a close to physiological pH. This makes **1a** an attractive probe for studies involving (de)protonation events on RNA, as there are no known isomorphous pyrimidine analogs with pK_a values at this range.^[21]

4.4. Sensitivity to Viscosity

The nucleobase moiety in **1a** and **1b** can be described as a molecular rotor, being comprised of a single bond linking two π systems.^[7] Such molecules can lose their excitation energy via a non-emissive rotational decay from a twisted excited state. Molecular crowding effects such as the one induced by viscous solvents can hinder formation of a twisted excited state, thereby limiting the non-emissive rotational decay process resulting in an increased emission intensity.^[43] The contribution of this non-emissive decay pathway can be studied by plotting the emission intensity as a function of sample viscosity.^[44] A practical approach to controlling sample viscosity is the use of binary mixtures of solvents with very low and very high viscosities that differ minimally in polarity.^[7,8,45] Suitable non-viscous and highly viscous solvents are methanol ($\eta_{20^\circ\text{C}} = 0.583$ cp), and glycerol ($\eta_{20^\circ\text{C}} = 1317$ cp),^[7, 46] respectively. They can be mixed in all ratios to control sample viscosity which can be calculated based on the weight fraction and viscosity values of its pure components.^[47]

Clearly, **1a** shows no fluorescence intensification upon increasing viscosity, suggesting that **1a** does not adopt a twisted excited state upon excitation (Figure 3a). This contrasts the significant sensitivity for viscosity observed for **1b**.^[25] The difference can perhaps be explained by their respective quantum yields in solvents of low viscosity (Table 1). Samples in non-viscous, protic solvents such as water and methanol display quantum yields of 0.20 and 0.50 for **1a**, but 0.05 and 0.04 for **1b**, respectively. The already much higher quantum yields for **1a** suggest that rotational relaxation might not be a major non-radiative pathway for this chromophore's electronic decay. The very low initial quantum yields of **1b**, however, benefit greatly from curtailment of this non-emissive decay pathway.

Explaining the distinct differences between the two related chromophores is challenging and must be sought in searching for differences in the relative ground state orientation of the thiophene (or furan) with respect to the nitrogenous nucleobase. In the reported crystal structure of the deoxy-ribose form of **1a**, the sulfur atom of the thiophene moiety, which is coplanar with the nucleobase, points away (“*anti*”) from the 4-carbonyl thereby facing (“*syn*”) the 6-aza nitrogen (Figure 4).^[48] This orientation is opposite to the one observed in our reported crystal structure of **8a**, in which the thiophene moiety, also coplanar with the nucleobase, is oriented with its sulfur atom pointing toward the 4-carbonyl.^[2,6] The crystal structure of **8b** shows again the opposite configuration, with the furan oxygen facing away from the 4-carbonyl (Figure 4).^[2] In lieu of a crystal structure of **1b**, we can only speculate on the orientation of its furan oxygen. While differences between solid and solution states could exist, the distinct orientational preferences of the thiophene and furan units in the crystal structures might also reflect their ground state orientation in solution. The electronic nature of the atom in the 6-position, CH (**8a** and **8b**) or N (**1a** and **1b**) seems, therefore, to influence the orientation of the 5-membered heterocycle. This, in turn, could have a profound influence on the excited state geometry and hence sensitivity toward viscosity.

5. Summary

The 6-aza-uridine motif, decorated at the 5-position with either a thiophene (**1a**) or furan (**1b**), is a synthetically accessible pyrimidine analog with highly desirable photophysical properties. The Stokes shift of **1a** is very sensitive to polarity. The pH sensitive fluorescence intensity starkly increases upon deprotonation with a pK_a value of 6.7–6.9, close to physiological pHs. Most importantly, the fluorescence quantum yield remains sufficient ($\Phi_{\text{Water(pH 2.55)}} = 0.13$) to excellent ($\Phi_{\text{dioxane}} = 0.80$) under all conditions and is thereby amongst the highest reported for isomorphous nucleoside probes.^[1] The robust fluorescence quantum yield in neutral protic solvents ($\Phi_{\text{water}} = 0.20$, $\Phi_{\text{MeOH}} = 0.50$, and $\Phi_{\text{glycerol}} = 0.66$) suggest minimal population of non-radiative decay pathways, which is arguably the cause for the lack of sensitivity to viscosity. The structurally related nucleoside **1b**, displays

relatively low quantum yields in protic solvents and sensitivity to viscosity, marked by a steep increasing fluorescence quantum yield with increasing medium viscosity. Interestingly, **1b** shows almost identical pH sensitivity to **1a**, but a weaker sensitivity to polarity. Despite their differences, both **1a** and **1b** exhibit more desirable photophysical properties than their reported 2'-deoxyuridine analogs (**8a** and **8b**), making the 6-aza motif one of the most attractive and promising emissive pyrimidines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the National Institutes of Health for their generous support (grant number GM 069773), and the National Science Foundation (instrumentation grant CHE-0741968).

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Figure 1.

a) Absorption (dashed line) and emission (solid line) spectra of **1a** in dioxane (bold black line, solid circles), water (bold black line, open circles), and binary mixtures of 10, 30, and 70 v% water in dioxane (black lines). Samples' concentration is $7.9 \times 10^{-6} \text{ M}$, emission is recorded after excitation at 335 nm. b) A plot of the Stokes shift (in kcal/mol) vs. $E_T(30)$ values (in kcal/mol) obtained from dioxane–water mixtures (data points with error bars) and linearization (grey line). Absorption and emission spectra of each sample are recorded in duplicate; only one set is shown.

Figure 2.

a) Absorption (dashed lines) and emission plots (solid lines), corrected for small differences in the O.D. at the λ_{ex} (335 nm) of **1a** at pH 0.61 (open circles), pH 10.99 (solid circles), pH 6.55 (open triangles) and intermediate pH values (black lines). b) Plot of the normalized emission intensity as function of sample pH (solid circles) fitted to a sigmoidal curve (grey line). The dashed lines illustrate a graphical determination of the pK_a value.

Figure 3.

Absorption (dashed lines) and emission (solid lines) curves for **1a**, corrected for small differences in the O.D. at the λ_{ex} (340 nm), in methanol (solid circles), glycerol (open circles) and mixtures thereof (black lines).

Figure 4.

Crystal structures of **8a**,^[6] **8b**,^[6] and the 2'-deoxy-ribose form of **1a**^[48]. Note: For clarity, the hetero atoms of the nucleobases are labeled.

Scheme 1.

a) Syntheses of **1a** and **1b**, reagents and conditions: *i*) semicarbazide hydrochloride (**3**) (1.1 equiv.), H₂O, 60°C, 17h, *ii*) add. 1 M NaOH aq. (3 equiv.), 60°C, 4h, *iii*) BSA (3 equiv.), CH₃CN, rt, 30', *iv*) β-D-ribofuranose 1-acetate 2,4,5-tribenzoate (**6**) (1.1 equiv.), TMSOTf (1.1 equiv.), CH₃CN, 85°C, 30', *v*) NH₃/MeOH, 60°C, 24h; and b) structures of reported 5-(thiophene-2-yl)-2'-deoxyuridine (**8a**) and 5-(furan-2-yl)-2'-deoxyuridine (**8b**).

Table 1

Selected spectroscopic properties of **1a** and **1b**.^[a]

Solvent	Absorption		Fluorescence			Brightness	Stokes shift	
	λ_{max} nm	$\epsilon \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$	λ_{max} nm	Φ	τ ns	$\Phi \times 10^3$	$\nu_{\text{abs}} - \nu_{\text{em}} \text{ cm}^{-1}$	
1a	Dioxane	335	1.3	415	0.80	5.4	10.4	6025
	Water	332	1.1	455	0.20	4.9	2.2	8492
	pH 2.55	336	1.2	462	0.13	3.0 ^b	1.6	8485
	pH 10.13	325	1.2	426	0.39	6.6 ^b	4.7	7588
	MeOH	334	1.1	433	0.50	6.6	5.5	7332
	Glycerol	339	1.0	444	0.66	7.9	6.6	7372
	Dioxane	327	1.2	414	0.60	5.6	7.2	6824
	Water	320	1.0	443	0.05	3.1	0.5	9107
1b	pH 2.55	329	1.1	485	<0.01	<1 ^b	<0.1	9861
	pH 10.13	319	1.1	446	0.11	2.8 ^b	1.2	9273
	MeOH	326	1.1	442	0.04	1.6	0.4	8585
	Glycerol	331	1.1	434	0.46	2.7	5.1	7574

^[a]The Stokes shifts are calculated with corrected emission maxima obtained using: Intensity [ν] = $\lambda^2 \times \text{Intensity} [\lambda]$.^[10]

^bValues for pH 2.55 and pH 10.13 (aq. 10 mM phosphate, 0.1 M NaCl buffers) represent the lower- and upper plateau values of the sigmoidal fit (Figure 2). All values represent averages, see SI for error analysis.

Table 2

Sensitivity to polarity^[a]

	Slope	Y-int.	Slope	Y-int.	R ²
	cm ⁻¹ /kcal mol ⁻¹	cm ⁻¹	-	kcal mol ⁻¹	
1a	93.7	2548	0.268	7.29	0.99510
8a	63.8	5188	0.182	14.83	0.86159
1b	75.4	4290	0.216	12.27	0.84211
8b	79.5	4437	0.227	12.69	0.96922

^[a]Polarity sensitivity equals the slope of the linearized Stokes shift vs. $E_T(30)$ plot. Values for 5-(thiophen-2-yl)-2dU **8a** and 5-(fur-2-yl)-2dU **8b** in cm⁻¹/kcal mol⁻¹ have been reported.[7, 25] A unitless value for polarity sensitivity is obtained by conversion of the Stokes shift value from cm⁻¹ to kcal mol and reploting it as a function of the sample $E_T(30)$ value (in kcal mol⁻¹). The adjusted R² represents the goodness of the linear fit.