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Functionalized Tricyclic Cytosine Analogues Provide Nucleoside Fluorophores with Improved Photophysical Properties and a Range of Solvent Sensitivities

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Abstract

Tricyclic cytosines (tC and tC^O frameworks) have emerged as a unique class of fluorescent nucleobase analogues that minimally perturb the structure of B-form DNA and that are not quenched in duplex nucleic acids. Systematic derivatization of these frameworks is a likely approach to improve on and diversify photophysical properties, but has not so far been examined. Synthetic methods were refined to improve on tolerance for electron donating and electron withdrawing groups, resulting in a series of eight new, fluorescent cytidine analogues. Photophysical studies show that substitution of the framework results in a pattern of effects largely consistent across tC and tC^O and provides nucleoside fluorophores that are brighter than either parent. Moreover, a range of solvent sensitivities is observed, offering promise that this family of probes can be extended to new applications that require reporting on the local environment.

Keywords

nucleosides; fluorescent probes; DNA; RNA; structure-activity relationships

Introduction

Fluorescent nucleoside analogues are used extensively as molecular probes for biophysical studies and in biotechnology.^[1–6] Especially for the former, there is a great need for new, improved probes with a range of fluorescence properties and minimal structural perturbation. The vast majority of available probes either dangle freely from the major groove of the duplex (e.g. Cy5-dC)^[2, 7] or are fluorescent nucleobase analogues.^[8–28] Most

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of the latter are either incompatible with Watson-Crick hydrogen bonding (e.g. pyrene deoxyriboside)^[1] or are almost completely quenched when stacked against neighboring bases (e.g. the popular, commercially available (deoxy)ribosides of 2-aminopurine and pyrrolocytosine).^[29, 30] The approach of using flexible tethers (as in the Cy5-dC example) is appealing because of the predictable fluorescence properties and the possibility to maintain a useful degree of polymerase compatibility.^[31] Applications of this approach are limited, however, by the large degree of structural perturbation, which can prevent normal interactions with proteins of interest. Moreover, the flexibility of the tether can lead to fluorophore intercalation and can limit the precision of distance measurements by FRET.^[32, 33] Another significant, unmet need is for a way to label nucleic acids for fluorescence microscopy with sufficiently small structural perturbation so that, e.g., uptake by competent bacteria can be tracked microscopically.

In the past decade, a very promising new class of analogues has emerged, based on a tricyclic cytidine (tC) scaffold (Figure 1).^[34–42] While originally developed as stabilizers for double-stranded nucleic acids (substitution of one tC for C in duplex oligonucleotides raises the T_m by an average of 3 °C),^[34] tC and tC^O were later found to be unique in that they retain their fluorescence ($\Phi_{em} \approx 0.2$) in duplex nucleic acids, varying little with the identity of neighboring bases.^[37] The photophysics of these compounds, as free nucleosides and in ss- and dsDNA, are well characterized.^[42] We have shown that both compounds are remarkably good substrates for A- and B-family DNA polymerases and T7 RNA polymerase, even to the point of being inserted faster than dCTP during DNA synthesis both by human pol α and Klenow fragment, albeit with approximately 10 % misincorporation of dtC^OTP across from A (fidelity of RNA synthesis with T7 RNA polymerase is greater).^[43, 44] More recently, Wilhelmsson et. al. have developed a nitro-tC^O analogue that is a non-emissive FRET acceptor and shown that it can be used with tC^O as a donor for inter-nucleobase FRET.^[41] The G clamp molecules are also members of the tC family.^[45, 46]

While the three nucleobases of the tC family have unique properties, they are also significantly limited in that the emissive compounds (tC and tC^O) have nearly overlapping spectra and they are around 50-fold less bright than fluorescein. This brightness is inadequate for use in most single-molecule fluorescence experiments or for labelling nucleic acids for fluorescence microscopy. Nitro-tC^O does not emit. Because it is difficult to predict, *a priori*, the photophysical properties of a molecule based on structure, we set out to design, synthesize, and characterize a new set of tC^O analogues incorporating electron-donating and withdrawing groups at varied positions and with further extensions to conjugation (Figure 2). The results show clear patterns in the substituent effects, changes to Φ_{em} and λ_{em} , and reveal a range of solvent sensitivities. (7-Cl)tC^O **1e** is now the brightest known member of the tC^O family in aqueous solution, and the strong solvent-sensitivity of the methoxy derivatives **1b**, **1c**, **2b**, and **2c** is expected make them useful as a new set of minimally perturbing and environmentally responsive fluorescence probes.

Results and Discussion

Synthesis of Fluorescent Nucleoside Analogues

Synthetic methods for the parent compounds **1a** (tC) and **2a** (tC^O) are established, but the substituent effects of the EDGs and EWGs necessitated re-optimization to improve yields and functional group tolerance. In our hands, the original route to tC^O worked poorly or not at all when applied to the synthesis of compounds **1b–g**, but our modified route provided adequate yields.^[34] Moreover, our re-optimization of the route to tC substantially improves the overall yield (from 10–15% to 43% for **10a** to **2a**), facilitating the synthesis of **2b,c**.^[47]

The original synthesis of tC^O (**1a**) entails the activation of the 4-OH tautomer of 3',5'-di-*O*-acetyl-5-bromo-2'-deoxyuridine as a mesitylsulfonyl ester, formation of a secondary amine by S_NAr of this mesylate, removal of the acetate protective groups, and ring closure by S_NAr of the aryl bromide, promoted by KF in ethanol (c.f. Figure 3).^[34] Two problems limited the application of this route to derivative synthesis. First, chloro and methoxy groups *meta* to *N* of 2-aminophenols attenuate the nucleophilicity, which has a detrimental effect on secondary amine formation. Second, chloro and methoxy groups *meta* to *O* attenuate the S_NAr reactivity with the aryl bromide, inhibiting ring closure.

An improved synthesis begins with the same 3',5'-di-*O*-acetyl-5-bromo-2'-deoxyuridine as starting material, but activates the O⁴ using Appel chemistry (PPh₃, CCl₄) for the conversion to a 4-Cl (Figure 3).^[48] The 4-Cl intermediate was used *in situ* for S_NAr with substituted 2-aminophenols in the presence of DBU, resulting in good yields of the corresponding secondary amines for all derivatives tested. Removal of the acetyl protective groups is required for ring closure, as previously reported by Matteucci,^[34] likely owing to the intermediacy of a 5',6-ether, itself resulting from reversible 1,4-addition of the 5'-OH to the uracil moiety. The attenuated reactivity of several of the targeted derivatives necessitated more forceful conditions for this step and the removal of competing nucleophiles. Nucleophilic deamination of the secondary amine (either by solvent or H₂O) is likely the main competing side reaction. The efficacy of ring closure could be improved significantly by switching from KF to CsF and changing solvents to *N*-methylpyrrolidinone, but the hygroscopicity of CsF made it difficult to handle without introducing water into the reaction mixture. Fortunately, a further change to KF, activated by 18-crown-6, in anhydrous diglyme at 120°C proved to offer a significant improvement, resulting in substantially improved yields of all derivatives in a reaction time of approximately 20 minutes. The formation of what are likely deaminated side products (as evidenced by ¹H NMR) was greatly minimized under these conditions.

The very different reactivity of aryl alcohols and aryl thiols necessitates a different route to X = S (tC) derivatives (Figures 1 and 4). Matteucci's and Wilhelmsson's syntheses of tC **2a** begins with 2-aminothiophenol,^[34, 47] but derivatives of this compound have very limited commercial availability and are highly sensitive to oxidative degradation. 4- and 5-Methoxy-2-aminothiophenol are accessible through ring opening of more readily available benzothiazole precursors, a reaction previously carried out using concentrated hydroxide. We found this procedure to be unreliable for methoxy starting materials **7b,c**, and a change to hydrazine provided significant improvement. Because of the tendency of the resulting 2-

amino-(7/8)-methoxythiophenols to undergo oxidative degradation, we performed a controlled, *in situ* oxidation to the disulfides **8b,c** using hydrogen peroxide, giving a product that could be purified chromatographically. A one-pot reduction of this disulfide using triethylphosphine and nucleophilic substitution with 5-bromouracil gave thioether compounds **9b,c**. The methoxy group *meta* to N attenuates its nucleophilicity, but condensation conditions more forcing than those of the original procedure nonetheless produced tricyclic nucleobases **10b,c**. The original ribosylation procedure uses the sodium salt of the nucleobase (from NaH) in a reaction with 3,5-di-*O*-(*p*-toluoyl)-2'-deoxyribofuranosyl chloride (Hoffer's chlorosugar)^[49] and yields only 10–15%, but we found that substantially better yields of the 2'-deoxyriboside could be obtained by instead activating the nucleobases **10a–c** as the TMS ether using BSA, followed by ribosylation *in situ*, Vorbrüggen's Silyl-Hilbert-Johnson method.^[50, 51] Yields were typically around 80% and consisted of a ~ 1:1 mixture of anomers. The pure β -anomers were separable in around 40% yield from **10**. Removal of the toluoyl groups using methoxide completed the synthesis of the fluorescent nucleoside analogues **2**. Yields were unfortunately significantly lower for compound **2c** because of an apparent high sensitivity to oxidative degradation of **8c** and **9c** and preferential formation of the α -anomer during 2'-deoxyribosylation.

Photophysical Measurements

In order to compare the effects of substituents and to determine how the new analogues compare with tC and tC^O, we carried out photophysical measurements in 1X PBS buffer, solvent mixtures, and 1,4-dioxane (Table 1 and Figure 5). The results show substituent effects that are consistent across the tC and tC^O derivatives. (7-Cl)tC^O (**1e**) is now the brightest known member of this family in an aqueous environment, and the solvent effects point to highly desirable properties for biophysical probe development.

In all cases, substitution of the tC and tC^O frameworks had little effect on λ_{abs} but a significant influence of λ_{em} . The addition of methoxy groups to either the 7- or 8-position of tC^O red-shifted λ_{em} by 27 and 26 nm respectively ((7-MeO)tC^O and (8-MeO)tC^O) in buffer. The position of this substituent had a stronger effect on λ_{em} of the tC framework, with red-shifts of 35 nm for (8-MeO)tC and only 5 nm for (7-MeO)tC. In 1,4-dioxane, the pattern of the effects is the same with the overall magnitude of the influence diminished. While the influence of methoxy substitution is consistent for tC and tC^O, the differences are largest at the 8-position, which is perhaps not surprising because this position is in direct resonance conjugation with the O or S atom.

Substitution of the tC^O framework with chloro groups ((7-Cl)tC^O and (8-Cl)tC^O, **1e** and **1d**, respectively) resulted in a slight blue-shift of λ_{em} by 4–5 nm in aqueous buffer, but in 1,4-dioxane, λ_{em} for (7-Cl)tC^O was red-shifted by 4 nm and λ_{em} for (8-Cl)tC^O was blue-shifted by 25 nm. Fluoro substitution at the 7 position of tC^O ((7-F)tC^O) gives a 10 nm red shift of λ_{em} in dioxane but nearly matches the emission of the parent in buffer. Tetracyclic compound tetC^O **1g** has λ_{em} blue-shifted by 4 nm in dioxane and 26 nm in buffer with respect to the parent tC^O.

Because the substituents affected λ_{em} much more than λ_{abs} , the Stokes shift is strongly correlated to λ_{em} ($r = 0.98$). As a result of its significantly red-shifted λ_{em} , (8-MeO)tC **2b** has the greatest Stokes shift of the tC family in water, 159 nm. Tetracyclic analogue **1g** has the smallest Stokes shift of the series of compounds in buffer, 73 nm.

Using mixtures of 1X PBS buffer and 1,4-dioxane, we measured the effect of solvent polarity on Stokes shift, calibrated to the $E_T(30)$ scale of Reichardt's dye (Table 1 and supporting information).^[18] The results show that substituents impart significant differences to polarity sensitivity. Substitution generally decreases the polarity sensitivity with the tC^O derivatives, whereas it increases polarity sensitivity of the tC compounds. (8-Cl)tC^O is now the least solvent-sensitive known member of the tC family, both in terms of this Stokes shift measurement and Φ_{em} . In contrast, (8-MeO)tC has the most solvent sensitive photophysical properties.

The introduction of substituents, including extending the conjugated framework, in general causes a decrease in the observed absorptivity of the compounds. An exception to this trend is seen with the chloro substituents in 1,4-dioxane. The decrease is greatest for the methoxy groups when the functionality is in the 7 position, but in buffer the smallest absorptivity is seen when a chloro group is in the 8 position. In contrast, the absorptivity of the methoxy analogues is relatively insensitive to solvent polarity.

Although tC and tC^O are known to maintain fluorescence in duplex oligonucleotides (tC becomes slightly brighter and tC^O slightly less bright), these analogues show variance in quantum yield depending on the solvent. tC and tC^O were found to have a 3-fold and 1.5-fold increase in quantum yield, respectively, when changing from buffer to 1,4-dioxane. Similar increases in Φ_{em} on going to non-hydrogen bonding solvents have been reported previously for tC.^[52] The presence of the chloro substituent, in either position on tC^O, attenuated solvent effects on quantum yield. The opposite is true when the substituents are methoxy groups. Although methoxy substituents attenuated the solvent sensitivity of ϵ , Φ_{em} for the analogues with methoxy groups is much more sensitive to solvent polarity than that for the corresponding parent compounds, with the greatest difference observed for 8-MeO-tC or -tC^O (**1b** and **2b**). The analogues with methoxy groups have a relatively high Φ_{em} in 1,4-dioxane, but much less so in buffer. Notable, (8-MeO)tC increases in fluorescence intensity by almost 30-fold on going from buffer to dioxane. The presence of a fourth aromatic ring (tetC^O) shows solvent sensitivity for Φ_{em} comparable to that of parent tC^O. In dioxane, (8-Cl)tC^O shows a greater quantum yield of emission than tC^O. In buffer, tetC^O, (7-Cl)tC^O, and (8-Cl)tC^O, all have greater Φ_{em} than the tC^O parent.

As a result of the combined effects of substitution on absorptivity and quantum yield, both chloro analogues (7-Cl)tC^O and (8-Cl)tC^O are brighter than the parent tC^O. (7-Cl)tC^O is now the brightest known member of the tC family in buffer, more than 40% brighter than tC^O. In 1,4-dioxane, the chloro analogues (8-Cl)tC^O and (7-Cl)tC^O are also the brightest known fluorophores of this family. The least bright analogues in each family are those which have the methoxy group in the 8 position ((8-MeO)tC and (8-MeO)tC^O), but these analogues are exciting prospective molecular probes because of the large increase in brightness on going to a less-polar environment. This property should be useful for probing molecular recognition

of nucleic acids and may also be useful for reporting on the environmental changes during hybridization of oligonucleotides to form a duplex.

Conclusion

While the chemical literature reports many examples of nucleobase-derived fluorophores, there is a dearth of data for substituent effects on the photophysical properties of the most important of these probes. In this work, we have designed and synthesized a new set of analogues of the tC family using synthetic procedures re-optimized for improved yields and access to greater structural diversity. Photophysical characterization of the analogues in buffer, 1,4-dioxane, and mixtures thereof reveals that substituents have similar effects whether attached to tC or tC^O. Generally, chloro compounds gave brighter fluorescence and methoxy compounds gave dramatically increased solvent sensitivity. Because the patterns of effects hold across tC and tC^O, future derivatives can be screened using whichever framework offers easier synthetic access, and then the most desired substituents can be added to the other framework, if desired, with predictable effect.

The results of this work encourage a full study of the compatibility of the new probes with DNA- and RNA-processing and polymerizing enzymes and a full photophysical characterization in single-stranded and duplex oligonucleotides. We anticipate that they will make highly valuable additions to the growing toolkit of fluorescent probes for the study of nucleic acid structure, recognition, and metabolism.

Experimental Section

Full details of the synthesis and characterization of new compounds, in addition to modified procedures for the synthesis of tC **2a** and tC^O **1a**, are provided in the supporting information. Photophysical measurements were performed at least in triplicate using samples dissolved in 1X PBS buffer, 1,4-dioxane (spectrophotometric grade), or mixtures thereof. Absorption spectra were recorded using a Varian Cary 100 Bio UV-visible spectrophotometer and fluorescence spectra were recorded using a Varian Cary Eclipse Fluorescence Spectrophotometer. Fluorescence spectra were corrected using published standard curves for the commercially available fluorophores 1,1,4,4-tetraphenyl-1,3-butadiene, Coumarin 153, and 4-(dicyanomethylene)-2-methyl-6-(*p*-dimethylaminostyryl)-4*H*-pyran.^[53] Absorption and emission spectra for all compounds **1** and **2** are included in the supporting information. Quantum yields were determined using the comparative method of Williams *et. al.* and a solution of quinine sulfate dissolved in 0.1 M H₂SO₄ as a reference with an accepted $\Phi_{em} = 0.54$.^[54] All quantum yield measurements were performed with tC **2a** as a second standard to ensure the accuracy of comparisons. Example plots for quantum yield measurements are shown in the supporting information. Polarity sensitivity was measured as the slope of a line defined by plotting Stokes shift against solvent polarity from the Dimroth-Reichardt $E_T(30)$ scale. The accuracy of this determination was in some cases limited by changes to the vibrational fine structure that occurred when changing solvents, sometimes manifest in abrupt changes to $\lambda_{max, em}$ (all relevant spectra are shown in the supporting information).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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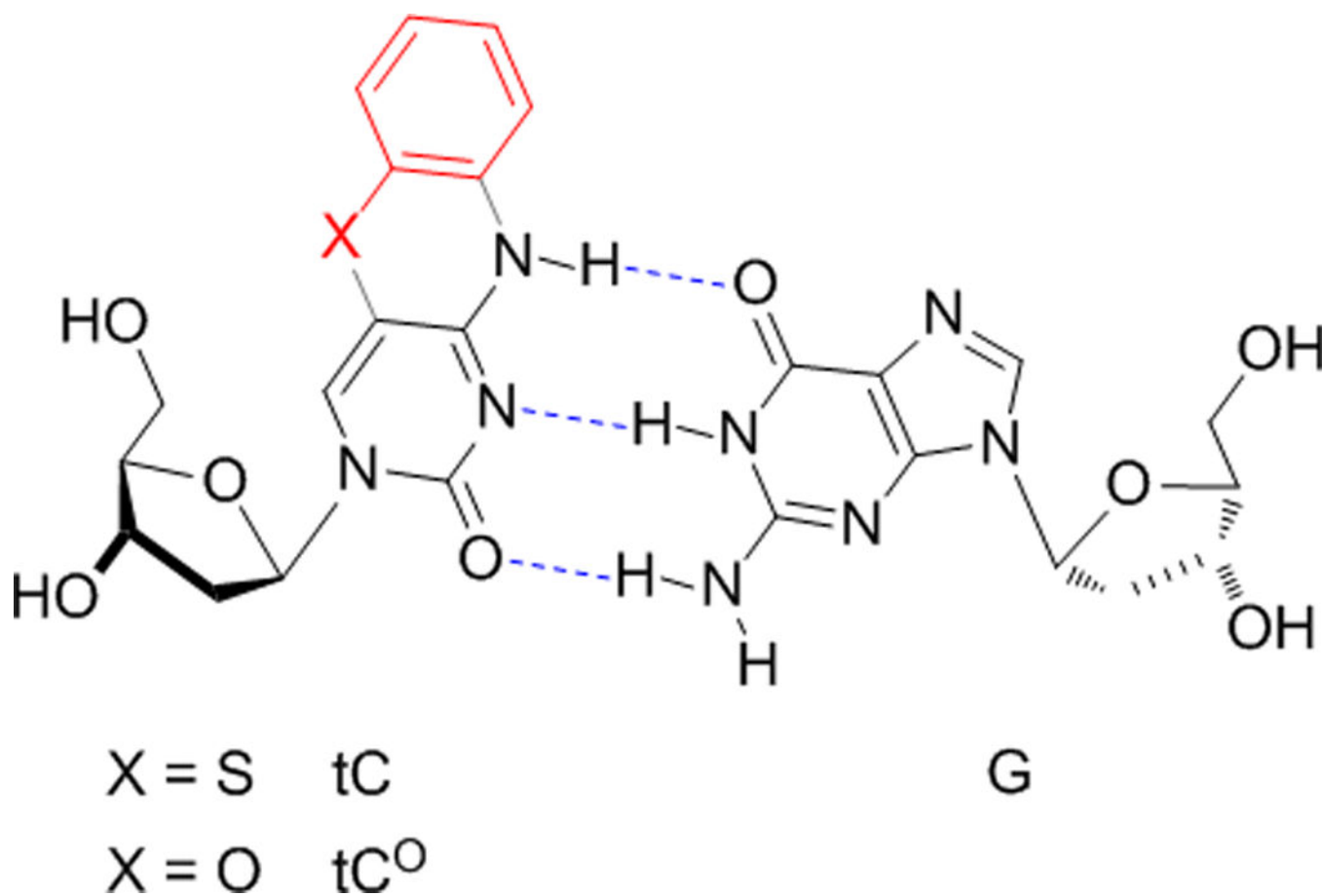
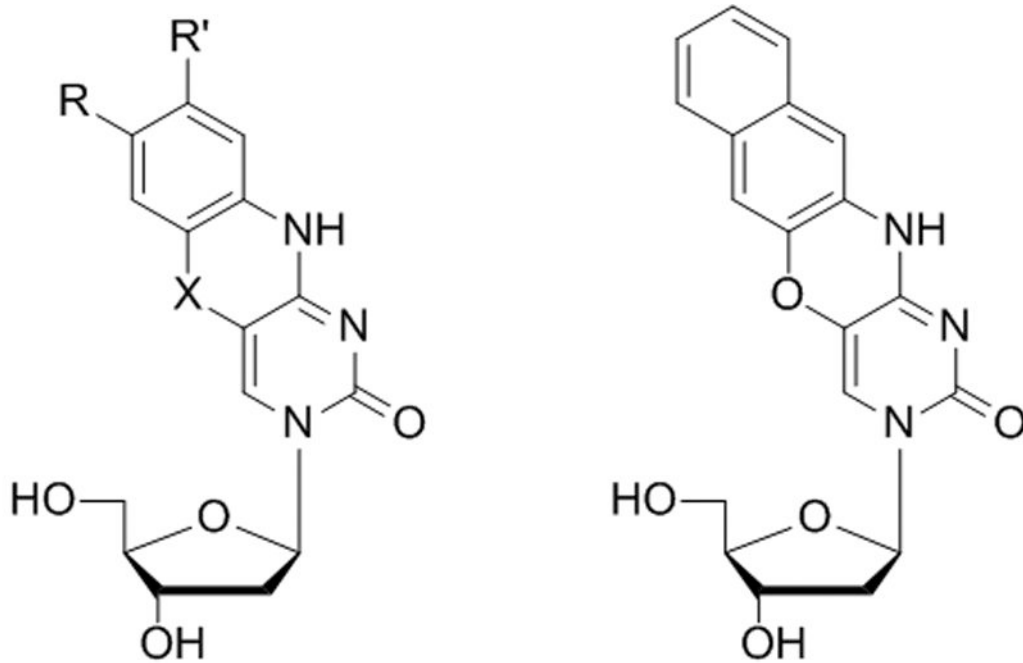
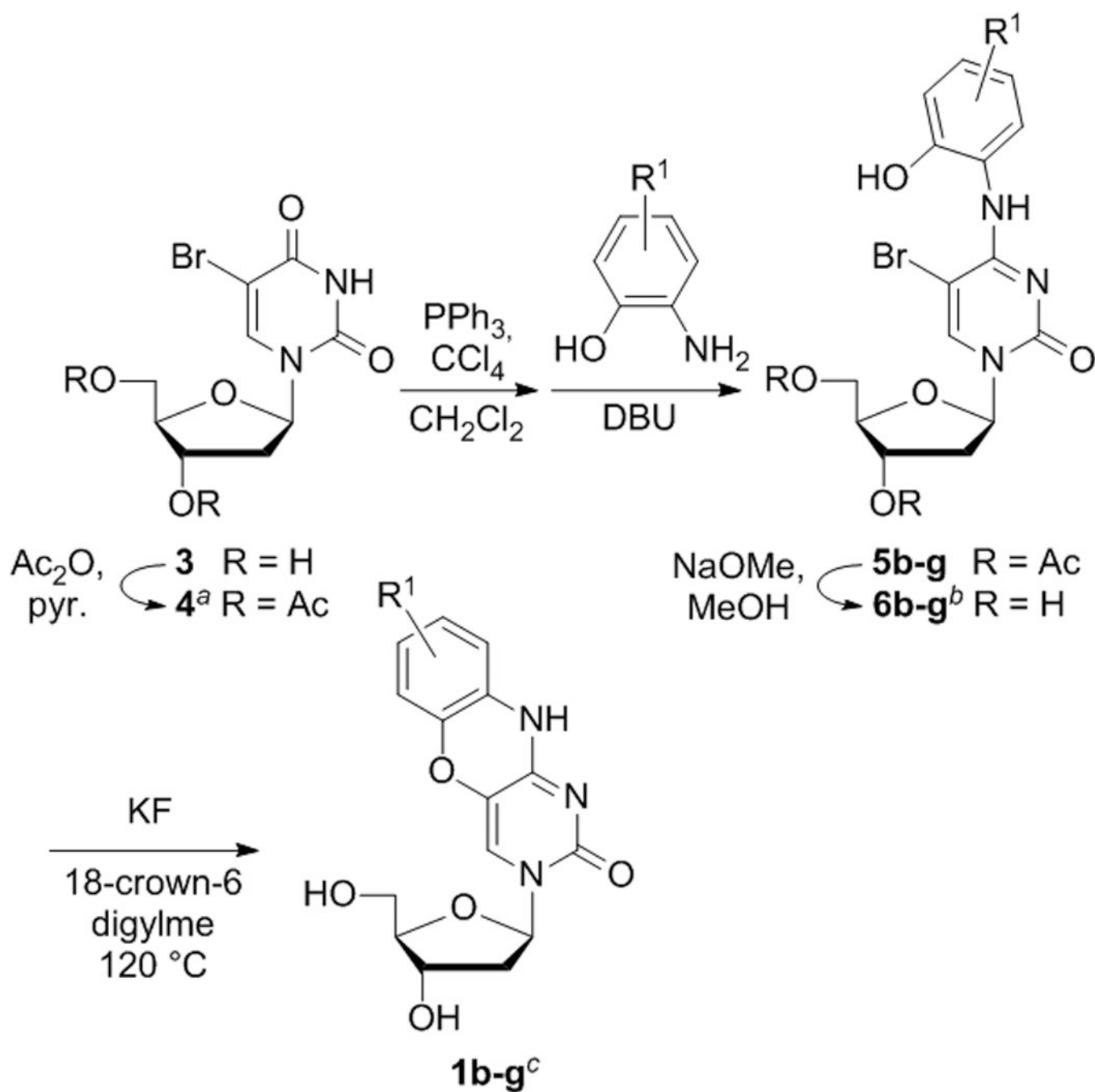


Figure 1.

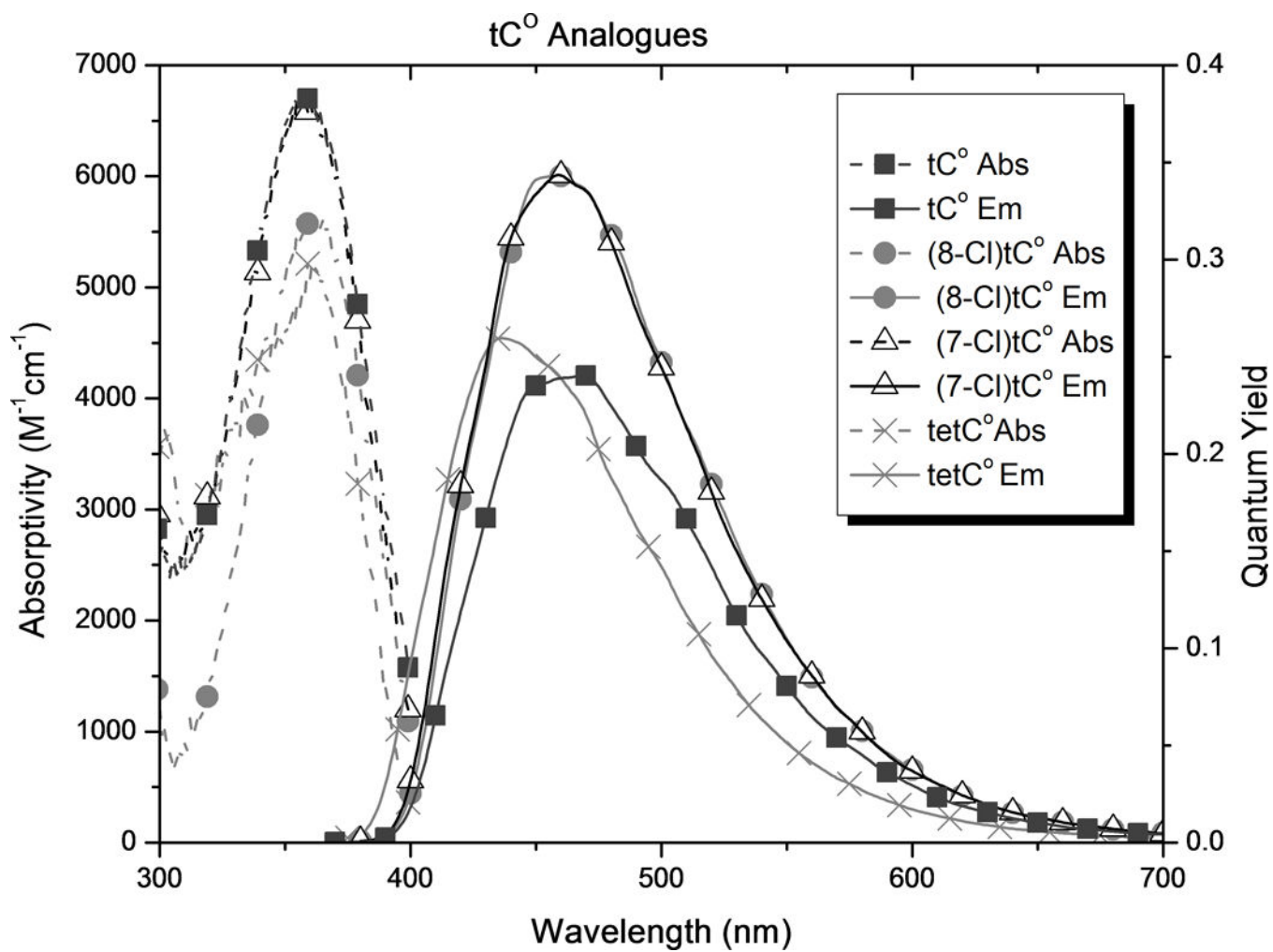


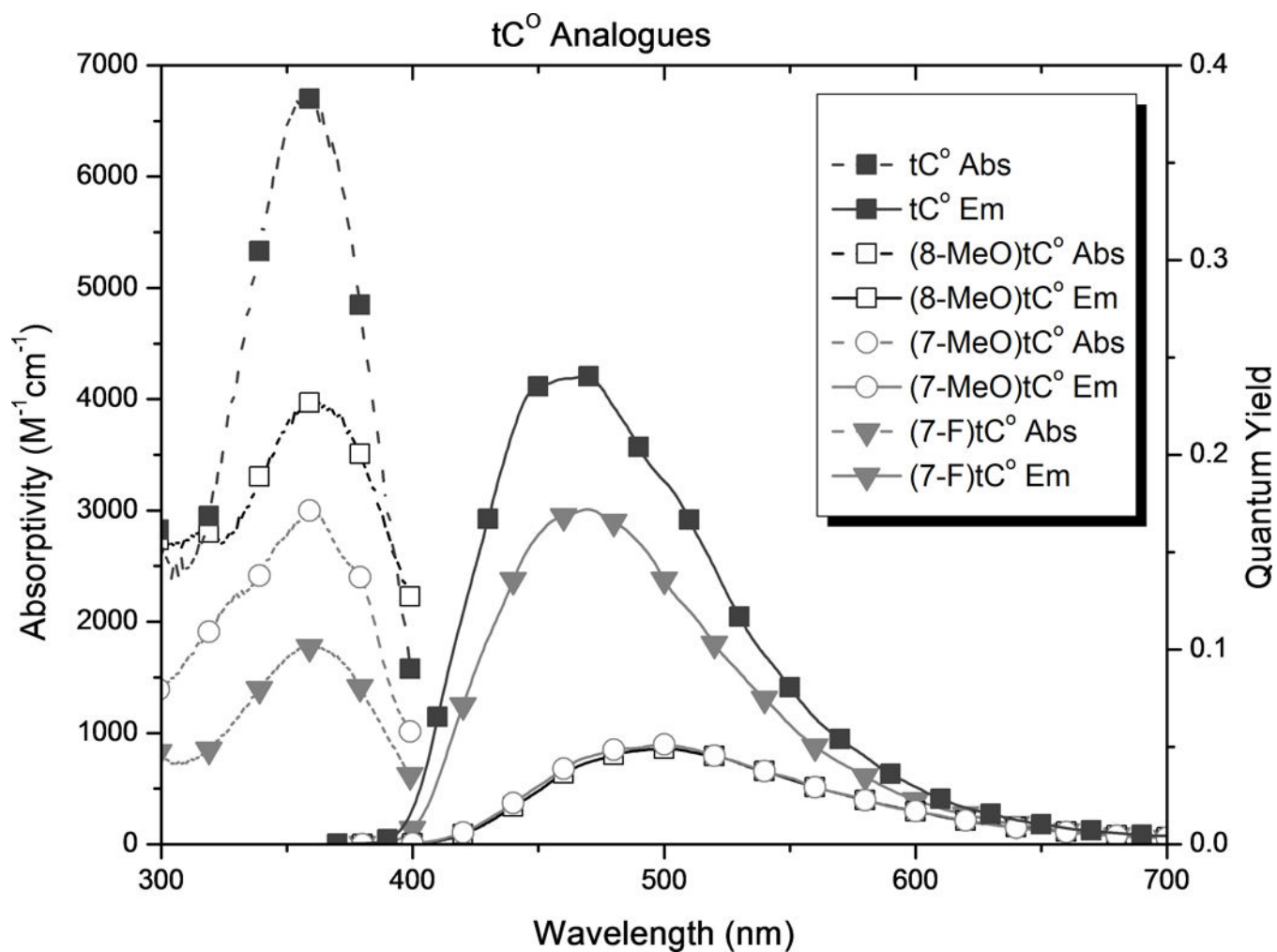
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1a	tC ⁰	O	H	H	
1b	(8-MeO)tC ⁰	O	H	OCH ₃	
1c	(7-MeO)tC ⁰	O	OCH ₃	H	
1d	(8-Cl)tC ⁰	O	H	Cl	
1e	(7-Cl)tC ⁰	O	Cl	H	
1f	(7-F)tC ⁰	O	F	H	
2a	tC	S	H	H	
2b	(8-MeO)tC	S	H	OCH ₃	
2c	(7-MeO)tC	S	OCH ₃	H	

Figure 2.
Tri- and tetracyclic cytidine analogues synthesized for this study.

**Figure 3.**

Synthesis of tC^{O} analogues. Yields are (a) 85%, (b) over two steps, **6b** 40%, **6c** 48%, **6d** 41%, **6e** 20%, **6f** 61%, **6g** 44%, (c) **1b** 20%, **6c** 11%, **1d** 24%, **1e** 3%, **1f** 11%, **1g** 53%. The parent tC^{O} **1a** was synthesized using the original procedure.^[34]





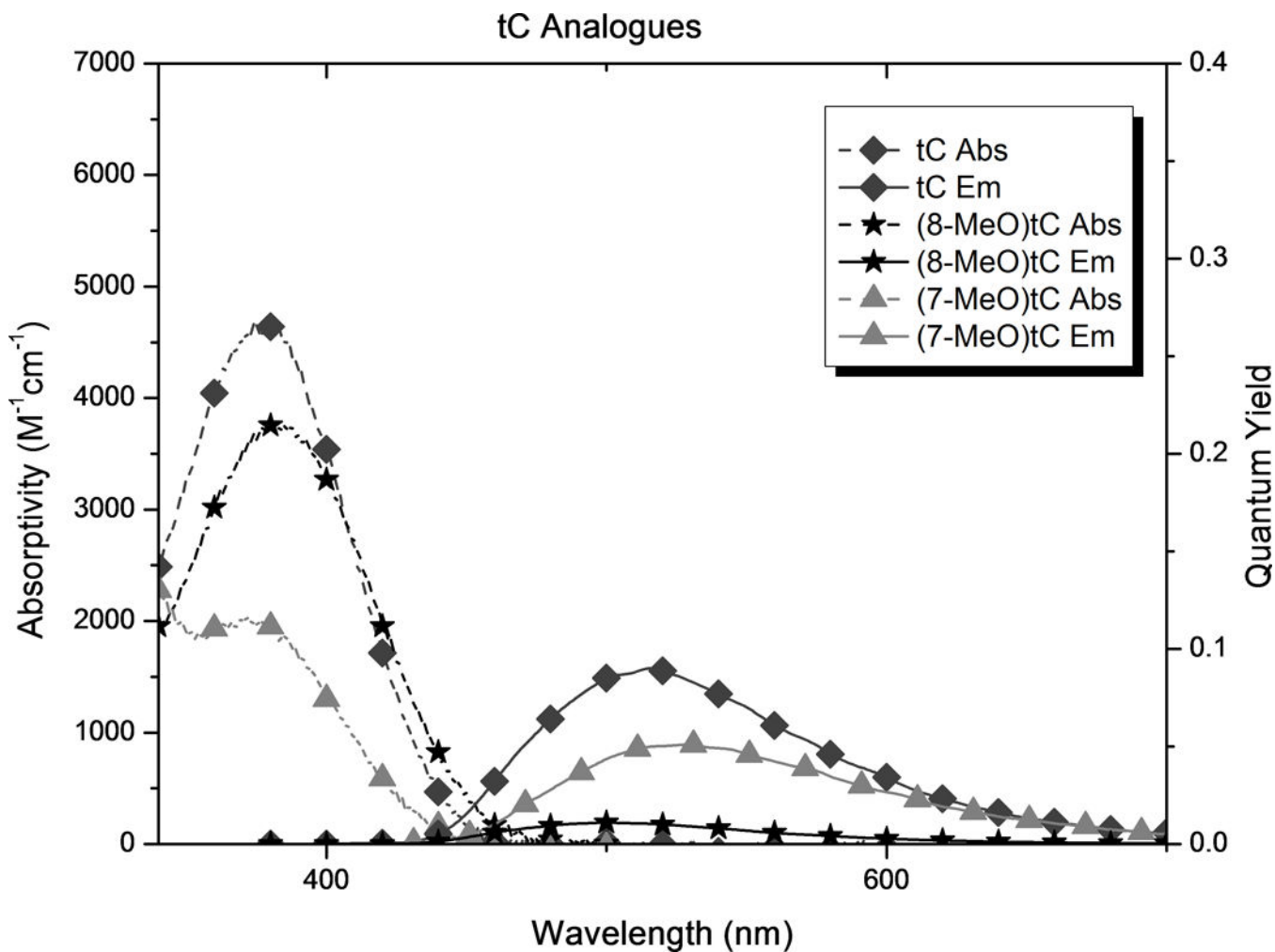


Figure 5. Spectroscopic properties of new analogues (see Figure 2) and comparison with tC and tC^O as measured in 1X PBS buffer.

Table 1

Photophysical properties of analogues **1–2** dissolved in 1X PBS buffer and in 1,4-dioxane.

compound	solvent	$\lambda_{\text{max,abs}} / \text{nm}$	$\lambda_{\text{max,em}} / \text{nm}$	Stokes shift / nm	$\epsilon / \text{L mol}^{-1} \text{cm}^{-1}$	Φ_{em}	brightness ($\epsilon \cdot \Phi_{\text{em}}$)	polarity sensitivity ^a
1a tC ⁰	dioxane	357	442	85	7400	0.46	3400	6
	1X PBS	355	460	105	6800	0.24	1600	
1b (8-MeO)tC ⁰	dioxane	360	449	89	4800	0.38	1800	5
	1X PBS	361	486	125	4000	0.05	200	
1c (7-MeO)tC ⁰	dioxane	358	449	91	2700	0.37	1000	2
	1X PBS	360	487	127	3000	0.05	150	
1d (8-Cl)tC ⁰	dioxane	366	417	51	8500	0.49	4200	2
	1X PBS	361	455	94	5600	0.35	2000	
1e (7-Cl)tC ⁰	dioxane	363	446	83	8700	0.46	4000	4
	1X PBS	357	456	99	6700	0.35	2300	
1f (7-F)tC ⁰	dioxane	366	452	86	2700	0.41	1100	5
	1X PBS	360	462	102	1800	0.17	300	
1g tetC ⁰	dioxane	362	438	76	6700	0.43	2900	7
	1X PBS	361	434	73	5300	0.27	1400	
2a tC	dioxane	369	472	103	4300	0.34	1500	3
	1X PBS	377	503	126	4700	0.09	400	
2b (8-MeO)tC	dioxane	372	490	118	3800	0.28	1100	7
	1X PBS	379	538	159	3800	0.01	40	
2c (7-MeO)tC	dioxane	372	477	105	2200	0.27	600	7
	1X PBS	379	508	129	2000	0.05	100	

[a] Expressed as the slope of Stokes shift plotted against solvent polarity as measured using the Dimroth-Reichardt $E_T(30)$ scale. Units are $10^4 \text{ cm}^{-1} / (\text{kcal} \cdot \text{mol}^{-1})$.