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Synthesis of a Fluorescent Xanthenic Derivative Useful for Labeling Amine Residues

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The 2,5-dioxopyrrolidin-1-yl-4-(3-hydroxy-6-oxo-6H-xanthen-9-yl)-3-methylbenzoate has been synthesized as an amine-reactive derivative able to yield stable covalently labeled biopolymers. The new derivative has been used to label polyribocytidilic acid (5′), poly(C), amine residues. **TG-II**-poly(C) exhibits monoexponential decay at the physiological pH range. In addition, both steady-state fluorescence intensity and fluorescence decay are also sensitive to solution pH. The large decrease in steady-state fluorescence upon hybridization allows it to be used as a nucleic acid probe in a homogeneous assay format. In summary, we report an efficient synthesis to obtain labeled RNA from commercially available materials in excellent yields.

In aqueous solutions, fluorescein can exist in one or more of four different prototropic forms (cation, neutral, monoanion, and dianion) depending on pH, although, in the physiological pH range, only the dianion and monoanion forms are important. Fluorescein and its derivatives have been widely employed as fluorescent labels because of their favorable spectral characteristics in aqueous media. In the visible range, the absorption maximum of the dianion is at 490 nm and its extinction coefficient is 8.36×10^4 M^{-1} cm^{-1}. The monoanion has two visible range maxima, one at 454 nm and the other at 474 nm, the extinction coefficients being 3.03×10^4 and 3.14×10^4 M^{-1} cm^{-1}, respectively. The emission spectra of both anion and dianion have their maxima around 515 nm. The dianion has a fluorescence quantum yield of 0.93, while for the monoanion it is 0.37.[1] Recently, various fluorescein derivatives named Tokyo Green (TG) have been synthesized.[2] Of these derivatives, 9-[1-(2-methoxy-5-methylphenyl)]-6-hydroxy-3*H*-xanthen-3-one (**TG-I**) and 9-[1-(2-methyl-4-methoxyphenyl)]-6-hydroxy-3*H*-xanthen-3-one (**TG-II**) are the most interesting as on/off fluorescent probes, since both are highly fluorescent when the xanthene moiety is in the anion form, but their quantum yields are near zero when the oxygen is either linked to another chemical group or simply protonated at acidic pH values. Moreover, their absorbances and emission maxima are hardly altered with respect to fluorescein.[3] The possibility of having "on/off" fluorescent probes showing similar absorption properties to fluorescein and only one electrical charge in their fluorescent prototropic form opens new prospects for the use of these fluorescein derivatives as analytical probes and pH indicators. **TG-II** is currently being used as an on/off fluorescent probe in some biological assays.[2,4]

Taking advantage of the spectral changes associated with the monoanion−dianion transition, fluorescein has been employed to detect DNA renaturation[5] and hybridization[6] in homogeneous assays. Homogeneous formats are simpler than heterogeneous formats, since they do not require any further treatment to detect the hybridized probe.[7–9] To establish the physicochemical basis of the methodology, a fluorescent-labeled polyribocytidylic acid (5′), poly(C), as a model of RNA probe and polyriboinosinic acid (5′), poly(I), as a model target, were used.[9] At neutral pH and millimolar salt concentrations, poly(C)−poly(I) forms a bihelical structure with Watson−Crick base pairing between the cytosine residues of one strand and the hypoxanthine residues of the other.[10] Homopolymers represent the least complex system for the study of the association of complementary strands, since this bimolecular second order reaction occurs quite rapidly, thus providing the simplest experimental system.[9]

Here, we synthesize the succinimidyl ester of **TG-II** as an amine-reactive derivative able to yield stable covalently labeled biopolymers, since carboxamides are more resistant to hydrolysis than bioconjugates from isothiocyanates, and we use this new derivative to label poly(C). Considering that the quantum yield of **TG-II** is near zero at acidic pH values, the shift in the apparent pK_a, as occurs in the hybridization reaction,[5] should result in a larger quenching of the steady-state fluorescence signal from the probe than when fluorescein is used, increasing the homogeneous assay sensitivity.

The synthesis of compound 2,5-dioxopyrrolidin-1-yl-4-(3-hydroxy-6-oxo-6H-xanthen-9-yl)-3-methylbenzoate was accomplished in five steps from commercially available xanthone (**1**). As shown in Scheme 1, xanthone (**1**) was protected as MEM by reaction with Mem-Cl using NaH as a base in THF. Compound **2** was converted into 4-carboxy-Tokyo Green methyl ester (**3**)[11] undergoing halogen metal exchange with *i*-PrMgCl.[12] This method, described by Knochel and co-workers, proved to be an efficient method for installation of the methyl benzoate moiety of **3**. Next, **3** was treated with LiOH in MeOH/H_2O to afford the free carboxylic compound **4**. In a 10 mL round-bottom flask, Tokyo Green-4-carboxylic acid **4** (1 equiv), DCC (1.1 equiv), and *N*-hydroxysuccinimide (2 equiv) were dissolved in dry THF. After stirring for 4 h at 23 °C, the urea byproduct was removed by filtration. The solution was concentrated in

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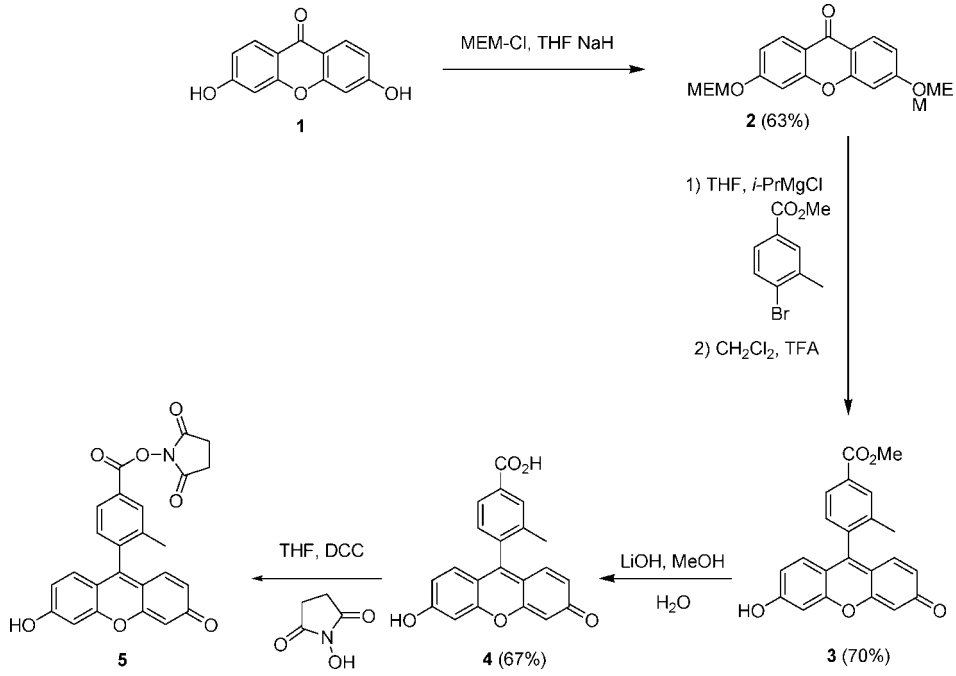
SCHEME 1: Synthesis of the 2,5-Dioxopyrrolidin-1-yl-4-(3-hydroxy-6-oxo-6H-xanthen-9-yl)-3-methylbenzoate



vacuo. Flash column chromatography ($MeOH/CH_2Cl_2$ 1:9) afforded the final compound **5** as an orange solid (45% yield). (NMR data: ^1H (300 MHz, CD_3OD): δ 8.09 (s, 1H), 8.04 (d, J=8.1 Hz, 1H), 7.31 (d, J=8.1 Hz, 1H), 7.09 (d, 2H), 6.76 (bs, 3H), 6.72 (bs, 1H), 2.74 (s, 4H), 2.13 (s, 3H). NMR data: ^{13}C (75 MHz, CD_3OD): 173.1, 171.1, 158.1, 154.2, 136.0, 135.3, 131.4, 130.9, 128.9, 127.0, 122.0, 114.6, 103.4, 25.1, 18.5.) (For synthesis and characterization of **2**, **3**, and **4**, see the Supporting Information).

To label poly(C), we first replaced the cytosine amino group at the N^4 position with another small, amine-containing, organic molecule using Shapiro and Weisgras's technique,[13] modified by Talavera et al.[5,6] This modification introduces a free amino group with a spacer arm to which further fluorescent labels can be added by using fluorophores with amine-specific reagent groups.[5,6,9] The modified poly(C) was precipitated from the dialysis bags by addition of NaCl and ethanol. The modified poly(C) was labeled with the succinimidyl ester of **TG-II** according to Reines and Schulman.[14] A 2 mg portion of modified poly(C) was suspended in 400 μL of 10 mM Tris−HCl 1 mM EDTA buffer, pH 7.35. To this, 100 μL of a 1.0 M HEPES buffer, pH 10.0, was added to maintain pH > 8.0, as the hydrolysis of free succinimidyl ester of **TG-II** can lower the pH. A 500 μL portion of a 2 mg/mL solution of recently obtained **TG-II** succinimidyl ester in DMSO, dissolved immediately before use, was added to the modified poly(C) solution dropwise. The reaction mixture was left to incubate at 37 °C for 2 h. The labeled poly(C) was purified by repeated precipitation with ethanol and NaCl until there was no detectable fluorescence from free dye in the remaining supernatant. The last step in the purification of the labeled material was by gel permeation through Sephadex G-25.

The extent of labeling was determined on labeled poly(C) by absorption spectroscopy. The labeling ratio of the sample used in this work is 2.1%. A labeling ratio of less than 3% does not significantly affect hybridization kinetics or the melting temperature of the poly(C)−poly(I) duplex.[9] The emission spectrum of **TG-II**-poly(C) in the hybridization buffer (20 mM Tris−HCl, 1 mM EDTA, 100 mM NaCl, pH 7.35) is slightly shifted to longer wavelengths with respect to free **TG-II**, but the overall emission profile is not altered.

Figure 1 shows the changes in fluorescence intensity and the slight spectrum shift which occur when **TG-II**-poly(C) is titrated with poly(I). The fluorescence decreases continuously with increasing concentration of poly(I) until saturation is reached at a molar ratio of around 1:1 in nucleotide bases of poly(I) to poly(C). After each addition of poly(I), the sample was incubated for 10 min before recording the fluorescence spectrum. The peak fluorescence intensity of **TG-II**-poly(C) decreases by 68% at saturating concentrations of poly(I), and this change in fluorescence intensity is independent of the initial concentration of **TG-II**-poly(C). As previously demonstrated, due to the high hybridization affinity of poly(C) for poly(I), the fraction of paired poly(C) bases increases linearly with the increase in the

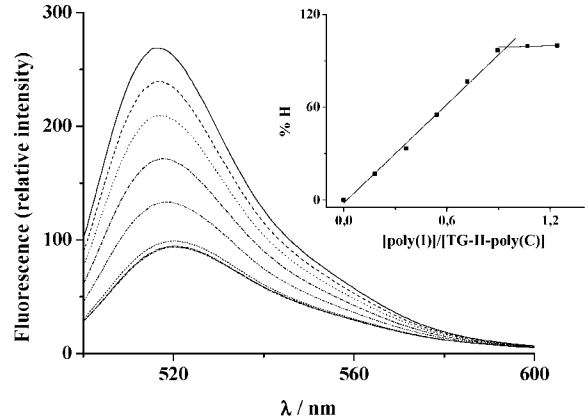


Figure 1. Emission spectra (λ_{ex} = 490 nm) of a solution of **TG-II**-poly(C) in hybridization buffer with an initial concentration of 4.68×10^{-5} M and different concentrations of poly(I) in the range $0-5.86 \times 10^{-5}$ M. The poly(I) concentration was increased by addition of 10 μL of 2.15×10^{-3} M poly(I) to 2500 μL of **TG-II**-poly(C) solution. The spectra were corrected for dilution effects. Inset: Percentage of **TG-II**-poly(C) that is hybridized with poly(I) vs [poly(I)]/[**TG-II**-poly(C)]. **TG-II**-poly(C) concentration was corrected for dilution effects during titration.

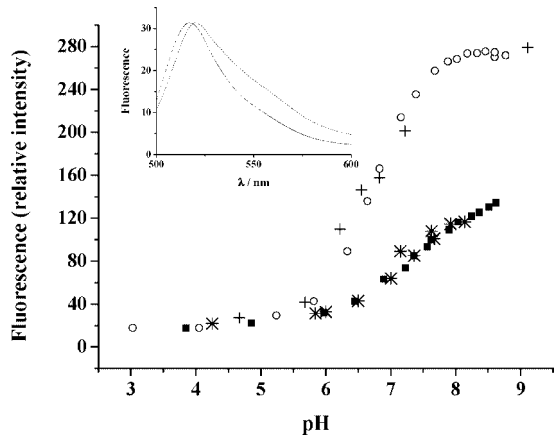


Figure 2. pH-dependent changes in steady-state fluorescence intensity of (○) **TG-II**-poly(C) and (■) **TG-II**-poly(C)−poly(I), both in 20 mM Tris−HCl, 1 mM EDTA, and 100 mM NaCl. (+) **TG-II**-poly(C) and (∗) **TG-II**-poly(C)−poly(I), both in unbuffered solutions (1 mM EDTA, 100 mM NaCl). $\lambda_{ex} = 490$ nm. $\lambda_{em} = 515$ nm. The **TG-II**-poly(C) concentration is 4.68×10^{-5} M. Inset: Normalized emission spectra of (—) **TG-II**-poly(C) and (•••) **TG-II**-poly(C)−poly(I) duplex in hybridization buffer. $\lambda_{ex} = 440$ nm.

poly(I) concentration.[9] Since no further quenching occurs above a poly(I)/**TG-II**-poly(C) ratio greater than 1.0, it can be assumed that the decrease in the fluorescence intensity upon hybridization is proportional to the amount of base pairing. Therefore, the following expression can be used to calculate the percentage of **TG-II**-poly(C) that is hybridized: $\%H = 100[(I_0 - I)/(I_0 - I_\infty)]$, where I_0 and I_∞ are the fluorescence intensities of **TG-II**-poly(C) at zero and saturating concentrations of poly(I). The inset in Figure 1 shows that $\%H$ does indeed increase linearly with poly(I) concentration and cuts off quite sharply at a molar ratio of 1:1 poly(I) to poly(C). This result supports the idea that the fluorescence intensity decreases linearly with the fraction of **TG-II**-poly(C) that is hybridized.

We also studied the effect of hybridization upon the anion/neutral equilibrium of **TG-II**, by comparing the pH titration profiles of **TG-II**-poly(C) and **TG-II**-poly(C)−poly(I) duplex, both in buffered (20 mM Tris−HCl, 1 mM EDTA, 100 mM NaCl) and nonbuffered solutions (1 mM EDTA, 100 mM NaCl). Figure 2 shows that the apparent pK_a of conjugated **TG-II** shifts from 6.7 when it is in a single-stranded structure to 7.3 when it is in a double-stranded structure. Thus, in the physiological pH range, the relative concentration of **TG-II** molecules in the anion form is far lower in **TG-II**-poly(C)−poly(I) than in **TG-II**-poly(C). The shift in pK_a can probably be attributed to the increase in local negative charge density from the phosphate groups in the polynucleotides, making it harder for the neutral to deprotonate to the anion.

Similarly to fluorescein and **TG-I**, the neutral emission from **TG-II** appears as a shoulder in the region around $\lambda = 550$ nm.[1,3,15] To corroborate the influence of the anion/neutral equilibrium in the hybridization, the emission spectra of **TG-II**-poly(C) and **TG-II**-poly(C)−poly(I) duplex solutions at pH 7.35 have been recorded with an excitation wavelength of 440 nm. At this wavelength, the absorbance of the neutral form is higher than that of the anion,[15] which allows us to excite the neutral preferentially. The inset in Figure 2 shows the normalized steady-state emission spectra from single and double strands of **TG-II**-labeled poly(C). It can be seen in the figure that the emission intensity around 550 nm increases in relation to the normalized peak when **TG-II**-poly(C) hybridizes to poly(I). This means that in bulk solution at pH 7.35 the hybridization with poly(I) increases the pK_a of **TG-II** and produces a more neutral form.

TABLE 1: Lifetimes, Normalized Weighting Coefficients, and Arithmetic Average Lifetime for the Decay Traces of TG-II-poly(C) and Fully Hybridized TG-II-poly(C)−poly(I) Duplex Solutions in the Hybridization Buffer at pH 7.35

sample	τ_1 (ns)	α_1	τ_2 (ns)	α_2	τ_{ave} (ns)
TG-II-poly(C)	4.03	1.00			4.03
TG-II-poly(C)−poly(I)	4.03	0.83	0.73	0.17	3.47

Finally, we recorded fluorescence decay traces, with the excitation polarizer in the vertical direction and the emission polarizer at 55° (magic angle), from **TG-II**-poly(C) and **TG-II**-poly(C)−poly(I) duplex solutions at pH 7.35 and 9.10, using a 488 nm excitation wavelength. At pH 7.35, the best fitting function was monoexponential for **TG-II**-poly(C) and biexponential for **TG-II**-poly(C)−poly(I) duplex. At pH 9.10, the fitting function was monoexponential in both cases. Table 1 shows the recovered lifetimes along with their normalized weighting coefficients, obtained by means of global analysis with linked lifetimes, from six decay traces with 20 000 counts each at the maximum, at pH 7.35. Since the lifetime values resemble those recovered for anion and neutral forms of free **TG-II** solutions,[15] we can assume that the larger lifetime corresponds to the anion form, and the shorter lifetime to the neutral form of **TG-II**. On this basis, and from the normalized weighting coefficients of the lifetimes (see Table 1), the neutral form appears at the physiological pH due to a shift of the apparent pK_a to higher values when **TG-II**-poly(C) hybridizes to **TG-II**-poly(C)−poly(I). Concomitantly, the arithmetic average lifetime decreases in fully hybridized **TG-II**-poly(C)−poly(I). At pH 7.35, rough calculations considering the pK_a value of 7.3 for the **TG-II**-poly(C)−poly(I) duplex result in an anion/neutral ratio of 1.10. Bearing in mind that the anion/neutral extinction coefficient ratio from free solutions of **TG-II** is 4.34 at 488 nm,[15] the excited anion/neutral ratio should be 4.77, in very good agreement with the 4.88 ratio calculated from the normalized weighting coefficients of the lifetimes.

In addition to the above considered shift on the apparent pK_a of conjugated **TG-II**, Figure 2 also shows a fluorescence decrease of around 48% at pH 9, in which the shift on the apparent pK_a has no effect. Since the recovered lifetimes of both **TG-II**-poly(C) and **TG-II**-poly(C)−poly(I) in the high pH range are the same (around 4.03 ns), static quenching must occur, whereby **TG-II** dyes in close proximity to poly(I) are quenched instantaneously. Adding this static quenching to the 14% decrease in the arithmetic average lifetime at pH 7.35 results in a 62% decrease, in good agreement with the 68% decrease in the steady-state fluorescence maximum intensity at saturating concentrations of poly(I) shown in Figure 1.

The effective labeling of the amine residues on modified poly(C) shows that 2,5-dioxopyrrolidin-1-yl-4-(3-hydroxy-6-oxo-6H-xanthen-9-yl)-3-methylbenzoate is suitable for giving stable covalently labeled biopolymers. As a first and simple application, this new fluorescent xanthenic derivative has been used to label poly(C) amine residues. **TG-II**-poly(C) yields monoexponential decay in the physiological pH range, a major simplification compared to other fluorescence RNA probes. In addition, both steady-state fluorescence intensity and fluorescence decay are also sensitive to solution pH. Finally, the large decrease in steady-state fluorescence upon hybridization with poly(I), together with the linear increase of $\%H$ with [poly(I)]/[**TG-II**-poly(C)], and the sharp leveling off at saturation, could

be suitable for the use of **TG-II**-poly(C) as a nucleic acid probe in homogeneous assays.

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Supporting Information Available: Synthesis and spectroscopic characterization (^{1}H) of compounds **2**, **3**, and **4**. Expression to calculate the dye-to-nucleotide base ratio. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Alvarez-Pez, J. M.; Ballesteros, L.; Talavera, E. M.; Yguerabide, J. *J. Phys. Chem. A* **2001**, *105*, 6320–6332.

(2) Urano, Y.; Kamiya, M.; Kanda, K.; Ueno, T.; Hirose, K.; Nagano, T. *J. Am. Chem. Soc.* **2005**, *127*, 4888–4894.

(3) Crovetto, L.; Paredes, J. M.; Rios, R.; Talavera, E. M.; Alvarez-Pez, J. M. *J. Phys. Chem. A* **2007**, *111*, 13311–13320.

(4) (a) Kamiya, M.; Kobayashi, T.; Hama, Y.; Koyama, Y.; Bernardo, M.; Nagano, T.; Choyke, P. L.; Urano, Y. *J. Am. Chem. Soc.* **2007**, *129*, 3918–3929. (b) Kobayashi, T.; Urano, Y.; Kamiya, M.; Ueno, T.; Kojima, H.; Nagano, T. *J. Am. Chem. Soc.* **2007**, *129*, 6696–6697. (c) Koide, Y.; Urano, Y.; Kenmoku, S.; Kojima, H.; Nagano, T. *J. Am. Chem. Soc.* **2007**, *129*, 10324–10325.

(5) Talavera, E. M.; Alvarez-Pez, J. M.; Ballesteros, L.; Bermejo, R. *Appl. Spectrosc.* **1997**, *51*, 401–406.

(6) Talavera, E. M.; Afkir, M.; Salto, R.; Vargas, A. M.; Alvarez-Pez, J. M. *J. Photochem. Photobiol., B* **2000**, *59*, 9–14.

(7) Morrison, L. E.; Stols, L. M. *Biochemistry* **1993**, *32*, 3095–3104.

(8) Yguerabide, J.; Ceballos, A. *Anal. Biochem.* **1995**, *228*, 208–220.

(9) Yguerabide, J.; Talavera, E. M.; Alvarez-Pez, J. M.; Afkir, M. *Anal. Biochem.* **1996**, *241*, 238–247.

(10) Arnott, S.; Hukins, D. W. L.; Dover, S. D.; Fuller, W.; Hodgson, A. R. *J. Mol. Biol.* **1973**, *81*, 107–122.

(11) Mottram, L. F.; Boonyarattanakalin, S.; Kovel, R. E.; Peterson, B. R. *Org. Lett.* **2006**, *8*, 581–584.

(12) Knochel, P.; Dohle, W.; Gommerman, N.; Kneisel, F. F.; Kopp, F.; Korn, T.; Sapountzis, I.; Vu, V. *Angew. Chem., Int. Ed.* **2003**, *42*, 4302–4320.

(13) Shapiro, R.; Weisgras, J. M. *Biochem. Biophys. Res. Commun.* **1970**, *40*, 839–843.

(14) Reines, S. A.; Schulman, L. H. *Methods Enzymol.* **1979**, *59*, 146–156.

(15) Paredes, J. M.; Crovetto, L.; Rios, R.; Alvarez-Pez, J. M.; Talavera, E. M. *J. Phys. Chem. A*, submitted for publication.

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