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Tsukuba-green: A Fluorescent Dye that Emits Green Fluorescence Useful for Live-cell Imaging

Naoko Senda,[1] Yoshihiro Miwa,*[2,3] Junko Tanaka,[3] Atsuya Momotake,[1] and Tatsuo Arai*[1]
[1]*Graduate School of Pure and Applied Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8571*
[2]*Department of Molecular Pharmacology, Institue of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8575*
[3]*Center for Tsukuba Advanced Research Alliance, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8577*

(Received December 21, 2009; CL-091129; E-mail: arai@chem.tsukuba.ac.jp)

A new fluorescent dye, 4-ethoxycarbonylmethyl-2-methyl-7-hydroxyquinoline (TG) was obtained unexpectedly from the $BiCl_3$-catalyzed Pechmann condensation. TG emits green fluorescence at 509 nm in water. For live-cell imaging applications, TG derivatives TG1–TG3 were prepared. Under physiological conditions, HEp-2 cells were immediately stained upon addition of a solution of TG3.

Fluorescent dyes enable the detection of particular components of complicated biomolecular processes.[1] Molecules that emit large Stokes shifted fluorescence are generally useful dyes because they can prevent contamination of scattering-light components into the fluorescence. Many traditional fluorescent probes, such as dansyl,[2] dapoxyl,[3] and DAPI[4] emit fluorescence with large Stokes shifts. The fluorescence usually is weak in polar solvents, especially water, and fluorescence quantum yields increase with decreasing solvent polarity. Similarly, conjugation to a biomolecule with a hydrophobic nature, such as a protein, DNA, or plasma membrane, enhances the fluorescence.

The fluorescence behavior of the new fluorescent dye, Tsukuba-green (TG), 4-ethoxycarbonylmethyl-2-methyl-7-hydroxyquinoline (Figure 1), is distinctive: TG emits blue and small Stokes-shifted fluorescence in organic solvents but green and large Stokes-shifted fluorescence peaking at 509 nm with quantum yield ($\Phi_f = 0.15$) in aqueous solution. TG was obtained unexpectedly from the $BiCl_3$-catalysed Pechmann reaction, and its 7-hydroxyquinoline (HQ) skeleton led to the consideration of its possible photochemical behavior due to the similarity of the excited-state dynamics of TG to HQ. The excited-state proton-transfer (ESPT) dynamics of cyclically H-bonded HQ–$(H_2O)_2$ complexes have been studied both experimentally[5] and theoretically.[6] However, TG possesses advantages as a fluorescent probe compared to the parent compound HQ. For example, photosensitivity, the product of the molar extinction coefficient and fluorescence quantum yield ($\varepsilon \cdot \Phi_F$) of TG is greater than that of HQ.

TG was considered a potential novel molecular probe for live-cell assays that could be modified by simple chemical reactions (Figure 1). Although HQ derivatives have been used as probes for in vitro tests,[7] they have not been applied for live-cell imaging, probably due to low photosensitivity and membrane permeability. However, the 7-acetyl derivative of TG (TG1) should enhance cell membrane permeability and eliminate fluorescence by inhibition of excited-state proton transfer. Immediately after passing through the membrane, rapid hydrolysis of the ester bond at the 7-position by intracellular esterase should release the highly fluorescent TG and reduce membrane permeability, resulting in effective intracellular fluorescence-staining. An *N*-methyl analog, TG2, was designed to promote the formation of zwitterions by reducing the pK_a of the 7-OH group to enhance both the molar extinction coefficient and the quantum yield of large-Stokes shifted fluorescence. TG3 was prepared by combining the two analogs to increase membrane permeability of the TG analog and to increase fluorescence efficiency of hydrolyzed product TG2.

This report describes the synthesis and fluorescence properties of TG and TG-related analogs as well as their application in live-cell imaging experiments.

As shown in Scheme 1, TG was obtained by the $BiCl_3$-catalyzed Pechmann condensation. De et al. reported that the $BiCl_3$-catalyzed reaction between *m*-aminophenol and ethyl 2-oxobutanoate yielded 7-amino-4-methylcoumarin (**1**) as a major product and 4-methyl-7-hydroxyquinolinone (**2**) as a minor product;[8] no other products were described (Scheme 1). However, when the same reaction was conducted according to the previously described method, the major product was **1** (80% yield) as expected, but the minor product was a bright green dye, instead of the reported product **2**. ^1H and ^{13}C NMR spectroscopy,

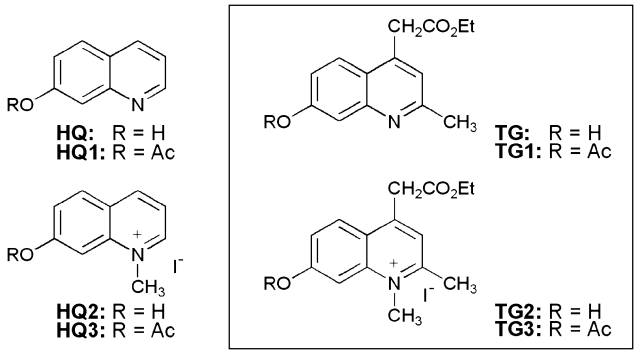
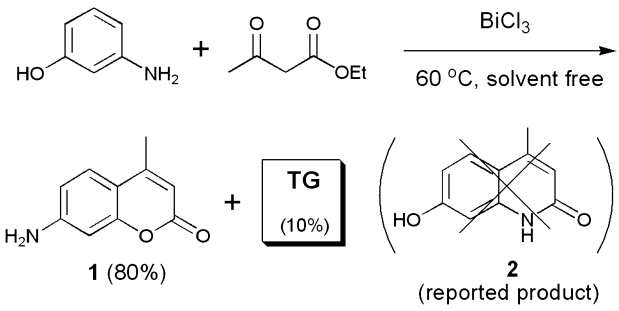


Figure 1. Chemical structures of TG and HQ derivatives.



Scheme 1.

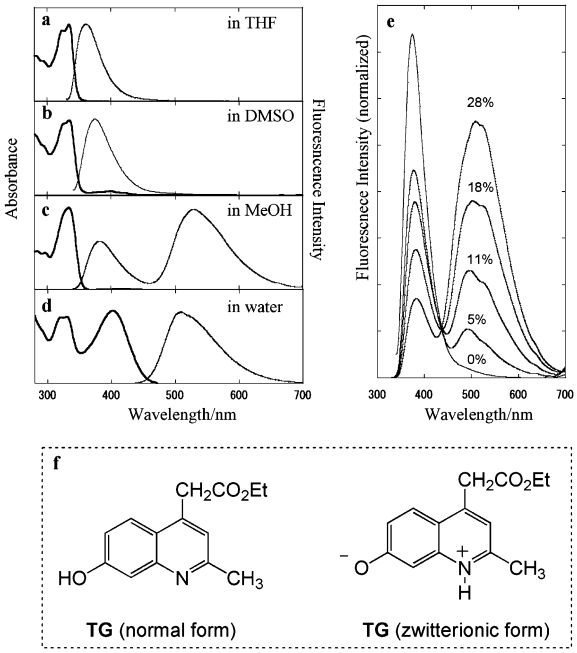


Figure 2. Absorption (solid line) and fluorescence (thin line) spectra of TG in (a) THF, (b) DMSO, (c) MeOH, (d) water (1% DMSO), and (e) upon addition of water in DMSO. Structures of TG in normal and zwitterionic form (f).

elemental analysis, and ESI-MS analysis revealed the structure of the bright green dye as 4-ethoxycarbonylmethyl-2-methyl-7-hydroxyquinoline (TG), which was reproducibly obtained in 10% yield.[16]

The fluorescence spectra of TG in various solvents are shown in Figures 2a–2d. TG emits blue fluorescence in THF ($\lambda_{max} = 360$ nm) and DMSO ($\lambda_{max} = 374$ nm), and dual fluorescence in MeOH ($\lambda_{max} = 382$ and 529 nm). The fluorescence band around 370 nm can be ascribed to the emission from excited state normal form and that around 500 nm is probably due to the excited state zwitterionic form (Figure 2f) produced by excited state proton transfer.[9] In water (1% DMSO) fluorescence appeared only at the longer wavelength of 509 nm (Figure 2d). The fluorescence at 374 nm decreased and that at 509 nm increased upon addition of water in DMSO solution (Figure 2e). The solvent dependence of the fluorescence indicates that a protic solvent is essential to observe a large Stokes-shifted green fluorescence for TG. Therefore, the fluorescence behavior of TG is distinctive in comparison with many traditional fluorescent probes such as dansyl, dapoxyl, and DAPI. In contrast to these fluorescent probes, TG exhibits greater fluorescence quantum yield in water than in organic solvents. In addition, TG exhibited fluorescence with a moderately large Stokes shift (5000 cm^{-1}) in water. These property differences may be advantageous for live-cell imaging, especially when used in combination with other established fluorescent probes in multicolor imaging. Furthermore, the fluorescence lifetimes of TG and its *N*-methylpyridinium derivative TG2 in water are 4.9 and 9.5 ns, respectively, which are longer than those of dansyl[10] or DAPI[11] in water. The photophysical properties of HQ and TG families are compared in Table 1. The fluorescence quantum yield of TG was more

Table 1. Absorption maximum, molar extinction coefficient, fluorescence maximum, fluorescence quantum yield, fluorescence lifetime, and product of the molar extinction coefficient and fluorescence quantum yield of HQ, TG, HQ2, and TG2 in water (1% DMSO)

	λ_{Abs}/nm (ε/M^{-1} cm^{-1})	λ_{F1}/nm	Φ_{F1}	τ_{F1}/ns	$\varepsilon \cdot \Phi_{F1}$
HQ	400 (2400)	526	0.06	2.7	140
TG	402 (4300)	509	0.15	4.9	650
HQ2	405 (10300)	517	0.19	7.4	2000
TG2	415 (12300)	503	0.34	9.5	4200

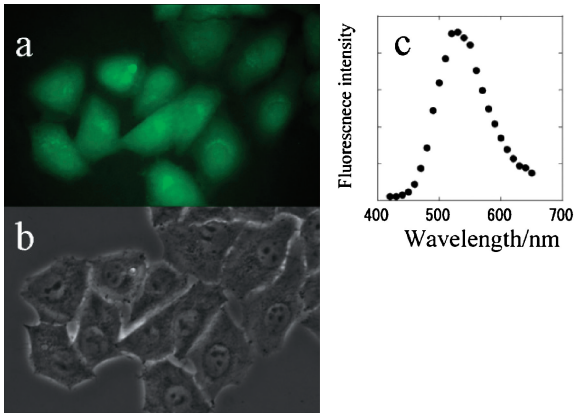


Figure 3. Fluorescence microscope images in HEp-2 cells 5 min after treatment with TG3: (a) fluorescence image with excitement at 460–490 nm and emission collected at wavelengths longer than 500 nm, (b) bright-field image, and (c) fluorescence spectra observed from HEp-2 cells treated with TG3.

than twice ($\Phi_f = 0.15$) that of the parent compound HQ ($\Phi_f = 0.06$), while TG and HQ showed similar absorption and fluorescence spectra in aqueous solution (Figure S1).[16]

7-Acetoxy analogs, TG1 and TG3, obtained by acetylation[12] of TG and TG2, respectively, were nearly nonfluorescent when excited by 400-nm light in aqueous solution, indicating that the 7-acetoxy group promotes nonradiative decay, and is favorable for bioimaging because of the nonfluorescence of the extracellular solution. *N*-Methylation[13] of TG dramatically improved photosensitivity. The ($\varepsilon \cdot \Phi_F$) value of TG2 increased by up to 8-fold by *N*-methylation of TG, and was 30-fold greater than that of HQ.

To demonstrate that TG-based substrates can be used for live-cell imaging, all of the compounds shown in Figure 1 were used with HEp-2 cells and a 10 mM solution of each derivative. Results showed that HEp-2 cells were stained only by the *N*-methyl-7-acetoxy derivative TG3, suggesting that not only 7-acylation but also *N*-methylation is necessary for a compound in the TG family to pass through the membrane. HEp-2 cells treated with a 10 mM solution of TG3 immediately developed strong green fluorescence from whole cells (Figure 3). In contrast, HEp-2 cells treated with 10 mM HQ3 resulted in blurring of the outline of the stained substrates. Furthermore intracellular fluorescence spectra observed from cells treated with TG3 solution showed close agreement with fluorescence

spectra of TG2 in a test tube (Figure 3c). The results indicate that TG3 effectively penetrated the cell membrane followed through hydrolysis by intracellular esterase to afford fluorescent species TG2, which was delocalized in the cells.

Flow cytometry using HEp-2 cells revealed the membrane permeability of each derivative. The cells were stained with a 30 mM solution of TG3 and HQ3 at 75 and 60%, respectively, for 2 h. Cells were not stained by the other derivatives.

TG-induced apoptosis was evaluated using cytofluorometric analysis of PI cell staining[14] and FSC/SSC plots.[15] Significant apoptosis was not observed in HEp-2 cells after treatment with a 10 mM solution of each compound for 12 h, suggesting that TG-based substrates could serve as useful fluorescent probes for live-cell imaging.

In summary, a new fluorescent dye TG was identified unexpectedly as a product of the $BiCl_3$-catalyzed Pechmann reaction. The TG emitted large Stokes-shifted fluorescence in water from the excited state zwitterionic form produced by excited-state proton transfer. The TG derivatives TG1–TG3 were prepared by simple chemical modification. The values of ($\varepsilon \cdot \Phi_F$) and the fluorescence lifetimes of TG and TG2 were greater than those of the model compounds HQ and HQ-2, respectively. In addition, TG3 was successfully applied for bioimaging. The immediate staining of HEp-2 cells after treatment of TG3 indicates that TG3 passed rapidly through the plasma membrane followed by hydrolysis via intracellular esterase to give fluorescent species TG2. Flow cytometry experiments revealed that 75% of HEp-2 cells were stained by TG3 treatment and no significant cytotoxicity of compounds in the TG family was observed. Further investigation into the usefulness in biological systems, e.g., modification of ester moiety at the 4-position with bioactive peptides or proteins, is underway.

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