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COMMUNICATION

Development of a fluorescein analogue, TokyoMagenta, as a novel scaffold for fluorescence probes in red region†

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We present a design strategy for fluorescence probes with a high off/on activation ratio in the red wavelength region, based on a novel fluorescein analogue in which the O atom at the 10 position of the xanthene chromophore is replaced with a Si atom. To demonstrate the usefulness of this strategy, we designed and synthesized a red-fluorescent probe for β-galactosidase, and showed that it works in live HEK293 cells.

Since the first fluorescence indicators for calcium ions were reported by Tsien *et al.*,[1] many fluorescence probes have been developed and have contributed greatly to biological and medical research.[2] In particular, fluorescein (Fig. 1) has many favorable characteristics, such as high water solubility, high fluorescence quantum yield and high molar extinction coefficient, and it has been utilized as the fluorescent core for a large number of probes, including, for example, fluorescence probes for metal ions[3] and reactive oxygen and nitrogen species.[4] However, fluorescence probes based on fluorescein and its derivatives, such as TokyoGreen (TG) derivatives (Fig. 1), have only 'green' fluorescence. Therefore, we set out to develop fluorescence probes in another color region, following the report that Si-substituted pyronine has 90 nm longer absorption and fluorescence wavelengths than pyronine Y,[5]

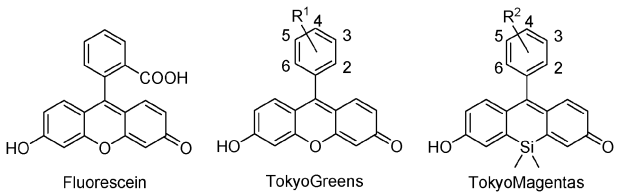
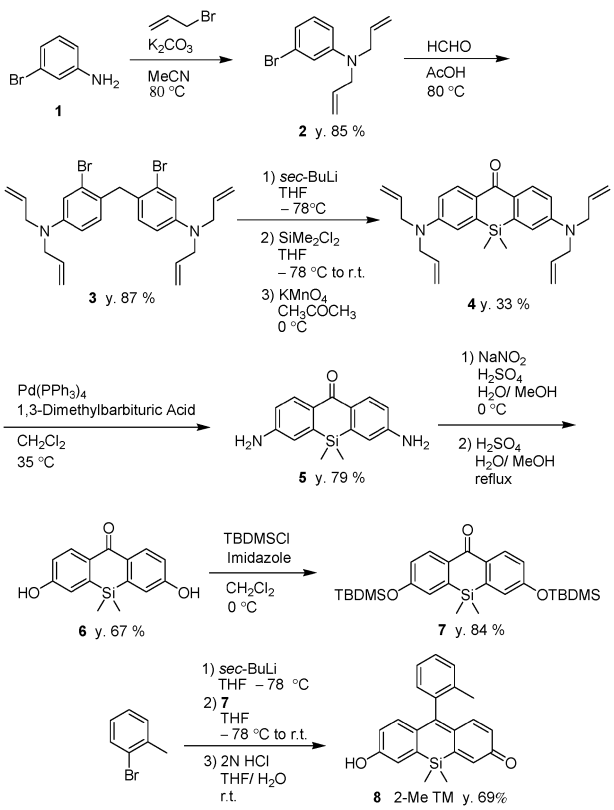


Fig. 1 Chemical structures of fluorescein, TokyoGreens and TokyoMagentas. R^1 = 2-Me: 2-Me TG. R^2 = 2-Me: 2-Me TM.

since we expected that they would be useful for dual-color imaging with various kinds of indicators having green fluorescence, such as GFP, fluorescein derivatives and so on.

Here, we describe the development of a novel fluorescein analogue, TokyoMagenta (TM) (Fig. 1), in which the O atom at the 10 position of the xanthene chromophore is replaced with a Si atom (Scheme 1). Our synthetic strategy was to synthesize the xanthene moiety from 3-bromoaniline (**1**). To synthesize the dimer **3**, a N atom is more suitable than an O atom because of its high electron-donating ability, so 3-bromoaniline was chosen as a starting material instead of 3-bromophenol. As a protecting group, we chose allyl because of its weak electron-withdrawing character. This results in less influence on the dimerization reaction, and also permits the



Scheme 1 Synthetic scheme of 2-Me TM.

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† Electronic supplementary information (ESI) available: Detailed descriptions of materials and general methods, and synthetic procedures. Data on the measurement of the pH profiles and pK_a plots of absorbance, fluorescence and excitation and the photobleaching test of **8**, fluorescence quantum yield of **9**, and the time-dependent fluorescence change of **9** in the presence of β-galactosidase. See DOI: 10.1039/c1cc00078k

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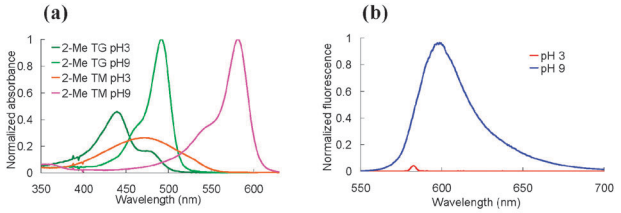

(c)

	ε	Φ_{fl}	λ_{abs} (nm)	λ_{fl} (nm)
Anion form	110000	0.42	582	598
Neutral form	29000	0.10	472	593

(d)

	2-Me TG	2-Me TM
Ratio value	67	986

Fig. 2 Photophysical properties of 2-Me TM. (a) pH-dependency of absorption spectra of 1 μM 2-Me TG and 2-Me TM in 0.1 M sodium phosphate buffer containing 1% DMSO. (b) pH-dependency of fluorescence spectra (Ex = 582 nm) of 2-Me TM under the same conditions as in (a). (c) Photophysical properties of 2-Me TM, measured in sodium phosphate buffer at pH 9 for the anion form and pH 3 for the neutral form. For the determination of fluorescence quantum yields, Rhodamine B in EtOH (Φ_{fl} = 0.65) was used as a fluorescence standard. (d) The value of the ratio of fluorescence intensity (anion form (pH 9)/neutral form (pH 3)) with excitation at the absorption maximum of the respective anion form (492 nm for 2-Me TG and 582 nm for 2-Me TM).

use of *sec*-butyllithium for the synthesis of Si-containing xanthone (**4**) by lithiation. Thus, diaminoxanthone (**5**) was synthesized, and then transformed into dihydroxyxanthone (**6**). Compound **7** was a key intermediate in the synthesis of TMs with various kinds of benzene moiety in one step. First, we synthesized 2-Me TM as a prototype.

Fig. 2 shows the photophysical properties of 2-Me TM in aqueous solution. A fascinating feature of 2-Me TM is that deprotonation of the hydroxyl group of the fluorophore triggered a 110 nm redshift of the absorbance (Fig. 2a). It is considered that this redshift is due to σ*–π* conjugation,[6] similar to that of Si-containing pyronine,[5] and the strength of the σ*–π* conjugation may be different between the neutral form and the anion form of TM. This redshift is very large compared to that of fluorescein derivatives; for example, 2-Me TG shows only a 52 nm redshift on deprotonation (Fig. 2a). Because of this large redshift, the fluorescence intensity of the anion form of 2-Me TM is much larger than that of the neutral form when excited at 582 nm (Fig. 2b). Thus, as 78% of 2-Me TM is present in the anion form at pH 7.4, which corresponds to the intercellular pH, fluorescence sensor probes with a high off/on ratio can be obtained simply by utilizing the large redshift of TM (Fig. 2c and d), without the need for additional controls, such as the photoinduced electron transfer (PeT)[7] or spiro cyclization strategies,[8] which are usually required for fluorescein-based sensor probes.

To examine whether hydroxyl group substitution alone can be used to develop fluorescence probes with a large off/on ratio based on the dramatic redshift of TM, we designed and synthesized 2-Me TM βgal as a fluorescence probe for β-galactosidase (Fig. 3a). The absorbance maximum of 2-Me

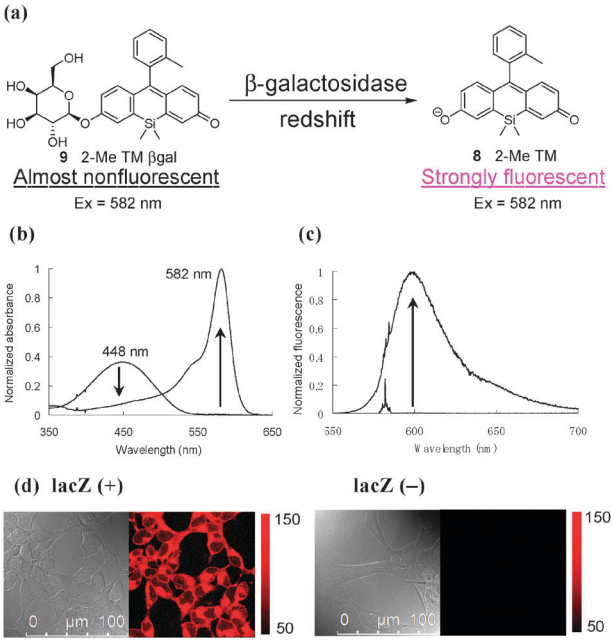

Fig. 3 (a) Reaction scheme of 2-Me TM βgal with β-galactosidase. (b) Absorption and (c) fluorescence spectra of 1 μM 2-Me TM βgal before and after reaction with 6 units β-galactosidase at 37 °C in 0.1 M sodium phosphate buffer (pH 7.4) containing 1% DMSO. (d) Visualizing β-galactosidase activity in live cells using 2-Me TM βgal. HEK293 cells (lacZ(+) or lacZ(−)) were incubated with 10 μM 2-Me TM βgal in DMEM containing 0.1% DMSO for 30 min. Bright field images (left) and fluorescence images (right). The excitation and emission wavelengths were 580 and 600–620 nm, respectively.

TM βgal was at a shorter wavelength than that of the neutral form of 2-Me TM, and 2-Me TM βgal could serve as a substrate for β-galactosidase. Further, 2-Me TM βgal showed a large redshift of the absorbance spectrum following the enzymatic reaction (Fig. 3b), and the fluorescence intensity upon excitation at 582 nm was also greatly increased (Fig. 3c). We next applied 2-Me TM βgal to live cells. When cultured HEK293 cells (lacZ(+) or lacZ(−)) were incubated with 10 μM 2-Me TM βgal for 30 min, a large fluorescence increment was observed in the intracellular region of lacZ(+) cells, but not lacZ(−) cells, on excitation at 580 nm (Fig. 3d).

In conclusion, we have developed a novel scaffold for fluorescence probes operating in the red wavelength region, *i.e.*, a Si-substituted fluorescein analogue, which we call TokyoMagenta. TM showed an extremely large change of its absorption spectrum upon deprotonation, and this phenomenon can be utilized to obtain red-fluorescent sensor probes with a high off/on ratio. This design strategy for controlling fluorescence intensity is noteworthy, because fluorescein-based probes require control by means of PeT or spiro cyclization strategies to achieve a high off/on ratio. Furthermore, since TMs can be highly activated without any additional strategy, their benzene moieties can be flexibly modified compared to TGs; *i.e.*, for example, modification of the benzene moiety of TGs is highly restricted because control of the oxidation potential is extremely important for the PeT strategy. Thus, it should be easy to design fluorescence sensor probes based on TMs for a range of applications by flexibly modifying the

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benzene moiety. Also, the switches of reported fluorescein-based sensor probes for bioimaging could be easily applied to TM to obtain equivalent probes operating in the red region, instead of green. Because fluorescein-based fluorescence probes have been widely utilized in biological and medical research, sensor probes based on TMs should also be useful in those fields as red-colored indicators, *e.g.*, for dual-color imaging.

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