

Supporting Information for:

**Development of Fluorescein Analogue, TokyoMagenta, as a Novel Scaffold  
for Fluorescence Probes in Red Region**

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## Methods

**General Methods.** General chemicals were of the best grade available, supplied by Tokyo Chemical Industries, Wako Pure Chemical, Aldrich Chemical Co., Alfa Aesar, Dojindo, GE Healthcare and Invitrogen, and were used without further purification. All solvents were used after appropriate distillation or purification. NMR spectra were recorded on a JEOL JNM-LA300 instrument at 300 MHz for  $^1\text{H}$  NMR and at 75 MHz for  $^{13}\text{C}$  NMR or a JEOL JNM-LA400 instrument at 400 MHz for  $^1\text{H}$  NMR and at 100 MHz for  $^{13}\text{C}$  NMR. Mass spectra (MS) were measured with a JEOL JMS-T100LC AccuToF using ESI.  $\delta$  values are given in ppm relative to tetramethylsilane. HPLC analysis was performed on an Inertsil ODS-3 (4.6  $\times$  250 mm) column (GL Sciences Inc.) using an HPLC system composed of a pump (PU-980, JASCO) and a detector (MD-2015 or FP-2025, JASCO). Preparative HPLC was performed on an Inertsil ODS-3 (10.0  $\times$  250 mm) column (GL Sciences Inc.) using an HPLC system composed of a pump (PU-2080, JASCO) and a detector (MD-2015 or FP-2025, JASCO).

**UV-Vis Absorption and Fluorescence Spectroscopy.** UV-visible spectra were obtained on a Shimadzu UV-1650. Fluorescence spectroscopic studies were performed on a Hitachi F4500. The slit width was 2.5 nm for both excitation and emission. The photomultiplier voltage was 700 V. Relative fluorescence quantum efficiency of 2-Me TM was obtained by comparing the area under the emission spectrum of the test sample excited at 540 nm with that of a solution of Rhodamine B in EtOH, which has a quantum efficiency of 0.65,<sup>SR1</sup> and 2-Me TM  $\beta$ gal was referred to fluorescein in 0.1 M NaOH, whose quantum efficiency is 0.85.<sup>SR2</sup>

**Fluorescence Imaging.** A confocal imaging system (TCS-SP5; Leica) equipped with an argon laser and a white light laser was used. Fluorescence images were taken with monitoring of fluorescence in the 600-620 nm channel. The excitation wavelength was 580 nm (white light laser). HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp.) supplemented with 10% (v/v) fetal bovine serum (Invitrogen Corp.), 1% penicillin and 1% streptomycin (Invitrogen Corp.) in a humidified incubator containing 5% CO<sub>2</sub> in air. For fluorescence microscopy, HEK293 cells were plated in a 35-mm

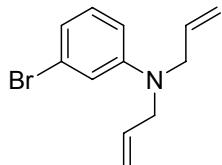
PDL-coated glass-bottomed dish (MatTek Corporation) in Dulbecco's modified Eagle's medium (DMEM).

For dye loading, the cells were incubated with 10  $\mu$ M **9** in DMEM for 30 min in an incubator.

### Synthetic procedures and characterizations

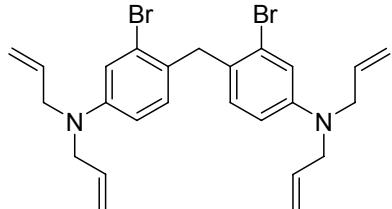
Elution in all HPLC analyses was done with a 20-min linear gradient from 20 % CH<sub>3</sub>CN / 0.1 % TFA aq. to 80 % CH<sub>3</sub>CN / 0.1 % TFA aq. (flow rate = 1.0 mL / min). UV, 460 nm.

#### 3-Bromo-N,N-diallylaniline (2)



To a suspension of K<sub>2</sub>CO<sub>3</sub> (22.0 g, 159 mmol) in MeCN were added 3-bromoaniline (8.71 mL, 80.0 mmol) and allyl bromide (23.7 mL, 280 mmol), and the mixture was stirred at 80 °C for 14 h. After cooling, the reaction mixture was filtered through a Celite pad, washed with AcOEt and evaporated to dryness. The residue was purified by column chromatography (silica gel, 1/40 AcOEt/hexane) to give pure **2** (17.1 g, 67.9 mmol, 85% yield). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ 3.87-3.90 (m, 4H), 5.11-5.15 (m, 2H), 5.17-5.18 (m, 2H), 5.75-5.88 (m, 2H) 6.58 (dd, 1H, *J* = 2.2, 8.1 Hz), 6.77-6.81 (m, 2H) 7.01 (t, 1H, *J* = 8.1 Hz); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): δ 52.7, 110.8, 115.0, 116.3, 119.0, 123.3, 130.2, 133.2, 150.0; HRMS (ESI<sup>+</sup>): m/z Found 252.0429, calculated 252.0388 for [M+H]<sup>+</sup> (+4.1 mmu).

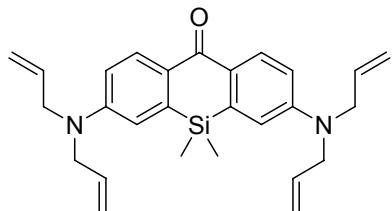
#### Bis(2-bromo-4-N,N-diallylaminophenyl)methane (3)



To a solution of compound **2** (17.1 g, 67.9 mmol) in AcOH (200 mL) was added 37% formaldehyde (10.2 g, 340 mmol), and the mixture was stirred at 80 °C for 75 min. After cooling to room temperature, the reaction mixture was neutralized with saturated NaHCO<sub>3</sub> aq. and NaOH aq., and extracted with CH<sub>2</sub>Cl<sub>2</sub>. The organic layer was washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness. The residue was purified by column chromatography (silica gel, 1/30 AcOEt/hexane) to give pure **3** (15.2 g, 29.5 mmol, 87% yield). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ 3.85-3.87 (m, 8H), 3.96 (s, 2H), 5.13-5.19 (m, 8H), 5.76-5.88 (m, 4H), 6.54

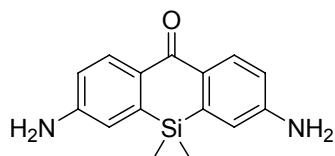
(dd, 2H,  $J = 2.9, 8.8$  Hz), 6.81 (d, 2H,  $J = 8.1$  Hz), 6.90 (d, 2H,  $J = 2.9$  Hz);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ):  $\delta$  39.7, 52.7, 111.7, 116.0, 116.2, 125.5, 126.9, 130.8, 133.5, 148.1; HRMS (ESI $^+$ ): m/z Found 517.0654, calculated 517.0677 for  $[\text{M}+\text{H}]^+$  (-2.3 mmu).

***N,N,N',N'-Tetraallyldiamino-Si-xanthone (4)***



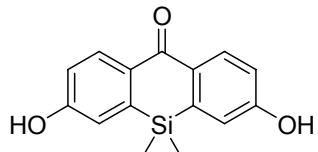
To a flame-dried flask flushed with argon, compound **3** (8.16 g, 15.8 mmol) and anhydrous THF (50 mL) were added. The solution was cooled to  $-78^\circ\text{C}$ , 1 M *sec*-BuLi (45 mmol) was added, and the mixture was stirred for 20 min. At the same temperature, a solution of  $\text{SiMe}_2\text{Cl}_2$  (2.9 mL, 30 mmol) in anhydrous THF (15 mL) was slowly added, and the mixture was warmed to room temperature, then stirred for 1 h. The reaction was quenched by addition of 2 N HCl aq., then the mixture was neutralized with  $\text{NaHCO}_3$ , and extracted with  $\text{CH}_2\text{Cl}_2$ . The organic layer was washed with brine, dried over  $\text{Na}_2\text{SO}_4$  and evaporated to dryness. The residue was dissolved in  $\text{CH}_3\text{COCH}_3$  (150 mL), and the solution was cooled to  $0^\circ\text{C}$ . To this solution,  $\text{KMnO}_4$  (6.88 g, 43.5 mmol) was added in small portions over a period of 2 h with stirring. The mixture was stirred for another 1 h at the same temperature, then diluted with  $\text{CH}_2\text{Cl}_2$  (200 mL), filtered through paper filter and evaporated to dryness. The residue was purified by column chromatography (silica gel,  $\text{CH}_2\text{Cl}_2$ ) to give pure **4** (2.23 g, 5.20 mmol, 33% yield).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ):  $\delta$  0.41 (s, 6H), 4.02 (d, 8H,  $J = 5.1$  Hz) 5.17-5.23 (m, 8H), 5.82-5.94 (m, 4H), 6.80-6.83 (m, 4H), 8.34 (d, 2H,  $J = 8.1$  Hz);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ):  $\delta$  -1.1, 52.8, 113.5, 114.8, 116.7, 130.0, 131.7, 133.1, 140.5, 150.2, 185.1; HRMS (ESI $^+$ ): m/z Found 429.2347, calculated 429.2362 for  $[\text{M}+\text{H}]^+$  (-1.5 mmu).

**Diamino-Si-xanthone (5)**



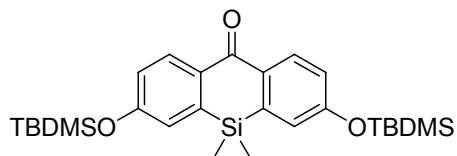
To a flame-dried flask flushed with argon, Pd(PPh<sub>3</sub>)<sub>4</sub> (35.0 mg, 0.0303 mmol) and 1,3-dimethylbarbituric acid (169 mg, 1.08 mmol) were added. A mixture of compound **4** (99.2 mg, 0.231 mmol) dissolved in CH<sub>2</sub>Cl<sub>2</sub> (10 mL) was further added and the solution was stirred at 35 °C for 16 h, then evaporated to dryness. The residue was suspended in saturated Na<sub>2</sub>CO<sub>3</sub> aq. and extracted with Cl<sub>2</sub>Cl<sub>2</sub>. The organic layer was washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness. The residue was purified by column chromatography (silica gel, 4/3 AcOEt/hexane) to give crude **5** (48.8 mg, 0.182 mmol, 79% yield). <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD): δ 0.40 (s, 6H), 6.76 (dd, 2H, *J* = 2.6, 8.4 Hz), 6.88 (d, 2H, *J* = 2.2 Hz), 8.13 (d, 2H, *J* = 8.8 Hz); <sup>13</sup>C NMR (75 MHz, CD<sub>3</sub>OD): δ -1.3, 116.6, 118.4, 131.0, 132.8, 142.6, 153.0, 187.5; HRMS (ESI<sup>+</sup>): m/z Found: 269.1108, calculated 269.1110 for [M+H]<sup>+</sup> (-0.2 mmu).

### Dihydroxy-Si-xanthone (**6**)



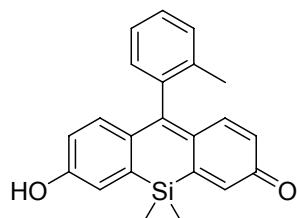
A solution of compound **5** (48.8 mg, 0.182 mmol) in MeOH / 6 N H<sub>2</sub>SO<sub>4</sub> (45 mL) was cooled to 0 °C. A solution of NaNO<sub>2</sub> (84.6 mg, 1.22 mmol) in H<sub>2</sub>O (2 mL) was slowly added, and the mixture was stirred at the same temperature for 1 h, then slowly added dropwise into boiling 1 N H<sub>2</sub>SO<sub>4</sub> (50 mL). The resulting mixture was refluxed for another 10 min, then allowed to cool to room temperature, and extracted with CH<sub>2</sub>Cl<sub>2</sub>. The organic layer was washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated. The residue was purified by column chromatography (silica gel, 1/20 MeOH/CH<sub>2</sub>Cl<sub>2</sub>) to give crude **6** (32.9 mg, 0.122 mmol, 67% yield). <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD): δ 0.45 (s, 6H), 6.95 (dd, 2H, *J* = 2.2, 8.8 Hz), 7.07 (d, 2H, *J* = 2.2 Hz), 8.26 (d, 2H, *J* = 8.8 Hz); <sup>13</sup>C NMR (75 MHz, CD<sub>3</sub>OD): δ -1.5, 118.4, 120.0, 133.3, 133.8, 143.1, 162.2, 187.6; HRMS (ESI<sup>-</sup>): Found 269.0674, calculated 269.0634 for [M-H]<sup>-</sup> (+4.0 mmu).

**3,6-DiOTBDMS-Si-xanthone (7)**



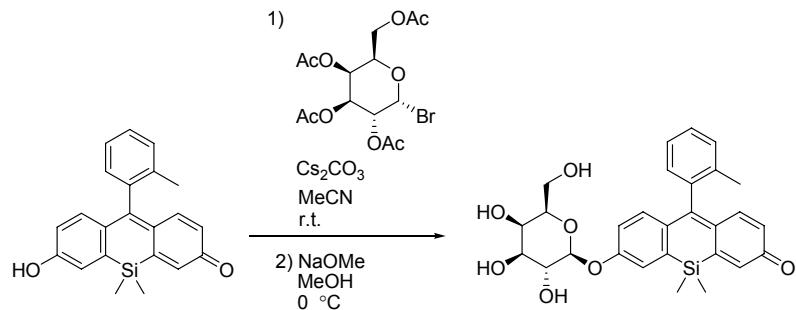
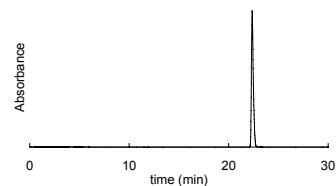
To a solution of compound **6** (32.9 mg, 0.122 mmol) and imidazole (85.5 mg, 1.26 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (20 mL) was slowly added TBDMSCl (185 mg, 1.23 mmol) dissolved in CH<sub>2</sub>Cl<sub>2</sub> (5 mL), and the mixture was stirred at room temperature for 14 h. H<sub>2</sub>O was added, and the mixture was extracted with CH<sub>2</sub>Cl<sub>2</sub>. The organic layer was washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness. The residue was purified by column chromatography (silica gel, 1/20 AcOEt/hexane) to give pure **7** (52.8 mg, 0.106 mmol, 84% yield). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ 0.26 (s, 12H), 0.46 (s, 6H), 1.01 (s, 18H), 6.98 (dd, 2H, *J* = 2.2, 8.8 Hz), 7.04 (d, 2H, *J* = 2.9 Hz), 8.37 (d, 2H, *J* = 8.8 Hz); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): δ -4.3, -1.6, 18.3, 25.6, 121.8, 123.7, 132.3, 134.5, 141.1, 158.7, 186.0; HRMS (ESI<sup>+</sup>): m/z Found 499.2480, calculated 499.2520 for [M+H]<sup>+</sup> (-4.0 mmu).

**2-Me TokyoMagenta (8)**



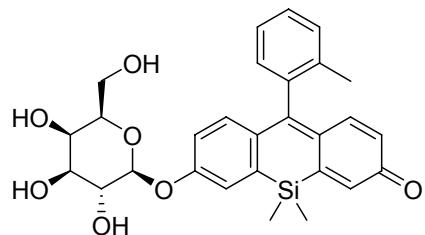
To a flame-dried flask flushed with argon, 2-bromotoluene (200 μL, 1.6 mmol) and anhydrous THF (5 mL) were added. The solution was cooled to -78 °C, 1 M sec-BuLi (1.0 mmol) was added, and the mixture was stirred for 20 min. At the same temperature, compound **7** (9.4 mg, 0.019 mmol) dissolved in anhydrous THF (5 mL) was slowly added. The mixture was warmed to room temperature then stirred for 1 h, and 2 N HCl aq. (10 mL) was added to it. Stirring was continued for 20 min, then the mixture was extracted with CH<sub>2</sub>Cl<sub>2</sub>. The organic layer was washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness. The residue was purified by HPLC to give pure 2-Me TokyoMagenta (**8**) (4.5 mg, 0.013 mmol, 69% yield). <sup>1</sup>H NMR (300 MHz, D<sub>2</sub>O): δ 0.46 (s 6H), 2.01 (s, 3H), 6.33 (dd, 2H, *J* = 2.9, 9.5 Hz), 7.01-7.09 (m, 5H), 7.27-7.46 (m, 3H); HRMS (ESI<sup>-</sup>): Found 343.1120 calculated 343.1154 for [M-H]<sup>-</sup> (-3.4mmu). HPLC

chromatogram after purification was as follows.



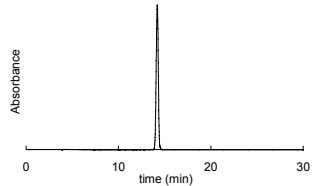
**Scheme S1** Synthetic scheme for 2-Me TokyoMagenta  $\beta$ gal (**9**)

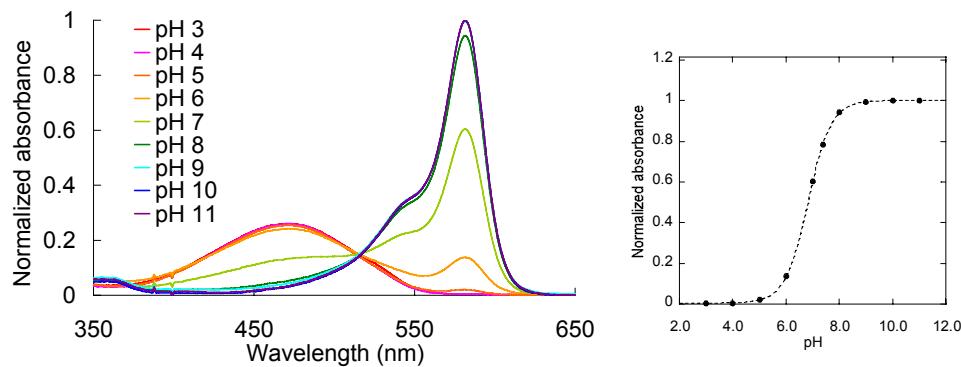
### 2-Me TokyoMagenta $\beta$ gal (**9**)



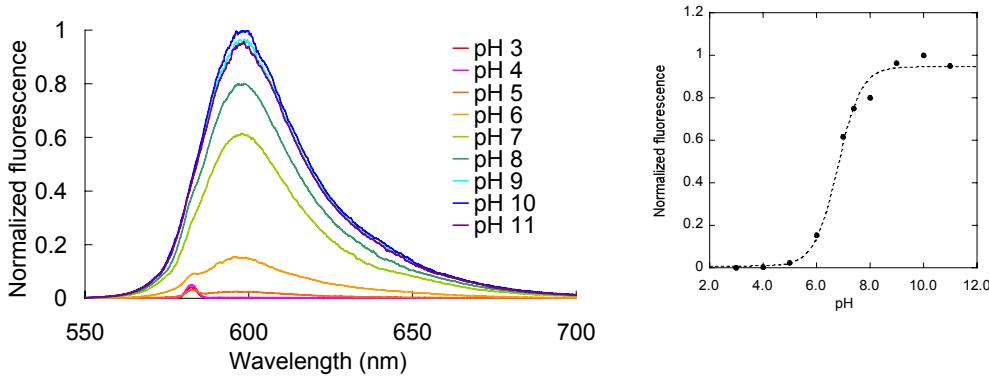
A mixture of 2-Me TokyoMagenta (**8**) (4.6 mg, 0.013 mmol), 2,3,4,6-tetra-*O*-acetyl- $\alpha$ -galactopyranosyl bromide<sup>SR3</sup> (80.8 mg, 0.197 mmol) and Cs<sub>2</sub>CO<sub>3</sub> (29.6 mg, 0.0909 mmol) in MeCN (3 mL) was stirred at room temperature under argon overnight. The inorganic precipitate was filtered off, and the filtrate was evaporated to dryness. The resulting residue was dissolved with MeOH (3 mL) and the solution was cooled to 0 °C. Then 15  $\mu$ L of 28 % NaOMe in MeOH was slowly added, and the mixture was stirred for 1 h. The reaction was quenched by addition of 0.2 N HCl aq., and the mixture was extracted with CH<sub>2</sub>Cl<sub>2</sub>. The organic layer was washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness. The residue was purified by HPLC to give pure 2-Me TokyoMagenta  $\beta$ gal (**9**) (2.4 mg, 0.0047 mmol, 36% yield). <sup>1</sup>H NMR (300.40 MHz, CD<sub>3</sub>OD):  $\delta$  0.51 (d, 3H, *J* = 1.8 Hz), 0.52 (d, 3H, *J* = 1.8 Hz), 2.04 (s, 3H), 3.59 (dd, 1H, *J* = 3.7, 9.5

Hz), 3.69-3.89 (m, 5H), 4.98 (dd, 1H,  $J = 3.3, 7.7$  Hz), 6.23 (dd, 1H,  $J = 2.2, 10.3$  Hz), 6.87-6.91 (m, 2H), 6.98-7.13 (m, 3H), 7.33-7.46 (m, 3H), 7.50 (d, 1H,  $J = 2.2$  Hz); HRMS (ESI $^+$ ): m/z Found 507.1826 calculated 507.1839 for [M+H] $^+$  ( $-1.3$  mmu). HPLC chromatogram after purification was as follows.

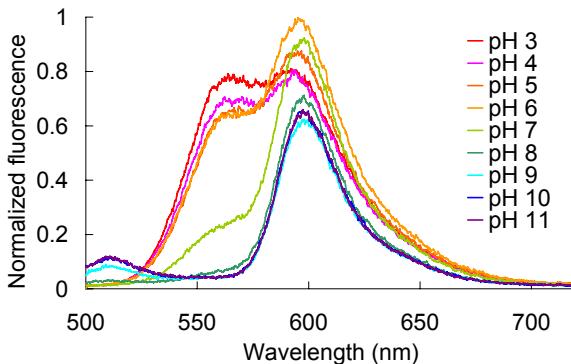




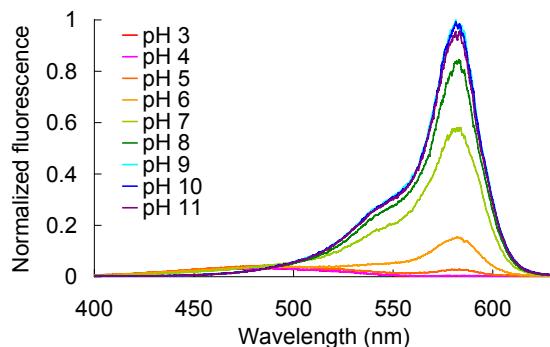
**Fig. S1** pH-dependent change of absorbance spectra of 1  $\mu$ M 2-Me TM in 0.1 M sodium phosphate buffer containing 1 % DMSO (left).  $pK_a$  Plot of 2-Me TM at 582 nm (right). The  $pK_a$  value was 6.8.



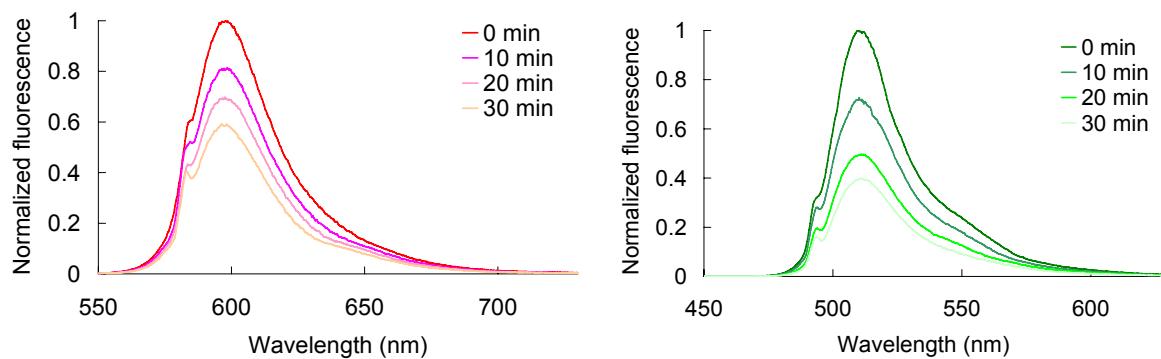
**Fig. S2** pH-dependent change of fluorescence spectra ( $Ex = 582$  nm) of 1  $\mu$ M 2-Me TM in 0.1 M sodium phosphate buffer containing 1 % DMSO (left). Plot of the normalized maximum fluorescence intensity against pH (right). The  $pK_a$  value was 6.8.



**Fig. S3** pH-dependent change of fluorescence spectra ( $Ex = 471$  nm) of 1  $\mu$ M 2-Me TM in 0.1 M sodium phosphate buffer containing 1 % DMSO.



**Fig. S4** pH-dependent change of excitation spectra ( $\text{Em} = 640 \text{ nm}$ ) of  $1 \mu\text{M}$  2-Me TM in  $0.1 \text{ M}$  sodium phosphate buffer containing  $1 \%$  DMSO.

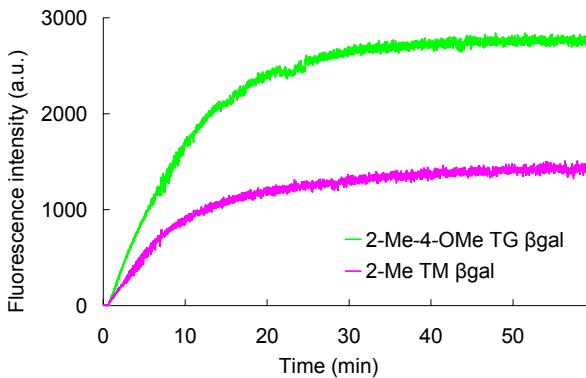


**Fig. S5** Photobleaching tests. Normalized fluorescence spectra of  $1 \mu\text{M}$  2-Me TM (left) and 2-Me TG (right) in  $0.1 \text{ M}$  sodium phosphate buffer (pH 7.4) containing  $1 \%$  DMSO. Samples were exposed to light (30 mW, 545-580 nm for 2 Me TM and 30 mW, 460-490 nm for 2-Me TG) for 0, 10, 20 and 30 min.

**Table S1** Fluorescence quantum yield of 2-Me TM  $\beta$ gal.

	$\Phi_{\text{fl}}$
2-Me TM $\beta$ gal	0.051

The fluorescence quantum yield was determined with fluorescein in 0.1 M NaOH aq. ( $\Phi_{\text{fl}} = 0.85$ )<sup>SR2</sup> as a standard.



**Fig. S6** Comparison of time-dependent fluorescence changes between 2-Me TM  $\beta$ gal and 2-Me-4-OMe TG  $\beta$ gal upon addition of  $\beta$ -galactosidase. Both experiments were performed at 37 °C in 0.1 M sodium phosphate buffer (pH 7.4) containing 1  $\mu$ M 2-Me TM  $\beta$ gal or 2-Me-4-OMe TG  $\beta$ gal and 0.1 % DMSO as a cosolvent. 1 unit (1.3  $\mu$ g) of  $\beta$ -galactosidase was added at 40 s.

#### Supporting references

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- SR2 Y. Urano, M. Kamiya, K. Kanda, T. Ueno, K. Hirose and T. Nagano, *J. Am. Chem. Soc.*, 2005, **127**, 4888.
- SR3 J. Montero, J. Winum, A. Leydet, M. Kamal, A. Pavia, and J. Roque, *Carbohydrate Research*, 1997, **297**, 175.