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Development of a Highly Specific Rhodamine-Based Fluorescence Probe for Hypochlorous Acid and Its Application to Real-Time Imaging of Phagocytosis

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Abstract: The tetramethylrhodamine (TMR) fluorophore is a useful platform for fluorescence probes, being applicable, for example, to biological investigations utilizing fluorescence microscopy, owing to its excellent photochemical properties in aqueous media. We have developed new TMR derivatives that show different dependences of their behavior upon the environment. Among them, HMTMR showed unique characteristics, and its putative spirocyclic structure was confirmed by X-ray crystallography. Utilizing this discovery, we have established a strategy to modulate the fluorescence of TMR by regulating the spirocyclization, and we have obtained a new fluorescence probe that can detect hypochlorous acid specifically. This probe, HySOx, can work in 99.9% aqueous solution at pH 7.4 and was confirmed to be able to detect hypochlorous acid being generated inside phagosomes in real time. HySOx is tolerant to autoxidation and photobleaching under bioimaging conditions. Regulation of the spirocyclization of rhodamines, as we describe here, provides a new approach to the rational development of novel fluorescence probes.

Introduction

The tetramethylrhodamine (TMR) fluorophore is a useful fluorescent molecule, being applicable to biological investigations utilizing fluorescence microscopy,[1,2] owing to its excellent photochemical properties in aqueous media,[3] including high fluorescence intensity, longer excitation and emission wavelengths (550−600 nm), high molar extinction coefficient (about 1×10^5 M^{-1} cm^{-1}), independence of pH and tolerance to photobleaching, and its wide use as a fluorescent tag.[4−6] However, it has not been widely employed in fluorescence probes, which are switched from a nonfluorescent to a fluorescent form upon interaction with analytes, in contrast to other common fluorophores such as fluorescein.[7] One of the reasons for this is probably the lack of a good strategy to regulate the fluorescence of the TMR fluorophore. Therefore, a novel and widely applicable strategy to regulate the fluorescence of the TMR fluorophore would potentially be applicable to the design of probes for a wide range of analytes.

Results and Discussion

We previously reported that the derivatization of fluorescein does not disrupt the photochemical properties when various functional groups are substituted for the carboxyl group at the 2 position.[8,9] We thought that a similar molecular design strategy might be applicable to the TMR fluorophore, so we synthesized various 2′-substituted TMR derivatives, including a new TMR derivative, hydroxymethyltetramethylrhodamine (HMTMR). To our surprise, the fluorescence of HMTMR showed a very interesting environmental dependence. HMTMR has a large absorbance and fluorescence in protic solvents but little in aprotic or basic solvents (Figure 1). This was found to be because of intramolecular spirocyclization at C9, deconjugating the TMR fluorophore. We obtained direct evidence of the spirocyclic form of HMTMR by X-ray crystallography (Figure 2). HMTMR in the dihydrofuran form crystallizes in a monoclinic system, space group $P2_1/m$ with $a = 6.6952(4)$ Å, $b =$

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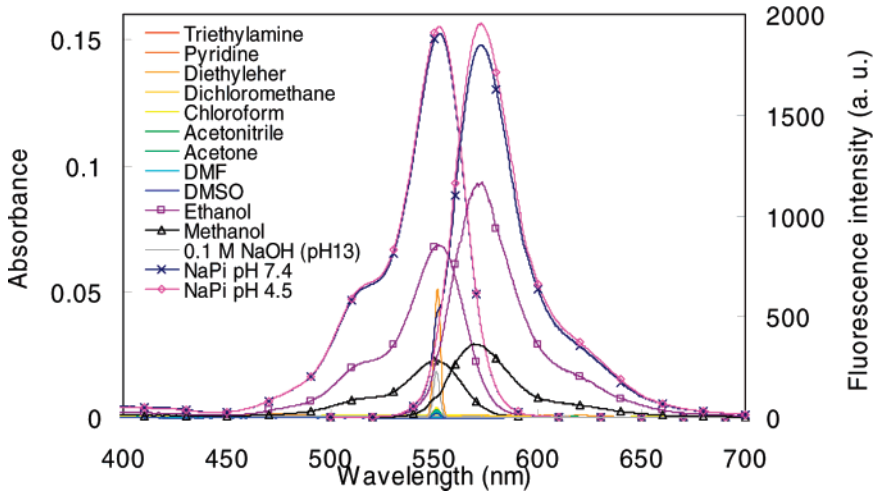

Figure 1. Absorption and emission spectra of HMTMR 1.0 μM in various solvents. Ex/em wavelengths were 550 nm/569 nm in MeOH and 552 nm/576 nm in other solvents.

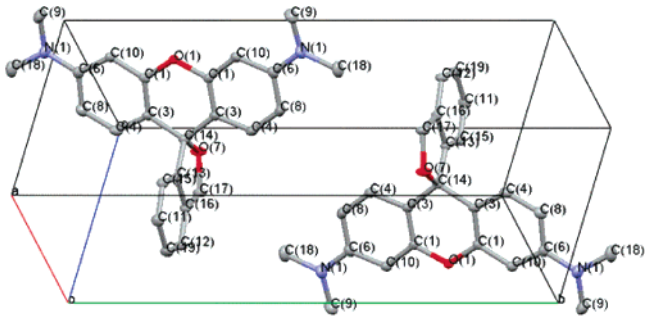

Figure 2. Perspective view of the crystal structure of HMTMR (50% probability ellipsoids) with the numbering scheme, excluding H atoms. An asymmetric unit consists of two independent molecules.

18.1555(14) Å, $c = 7.9560(6)$ Å, $\beta = 96.612(3)°$, $V = 960.66(12)$ Å^3, and $Z = 2$. Among similar spirocyclic compounds, rhodamine B lactone and rhodamine B lactam derivatives have been successfully determined by X-ray crystallography.[10,11] Rhodamines in a spirocyclic form have neither absorbance nor fluorescence in the visible region (Figure 3). So, regulation of the equilibrium between the spirocyclic form and the open circular form provides a strategy for controlling the fluorescence. Rhodamine spirolactams have already been applied as a platform for fluorescence probes, but they are available only for heavy metal cations such as Hg^{2+}, Cu^{2+}, and Fe^{3+} so far and do not work in physiological media,[12−14] with only a few exceptions.[15,16] Here, we have designed and developed a new fluorescence probe that works by regulating novel spirocyclization in physiological media.

The initial requirements for a practical fluorescence probe based on control of spirocyclization are that the probe molecule itself should be in almost 100% spirocyclic and nonfluorescent form, while the product of the interaction between probe molecule and analytes should be in an almost 100% open circular and fluorescent form. On the basis of this strategy, we designed and synthesized a thiol analogue of HMTMR to improve the stability of the spirocyclic structure in aqueous media at physiological pH. The thiol analogue, HySOx, was almost completely colorless and nonfluorescent in aqueous solutions over a wide range of pH values (Figure 4). HySOx can be isolated as a colorless solid from various organic solvents. The TMR fluorophore (e.g., tetramethylrhodamine methyl ester (TMRM)) shows almost constant, pH-independent high absorbance and fluorescence in water.[17] In contrast, however,

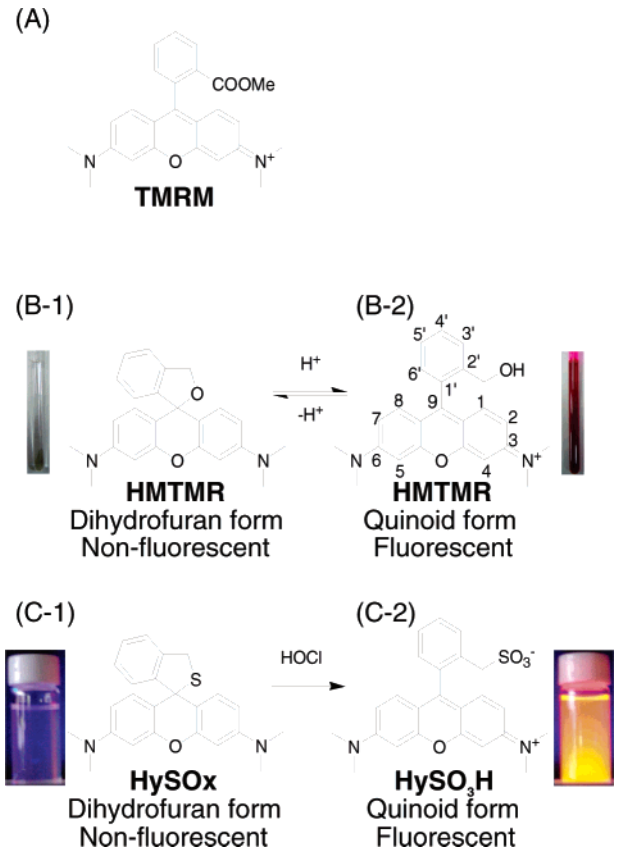

Figure 3. Structures of tetramethylrhodamines. (A) TMRM. (B-1) HMTMR 25 mM in DMSO-d_6. (B-2) TFA 25 mM (final) was added to sample (B-1). (C-1) HySOx 40 μM in sodium phosphate buffer containing 20% DMF. (C-2) NaOCl solution 40 μM (final) was added to sample (C-1).

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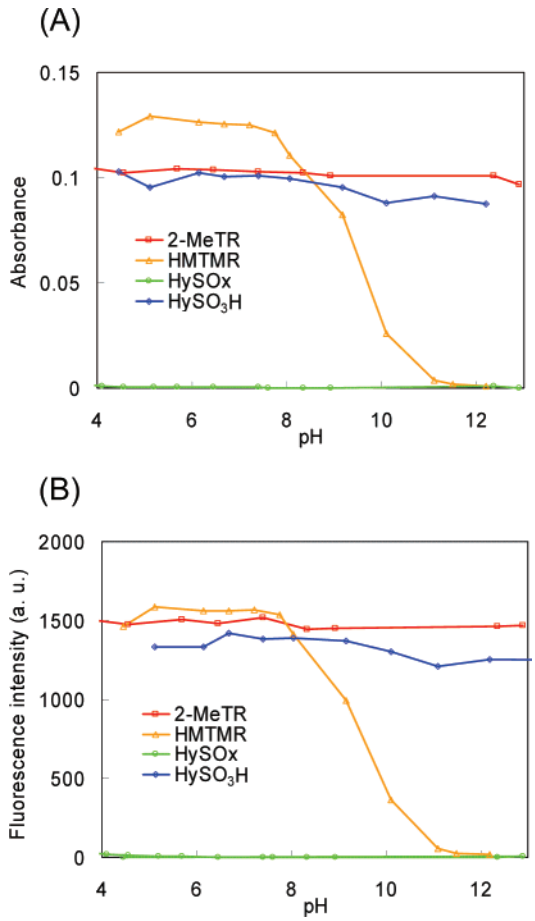

Figure 4. pH profiles of 1.0 μM TMRM, HMTMR, HySOx, and $HySO_3H$ in 0.10 M sodium phosphate buffer at various pH values in the presence of 1.0% DMF as a cosolvent. (A) Absorbances of TMRM (554 nm), HMTMR (554 nm), HySOx (552 nm), and $HySO_3H$ (555 nm). (B) Fluorescences of TMRM (ex/em = 554/577 nm), HMTMR (554/572 nm), HySOx (552/575 nm), and $HySO_3H$ (555/575 nm).

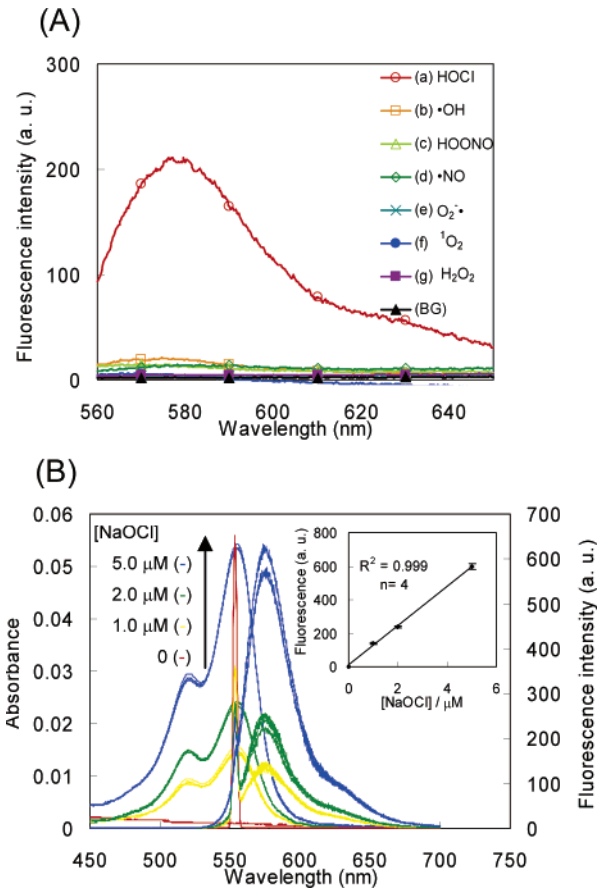

Figure 5. (A) Emission spectra of 2.0 μM HySOx after addition of various ROS in 0.10 M sodium phosphate buffer at pH 7.4, containing 0.10% DMF as a cosolvent. (a) NaOCl solution 5.0 μM, (b) 100 μM H_2O_2 and $Fe(ClO_4)_2$ 50 μM (Fenton reaction to generate hydroxyl radical), (c) 5.0 μM sodium peroxynitrite solution, (d) 5.0 μM NOC13 (nitric oxide donor), (e) 10 μM KO_2, (f) 1.0 μM Rose Bengal and light irradiation (2.3 mW/cm^2) at 550 nm for 10 min, (g) 100 μM H_2O_2, (h) exposure to room light for 30 min and (BG) background. Fluorescence increase was plotted in sample f to cancel the fluorescence of Rose Bengal. Fluorescence intensity was plotted in the others. (B) Absorption and emission spectra of 5.0 μM HySOx after addition of NaOCl in 0.10 M sodium phosphate buffer at pH 7.4 in the presence of 0.10% DMF as a cosolvent. Four spectra obtained from four individual experiments under each condition were overlaid. In the inset, the relation between dose of NaOCl and fluorescence intensity is shown.

HMTMR is quite dependent upon pH, existing in a spirocyclic form under basic conditions and showing lower absorbance and fluorescence at higher pH values. HySOx favors a spirocyclic form more strongly than HMTMR because HySOx has a thiol group, which is more nucleophilic than the hydroxyl group of HMTMR. Thus, by regulating the nucleophilicity of the function at the 2′ position, we obtained HMTMR, which has an open/closed equilibrium and is environmentally dependent, and HySOx, which favors a spirocyclic form independently of the environment and is completely nonfluorescent.

The thiol group works not only as a cyclization enhancer but also as a center of redox reaction. So, we tested the utility of HySOx as a fluorescence probe for reactive oxygen species (ROS). Various important ROS generated in organisms (i.e., (a) hypochlorous acid (HOCl), (b) hydroxyl radical, (c) peroxynitrite, (d) nitric oxide, (e) superoxide, (f) singlet oxygen, and (g) hydrogen peroxide) were added to a solution of HySOx in 0.10 M sodium phosphate buffer at pH 7.4, containing 0.10% DMF (Figure 5). The fluorescence intensity increased quickly (within seconds; see Supporting Information) and quantitatively upon sodium hypochlotrite (NaOCl) addition. The absorbance also increased. HySOx responded quickly in aqueous solutions at pH 7.4 and more slowly at higher pH to the addition of NaOCl (HOCl/$^-$OCl (pK_a = 7.6)) (see Supporting Information), which reveals that HySOx detects HOCl but not the hypochlorite anion ($^-$OCl). Many oxidizing reagents are known to oxidize dihydrothiophene substrates in organic synthesis conditions.[18,19] Indeed, the fluorescence of the HySOx solution at pH 7.4 increased slowly after the addition of *meta*-chloroperoxybenzoic acid (mCPBA) (see Supporting Information, Figure S8). However, HySOx is able to detect HOCl specifically among various reactive oxygen species in aqueous solution. There are other fluorescence probes that can detect HOCl, but these probes detect other ROS, too.[20] HySOx seems to be the only one that can detect HOCl specifically under biological conditions. We think that the application of rhodamine spirocycles has great potential for developing novel probes.

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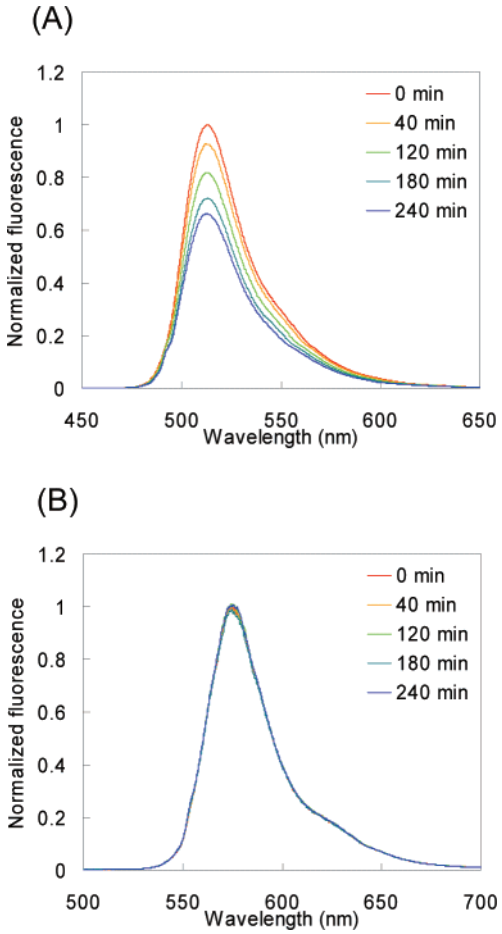

Figure 6. Fluorescence spectra of (A) fluorescein and (B) $HySO_3H$ in 0.10 M sodium phosphate buffer at pH 7.4. Samples were exposed to light (1.7 mW/cm^2, (A) 493 nm and (B) 555 nm) for 0, 40, 120, 180, and 240 min. Fluorescein and $HySO_3H$ were excited at 493 and 555 nm, respectively.

Table 1. Photochemical Properties of Rhodamines[a]

	abs_{max} (nm)	em_{max} (nm)	molar extinction coefficient (10^5 M^{-1} cm^{-1})	fluorescence quantum efficiency (Φ_{fl})
TMRM	554	577	1.1	0.34
HMTMR	552	574	1.2	0.32
HySOx	n.d.[b]	n.d.	n.d.	n.d.
$HySO_3H$	555	575	1.0	0.34

[a] Measured in 0.1 M sodium phosphate buffer at pH 7.4. [b] Not detectable.

We also separated the main fluorescent product of the reaction between HySOx and HOCl and identified it as the sulfonate derivative, $HySO_3H$. $HySO_3H$ showed a high tolerance to photobleaching (Figure 6), which is a very important feature for application to bioimaging. This is due to the high tolerance of the TMR fluorophore to photobleaching. The photochemical properties of $HySO_3H$ are listed in Table 1. The molar extinction coefficient is 1.0×10^5 at pH 7.4, which means that $HySO_3H$ is in an almost completely open circular form because the sulfonic group is not nucleophilic enough to be involved in cyclization. The absorbance and fluorescence of $HySO_3H$ are enormously larger than those of HySOx and are quite independent of pH, as shown in Figure 5. These results support the idea that spirocyclization can be regulated by altering the nuclephilicity of the function at the 2' position to afford TMR derivatives with different pH dependences.

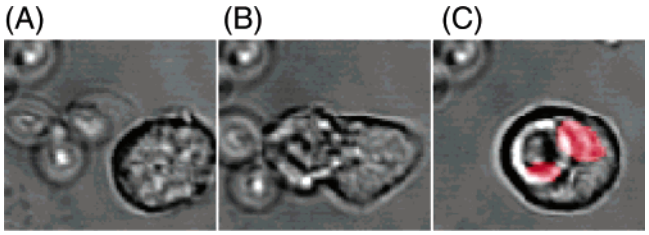

Figure 7. Fluorescence microscopy imaging of porcine neutrophil by confocal microscopy. (A) Zymosan particles are near the neutrophil. (B) The neutrophil engulfs the zymosan. (C) Phagocytosis is complete.

Ⓦ A video of this imaging is available.

We next applied HySOx to visualize phagocytosis by porcine neutrophils, with opsonized zymosan derived from *Saccharomyces cerevisiae* as a target. HySOx detected HOCl generated inside the phagosomes just after the completion of phagocytosis (Figure 7). It is reported that the inside of the phagosome is acidic.[21] We also confirmed that HySOx is able to detect HOCl specifically at pH 6.0 (see Supporting Information, Figure S9). In the imaging of phagocytosis, there was little fluorescence increase due to autoxidation under laser excitation before phagocytosis began. Further, there was no fluorescence decrease due to photobleaching after membrane fusion following phagocytosis. In contrast, it is well-known that ROS probes such as dihydrodichlorofluorescein (DCFH) become fluorescent due to autoxidation simply upon exposure to excitation light.[22] Further, photobleaching is a very common problem in fluorescence microscopy. Therefore, HySOx has very superior properties as a probe, being tolerant to autoxidation due to its high specificity for HOCl and producing a fluorescent product, $HySO_3H$, that is photostable and pH independent owing to the TMR fluorophore.

Conclusion

We discovered a spirocyclic form of HMTMR and confirmed its structure by X-ray crystallography. On the basis of this discovery, we developed a novel fluorescence probe specific for HOCl by controlling the nucleophilicity of the attacking group involved in the spirocyclization of TMR derivatives. Our study extends the applications of rhodamine spirocycles to a new area, not simply heavy metal cations. HySOx can detect HOCl specifically among various important ROS generated in organisms under physiological conditions. HySOx showed a quick and quantitative response to the addition of HOCl. The properties of HySOx and the main fluorescent product, $HySO_3H$, are independent of pH. Furthermore, HySOx is tolerant to autoxidation, and $HySO_3H$ is highly photostable. These excellent properties mean that HySOx should be a very useful fluorescence probe for biological phenomena, such as phagocytosis. We successfully visualized the generation of HOCl inside phagosomes in real time. HySOx will bring new insights into the behavior of HOCl in various biological phenomena.

Experimental Procedures

Materials. General chemicals were of the best grade available, supplied by Tokyo Chemical Industries, Wako Pure Chemical, Aldrich Chemical Co., Dojindo, Acros Organics, and Lancaster Synthesis, and were used without further purification. Metahnol, DMSO, DMF, acetone, acetonitrile, chloroform, dichloromethane, and diethylether used

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in spectroscopic analysis were of fluorometric grade (Dojindo). The other solvents were used after appropriate distillation or purification. Zymosan A unlabeled (*S. cerevisiae*) and opsonizing reagent were purchased from Invitrogen.

Instruments. NMR spectra were recorded on a JEOL JNM-LA300 instrument at 300 MHz for ^{1}H NMR and at 75 MHz for ^{13}C NMR. Mass spectra (MS) were measured with a JEOL JMS-T100LC AccuToF for ESI. UV−vis spectra were obtained on a Shimadzu UV-1650. Fluorescence spectroscopic studies were performed on a Hitachi F4500. The photobleaching experiment was carried out with BPS-X500B (Bunkokeiki).

Fluorometric Analysis. The slit width was 2.5 nm for both excitation and emission. The photon multiplier voltage was 700 V. Relative fluorescence quantum efficiency of TMR derivatives was obtained by comparing the area under the emission spectrum of the test sample excited at 545 nm with that of a solution of rhodamine B in EtOH, which has a quantum efficiency of 0.97.[23]

HPLC. HPLC analyses were performed on an Inertsil ODS-3 (4.6 mm × 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 mm × 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).

Fluorescence Microscopy. We used an U-LH100HG confocal laser scanning unit (Olympus) coupled to an IY81FVBF inverted microscope with a PlanApo × 60 objective lens (Oympus). The excitation wavelength was 543 nm, and the emission was filtered using a 560 nm barrier filter.

Phagocytosis. Porcine neutrophils (1×10^6 cells/mL) were seeded onto a glass-bottomed dish (Matsunami, D110110) according to the reported method.[22] Opsonized zymosan was prepared according to the instructions supplied by Invitrogen. The resulting suspension in Krebs-Ringer phosphate buffer (114 mM NaCl, 4.6 mM KCl, 2.4 mM $MgSO_4$, 1.0 mM $CaCl_2$, 15 mM NaH_2PO_4/Na_2HPO_4, pH 7.4) was diluted 50-fold. The neutrophils on the dish were loaded with HySOx (5.0 μM) by incubation for 30 min at room temperature and then washed with 0.5 mL of KRP buffer twice. Observation was started at time = 0. The fluorescence images were acquired every 15 s. Just after the first capture, the opsonized zymosan suspension (5.0 μL) was added to the dish. Each capture required 3.24 s irradiation with the excitation laser.

X-ray Crystallography. HMTMR was dissolved in a minimal amount of CH_2Cl_2. Some Et_3N was added to this solution. The resultant solution was evaporated at 200 mmHg so as to remove CH_2Cl_2, and the remaining solution was left at room temperature. The crystal was collected, washed with Et_3N, and dried, affording a colorless prism. A colorless prism crystal having approximate dimensions of 0.1 mm × 0.1 mm × 0.1 mm was mounted on a glass fiber. All measurements were made with a Rigaku RAXIS RAPID imaging plate area detector using graphite-monochromated Mo Kα radiation. Indexing was performed from three oscillations that were exposed for 90 s. The crystal-to-detector distance was 127.40 mm. The data were collected at a temperature of −174 + 1 °C to a maximum 2θ value of 55.0°. A total of 44 oscillation images was collected. A sweep of data was done using ω scans from 130.0 to 190.0° in 5.0° steps, at $\chi = 45.0°$ and $\phi = 0.0°$. The exposure rate was 120.0 s/deg. A second sweep was performed using ω scans from 0.0 to 160.0° in 5.0° steps, at $\chi = 45.0°$ and $\phi = 180.0°$. The exposure rate was 120.0 s/deg. The crystal-to-detector distance was 127.40 mm. The readout was performed in the 0.100 mm pixel mode.

Synthesis. 1-Bromo-2-[(1,1-dimethylethoxy)methyl]benzene (1a). This compound was prepared according to ref 24.

1-Bromo-2-[[(1,1-dimethylethyl)thio]methyl]benzene (1b). This compound was prepared according to ref 25.

3,6-Bis(*N*,*N*-dimethylamino)xanthone (2). A mixture of pyronin G (5.00 g), potassium cyanide (2.79 g), and water (50 mL) was refluxed for 18 h, then cooled to room temperature, filtered, and washed with water. The resulting residue was suspended in 2.0 M aq HCl (100 mL). The suspension was poured dropwise onto a solution of $FeCl_3 \cdot 6H_2O$ (13.5 g) in 2.0 M aq HCl (100 mL). The reaction mixture was heated at 90 °C for 12 h, then cooled to room temperature, filtrated, and washed well with water. The residue was suspended in saturated aq $NaHCO_3$ (500 mL), refluxed for 3 days, cooled to room temperature, filtered, and washed with water. The resulting residue was crystallized from ethanol to give slightly brown granules (1.01 g) (22%). ^{1}H NMR (300 MHz, ($CDCl_3$): δ 3.07 (12H, s), 6.46 (2H, d, J = 2.55 Hz), 6.67 (2H, dd, J = 8.97 Hz, 2.55 Hz), 8.12 (2H, d, J = 8.97 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 40.1, 97.0, 108.9, 112.0, 127.6, 154.3, 158.1. HRMS (ESI-Tof) *m*/*z* Found 283.1422 $(MH)^+$, calculated 283.1447 for $C_{17}H_{19}N_2O_2$ (−2.45 mmu).

3,6-Bis(dimethylamino)-9-[2-[(1,1-dimethylethoxy)methyl]]xanthylium Chloride (3a). A solution of **1a** (0.85 g) in THF (20 mL) was sealed in a two-necked flask under an Ar atmosphere. Then, 1.5 M *t*-butyllithium solution in *n*-pentane (3 mL) was added dropwise into the flask at −80 °C. The reaction mixture was stirred for 10 min. A solution of **2** (0.56 g) (2 mmol, 1 equiv) in THF (200 mL) was added to the flask. The mixture was brought to room temperature and stirred for 10 h. The reaction was stopped by the addition of concd HCl (3 mL). THF was removed by evaporation. The resulting red residue was dissolved in CH_2Cl_2 and washed with brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was dissolved in a minimal amount of CH_2Cl_2 and charged onto a silica gel column packed with CH_2Cl_2. The column was eluted stepwise with AcOEt, AcOEt−MeOH (10:1), CH_2Cl_2, and CH_2Cl_2−MeOH (10:1). The organic solvents were removed by evaporation to give a deep purple solid 0.45 g (48%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.78 (9H, s), 3.39 (12H, s), 4.14 (2H, s), 6.92 (2H, d, J = 2.38 Hz), 6.95 (2H, dd, J = 9.35, 2.38 Hz), 7.18 (1H, d, J = 6.97 Hz), 7.20 (2H, d, J = 9.35 Hz), 7.5 (1H, m), 7.6 (2H, m). ^{13}C NMR (75 MHz, $CDCl_3$): δ 26.9, 41.2, 62.5, 73.3, 96.7, 113.9, 114.1, 127.9, 129.1, 129.6, 130.2, 131.4, 131.9, 137.6, 157.3, 157.5, 158.6. HRMS (ESI-Tof) *m*/*z* Found 429.2570 $(M-Cl)^+$, calculated 429.2542 for $C_{28}H_{33}N_2O_2$ (2.74 mmu).

***N*,*N*,*N*′,*N*′-Tetramethyl-spiro[benzo[*c*]furan-1(3H),9′-[9H]xanthene]-3′,6′-diamine (HMTMR).** A solution of **3a** (50 mg) in 2,2,2-trifluoroethanol (3 mL) was stirred at 0 °C. To this mixture, drops of trifluoromethane sulfonate were added at 0 °C. The reaction mixture was diluted in CH_2Cl_2 and washed with brine. The organic layer was concentrated as much as possible and then charged onto a silica gel column packed with CH_2Cl_2. The column was eluted with CH_2Cl_2 to wash out the byproducts and then with AcOEt to extract the product. AcOEt was removed by evaporation, to give a slightly red solid (19 mg) (33%). Further purification was performed by recrystallization from Et_3N to give colorless prisms. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.89 (12H, s), 5.16 (2H, s), 6.39 (2H, d, J = 2.38 Hz), 6.44 (2H, dd, J = 8.80, 2.57 Hz), 6.66 (2H, d, J = 8.62 Hz), 6.72 (1H, d, J = 7.52 Hz), 7.22 (1H, t, J = 7.43 Hz), 7.33 (1H, t, J = 6.79 Hz), 7.42 (1H, d, J = 7.52 Hz). ^{13}C NMR (75 MHz, DMSO-d_6): δ 39.3, 71.0, 97.9, 108.5, 112.9, 121.0, 123.2, 127.6, 128.0, 129.2, 138.9, 145.6, 150.9, 150.9. HRMS (ESI-Tof) *m*/*z* Found 373.1879 $(MH)^+$, calculated 373.1916 for $C_{24}H_{25}N_2O_2$ (−3.67 mmu).

3,6-Bis(dimethylamino)-9-[2-[(1,1-dimethylethyl)thio]methyl]-xanthylium Chloride (3b). A solution of **1b** (0.59 g) (2.4 mmol, 4.0 equiv) in THF (25 mL) was sealed in a two-necked flask under an Ar atmosphere. A 1.5 M *t*-butyllithium solution in pentane (2 mL) (3 mmol, 5.0 equiv) was added dropwise into the flask at −80 °C, and the mixture was stirred for 10 min. A solution of **2** (0.28 g) (1 mmol, 1 equiv) in

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THF (100 mL) was added to the flask. The mixture was brought to room temperature and stirred for 10 h. The reaction was stopped by the addition of concd HCl (5 mL). THF was removed by evaporation. The resulting red residue was dissolved in CH_2Cl_2, and the solution was washed with brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was dissolved in a minimal amount of CH_2Cl_2 and charged onto a silica gel column packed with CH_2Cl_2. The column was eluted stepwise with AcOEt, AcOEt−MeOH (10:1), CH_2Cl_2, and CH_2Cl_2−MeOH (20:1). The organic solvents were removed by evaporation to give a deep purple solid (48 mg) (10%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.94 (s, 9H), 3.32 (s, 12H), 3.35 (s, 2H), 6.81 (d, 2H, J = 2.37 Hz), 6.91 (dd, 2H, J = 9.51, 2.37 Hz), 7.10 (dd, 1H, J = 7.77, 0.99 Hz), 7.14 (d, 2H, J = 9.51 Hz), 7.39 (td, 1H, J = 7.37, 1.35 Hz), 7.49 (td, 1H, J = 7.50, 1.29 Hz), 7.55 (dd, 1H, J = 7.70, 1.10 Hz). ^{13}C NMR (75 MHz, ($CDCl_3$): δ 30.1, 30.8, 41.0, 43.0, 96.5, 113.7, 114.2, 127.3, 129.3, 130.3, 130.7, 131.4, 131.7, 136.4, 157.1, 157.3, 157.4. HRMS (ESI-Tof) m/z Found 445.2279 $(M-Cl)^+$, calculated 445.2314 for $C_{28}H_{33}N_2OS$ (−3.43 mmu).

***N,N,N′,N′*-Tetramethyl-spiro[benzo[*c*]thiophene-1(3H),9′-[9H]xanthene]-3′,6′-diamine (HySOx).** Compound **3b** (48 mg) (0.10 mmol, 1 equiv) was refluxed in TFA (10 mL) for 1 day. TFA was removed by evaporation. The resulting residue was dissolved in CH_2Cl_2, and the solution was washed with brine. The organic layer was evaporated to dryness. The resulting residue was purified by silica gel column chromatography with CH_2Cl_2. Elution was carried out as fast as possible in the dark. Then, CH_2Cl_2 was removed by evaporation in the dark to give a colorless oil. After 1 day's storage at −20 °C, a colorless solid appeared. The remaining CH_2Cl_2 was removed by evaporation to give a colorless solid (24 mg) (62%). ^{1}H NMR (300 MHz, DMF-d_7/CD_3-CN = 1:1): δ 2.82 (s, 12H), 4.42 (s, 2H), 6.24 (d, 2H, J = 2.57 Hz), 6.35 (dd, 2H, J = 8.99, 2.57 Hz), 6.68 (d, 1H, J = 7.70 Hz), 6.74 (d, 2H, J = 8.99 Hz), 7.10 (t, 1H, J = 7.43 Hz), 7.19 (td, 1H, J = 7.43, 1.22 Hz), 7.34 (d, 1H, J = 7.70 Hz). ^{13}C NMR (75 MHz, DMF-d_7/CD_3CN = 1:1): δ 38.3, 40.9, 63.9, 99.2, 110.1, 116.7, 126.0, 127.5, 128.3, 128.8, 131.8, 141.4, 151.0, 152.0, 152.1. HRMS (ESI-Tof) m/z Found 389.1685 $(MH)^+$, calculated 389.1688 for $C_{24}H_{25}N_2OS$ (−0.22 mmu).

9-(2-Sulfoxymethylphenyl)-3,6-bis(dimethylamino)xanthylium Inner Salt ($HySO_3H$). Sodium hypochlorite solution (0.1 mmol) was added to a mixture of HySOx (120 mg), DMF (30 mL), and 0.1 M sodium phosphate buffer at pH 7.4 (270 mL). The mixture was stirred at room temperature, acidified with concd HCl, and saturated with NaCl. The aqueous layer was extracted with CH_2Cl_2. The organic layer was evaporated to dryness. The resulting residue was purified by silica gel column chromatography with CH_2Cl_2−MeOH (10:1). Further purification was carried out by HPLC with a linear gradient (eluent, 0 min, 8% CH_3CN/0.1% TFA aq ∼90 min, 80% CH_3CN/0.1% TFA aq; flow rate = 5.0 mL/min) to give 31 mg of a purple solid (23%). ^{1}H NMR (300 MHz, CD_2Cl_2): δ 3.27 (s, 12H), 3.72 (s, 2H), 6.77 (d, 2H, J = 2.38 Hz), 6.90 (dd, 2H, J = 9.54, 2.38 Hz), 7.14 (dd, 1H, J = 7.61, 1.19 Hz), 7.38 (d, 2H, J = 9.54 Hz), 7.45 (td, 1H, J = 7.66, 1.22 Hz), 7.61 (td, 1H, J = 7.57, 1.41 Hz), 7.99 (d, 1H, J = 7.34 Hz). ^{13}C NMR (100 MHz, CD_2Cl_2): δ 30.1, 41.1, 54.9, 70.9, 96.4, 114.1, 114.9, 127.0, 129.2, 130.1, 132.5, 132.7, 133.6, 157.8, 158.3. HRMS (ESI-Tof) m/z Found 437.1569 $(MH)^+$, calculated 437.1535 for $C_{24}H_{25}N_2O_4S$ (3.36 mmu).

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Supporting Information Available: Spectra of HMTMR, HySOx, and $HySO_3H$ at various pH's; fluorescence time course of reactions between HySOx and NaOCl; and HPLC chromatograms of HMTMR, HySOx, and $HySO_3H$ (PDF); X-ray crystallographic data for HMTMR (CIF). This material is available free of charge via the Internet at http://pubs.acs.org.

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