Fluorescent Probes

Fluorescent Probes for Hydrogen Peroxide Based on a Non-Oxidative Mechanism**

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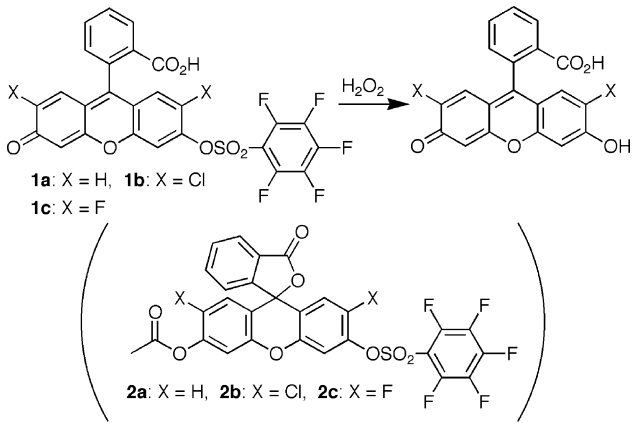
Reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^{\cdot}$) are important mediators of pathological processes in various diseases.[1] Detection by fluorescent probes is one of the most useful methods for evaluating the roles of ROS in pathological processes. 2′,7′-Dichlorofluorescin (DCFH) and its diacetyl derivative (DCFH-DA)[2] have been widely used as fluorescent probes for measuring cell-derived H_2O_2,[3] but these compounds suffer from the major drawback that they are poorly selective toward H_2O_2. Researchers have demonstrated that oxidation of DCFH to dichlorofluorescein is also induced by peroxidase[4] and other hemoproteins[5] as well as by hydroperoxides in the presence of peroxidase,[6] nitric oxide,[7] and peroxynitrite.[8] Therefore, the fluorescent response based on the oxidation of DCFH provides an index, not for cell-derived H_2O_2, but for the total oxidants present in biological systems. This limitation stems from its mechanism of fluorescence, which is based on oxidation. Dihydro derivatives of fluorescent compounds such as dihydrorhodamine 123[3c,g] and *N*-acetyl-3,7-dihydroxyphenoxazine (Amplex Red)[9] have been shown to function as probes for detecting H_2O_2. However, their mechanism of action is similar to that of DCFH, which implies that low selectivity toward H_2O_2 is a shortcoming that must be accepted when utilizing these probes. In fact, dihydrorhodamine 123 was shown to react with various ROS,[3c,7b] and although Amplex Red seems to have high selectivity toward H_2O_2, peroxidase is essential for its fluorescence, similar to the case of DCFH. Thus, developing probes for H_2O_2 based on a non-oxidative fluorescence mechanism, which would allow the highly specific and peroxidase-independent detection of H_2O_2 under the complicated oxidative circumstances found in biological systems, is a worthwhile goal.

Recently, we found that perhydrolysis of acyl resorufins is a useful reaction that acts as a fluorescent indicator for H_2O_2 assays.[10] The method is based on simple deprotection, not on oxidation, thus allowing acyl derivatives of fluorescent compounds such as resorufin and fluorescein to work as probes for detecting cell-derived H_2O_2 with higher selectively than that provided by DCFH and its analogues. Unfortunately, the competition between perhydrolysis and hydrolysis of acyl resorufins and fluoresceins in biological systems was not altered in a manner favorable towards H_2O_2-based deacylation.

We thus designed pentafluorobenzenesulfonyl fluoresceins (**1a–c**, Scheme 1) as selective fluorescent probes for



Scheme 1. Fluorescent probes and their reactions that produce the fluorescence used in this study.

H_2O_2 but would eliminate, or at least significantly reduce, competition from hydrolysis reactions of the acetyl derivatives. These compounds were chosen for the following reasons: sulfonates are more stable to hydrolysis than are esters; fluoresceins have high fluorescence quantum yields in aqueous solution; and the pentafluorobenzene ring enhances the reactivity of the sulfonates toward H_2O_2. A solution of **1a** (10 mM), **1b** (2 mM), or **1c** (2 mM) in EtOH was diluted 400 times with 2-[4-(hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer (pH 7.4, 10 mM) and the suitability of **1a–c** as probes for H_2O_2 were evaluated. The results are summarized in Table 1. As apparent from the estimated values of the relative quantum efficiencies, sulfonylation markedly quenched the fluorescence of the original fluoresceins. Compounds **1a–c** all fluoresced on reaction with H_2O_2, and perhydrolysis of **1b** and **1c** was much faster than that of **1a**. The rate constants of the reactions were comparable to or faster than those for the alkaline hydrolysis of ethyl benzoates.[12] Treatment of each of the solutions (150 μL) containing the probe compounds with H_2O_2 in water (10 μL) at 25 °C for 60 min in a 96-well microplate assay resulted in the rate of perhydrolysis of these compounds

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Table 1: Characteristics of **1a–c** as fluorescent probes for H_2O_2.[a]

	Relative quantum efficiency[b]	k for reaction with H_2O_2 [$\times 10^2$ M^{-1} s^{-1}]		Detection limit [pmol]		Decomposition [%] after 1 h in blank solution	
		25 °C	37 °C	25 °C	37 °C	25 °C	37 °C
1a	0.003	2.7	6.3	46.0	9.2	1.1	2.6
1b	0.008	14	23	23.1	231	2.8	7.7
1c	0.010	15	25	4.6	4.6	2.8	7.8

[a] All data were obtained in pH 7.4 HEPES buffer with each of the probes (**1a**: 25 μM; **1b** and **1c**: 5 μM). [b] Obtained by comparing the area under the corrected emission spectrum of the test sample at 492 nm excitation with that of a solution of fluorescein in 0.1 M sodium hydroxide, which has a quantum efficiency of 0.85 according to the literature.[11]

Table 2: Comparison of the fluorescent responses observed from the reactions of **1a–c** with various reactive oxygen species.

	Relative fluorescence intensity[a]		
	1a	**1b**	**1c**
blank	100	100	100
H_2O_2	150	248	239
HO·	82	87	89
*t*BuOOH	107	123	110
$ONOO^-$	105	124	110
NO·	117	216	155
$O_2^{-\cdot}$	141	341	324
$O_2^{-\cdot}$+catalase	91	160	127
$O_2^{-\cdot}$+SOD	134	374	341

[a] All data were obtained after incubation at 25 °C for 1 h.

producing fluorescent responses that were dependent on the concentration of H_2O_2. Linear calibration curves were obtained from the detection limits shown in Table 1 up to concentrations of 92.3 nmol, with correlation coefficients being greater than 0.997. Decomposition of **1a–c** to the corresponding fluoresceins in blank buffer solutions was relatively slow at 25 °C, but much faster at 37 °C. However, the concentration range over which **1c** functioned was the same at both temperatures, while the detection limit for **1a** was much lower at 37 °C than at 25 °C. The effect of the pH value on the reaction of **1c** with H_2O_2 was also examined. The rate of perhydrolysis of **1c** decreased strikingly below pH 6.6. However, **1c** still functioned well as a fluorescent probe at pH 6.6, although the fluorescent intensities produced were about 20 % of those observed at pH 7.4.

The fluorescent responses from the reaction of solutions of **1a** (25 μM), **1b** (5 μM), or **1c** (5 μM) in HEPES buffer (150 μL) with H_2O_2 (0.92 mM, 10 μL) in a 96-well microplate at 25 °C for 1 h were compared to those of reactions with HO·, *t*BuOOH (1 mM, 10 μL), NO, $ONOO^-$, and $O_2^{-\cdot}$. The Fenton reaction between H_2O_2 (0.92 mM, 10 μL) and Fe^{2+} ions (5 mM, 10 μL) was used as the source of HO·. The reaction with NO· or $ONOO^-$ was carried out in the presence of 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5)[13] or 3-morpholinosydnonimine (SIN-1)[14] (1 mM, 10 μL each), respectively. $O_2^{-\cdot}$ was generated by the enzymatic reaction of hypoxanthine (HPX; 1 mM, 10 μL) with xanthine oxidase (XO; 0.26 U mL^{-1}, 10 μL). The results are summarized in Table 2. The reactions of **1a–c** with HO·, *t*BuOOH, and $ONOO^-$ resulted in much smaller responses than did reactions with H_2O_2. Compounds **1a** and **1c** showed enhanced fluorescence on reaction with NO·, the extent of which was about one third of that with H_2O_2, while NO· induced a larger increase in the fluorescence response of **1b**. The fluorescent responses from the reactions of **1a–c** with enzymatically generated $O_2^{-\cdot}$ were mainly eliminated by addition of catalase (5000 U mL^{-1}, 10 μL), but was maintained or increased by the presence of superoxide dismutase (SOD; 1000 U mL^{-1}, 10 μL). These results suggest that these sulfonylated fluoresceins, especially **1a** and **1c**, act as fluorescent probes with high selectivity toward H_2O_2 over HO·, *t*BuOOH, $ONOO^-$, and $O_2^{-\cdot}$, although these probes do produce fluorescent responses toward NO· to some extent. It should be noted here that incubation of **1a–c** in the presence of horseradish peroxidase did not bring about any fluorescent responses.

Oxidative stress can be induced in green algae by incubation with suitable reagents in the light. Stimulation with Cu^{2+} ions causes intracellular formation of various ROS, such as $O_2^{-\cdot}$, H_2O_2, and HO·.[15] Cells also undergo oxidative stress upon generation of $O_2^{-\cdot}$ or $^1O_2$ through specific activation by paraquat (PQ) or methylene blue (MB), respectively.[16] Thus, experimental models using *Chlamydomonas reinharadtii*, a freshwater green alga, were informative for evaluating the applicability of the present probes to cell systems. Their acetyl derivatives **2a–c** (Scheme 1) were used to load the algal cells with **1a–c**. It was confirmed by a similar microplate assay that esterase was essential for **2a–c** to function as probes for detecting H_2O_2. In addition, these acetyl derivatives were considerably less susceptible to simple hydrolysis than **1a–c** and led to almost no fluorescent responses after incubation in blank buffer solutions, even at 37 °C. Figure 1 summarizes the results obtained when cells treated with **2a–c** (25 μM) or DCFH-DA (50 μM) for 30 minutes at 25 °C in the dark were incubated in a 96-well microplate for 60 minutes in the light or dark in the presence of Cu^{2+} ions, PQ, or MB. Fluorescent responses, which

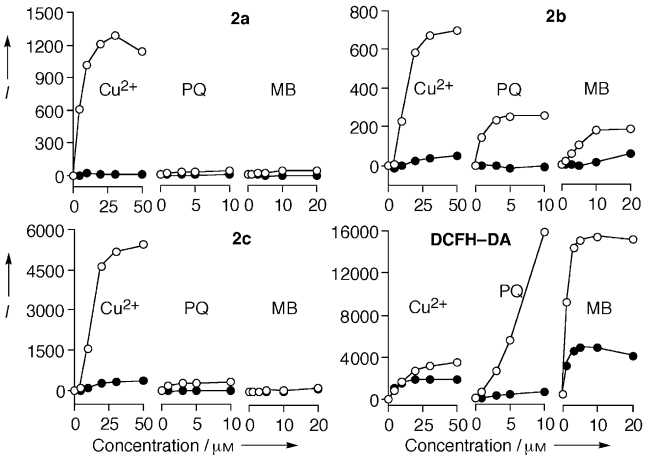

Figure 1. Fluorescence intensities *I* measured for *Chlamydomonas reinharadtii* loaded with **2a–c**, or DCFH-DA after incubation in the presence of Cu^{2+} ions, paraquat (PQ), or methylene blue (MB) in the light (○) or the dark (●) at 25 °C for 60 min.

depended on the concentration of the latter species, were only produced in the cells loaded with **2a** and **2c** upon incubation with Cu^{2+} ions in the light. When the high H_2O_2-selectivity of **1a** and **1c** is taken into consideration, these results demonstrate that **2a** and **2c** permeate the cells and are transformed into **1a** and **1c**, respectively, which then detect the oxidative stress arising from intracellular formation, not of $O_2^{-\cdot}$ and $^1O_2$, but of H_2O_2 on stimulation by Cu^{2+} ions in the light. Loading with **2b** also enabled detection of Cu^{2+}-dependent oxidative stress, but its specificity toward the stimulus was poorer than those of **2a** and **2c** for reasons that are not clear. In contrast to the actions of **2a–c**, DCFH-DA effectively detected the oxidative stress caused by PQ and MB, and also detected ROS generated on activation by Cu^{2+} ions. These results are consistent with the usefulness of DCFH as a probe for providing an index for total oxidants and thus confirming that **2**, especially **2a** or **2c**, can serve as a probe for cell systems without loss of selectivity.

These results demonstrate that **1a–c** serve as novel fluorescent probes with a non-oxidative mechanism that has a high selectivity toward H_2O_2 over $HO^{\cdot}$, *t*BuOOH, $ONOO^-$, $O_2^{-\cdot}$, and $^1O_2$. These new probes and their analogues facilitate the measurements of cell-derived H_2O_2 and elucidate the dynamic functions of oxidative stress, not only in algal cells, but also in phagocytes and vascular endothelium cells, although additional molecular design might be required for improving sensitivities toward H_2O_2. Further studies along these lines are currently under way.

Experimental Section

The syntheses of **1** and **2** are described in the Supporting Information.

Evaluation of the H_2O_2-selectivity of **2** with algal cells: The probes (**2**) were dissolved in DMSO to obtain 10 mM stock solutions. The cells of *Chlamydomonas reinhardtii* (IAM C-238), subcultured under conditions previously reported,[17] were inoculated into modified Bristol medium (MBM, 3 mL) and loaded with **2** (7.5 μL as DMSO solutions) in the dark at 25 °C for 30 min. The probe-loaded cell suspensions (50 μL) were inoculated into each well of a 96-well tissue culture plate containing solutions (50 μL) of $CuCl_2$, PQ, or MV in MBM at the indicated concentrations, and incubated in the light or the dark. The fluorescence of the cells was measured after 60 min with a CytoFluor II multiwell fluorescence plate reader (PerSeptive Biosystems Inc., USA), with excitation and emission filters set at 485 ± 20 and 530 ± 25 nm, respectively.

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