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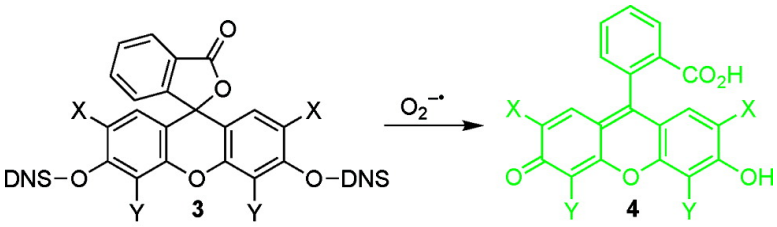

(DNS = 2,4-dinitrobenzenesulfonyl)

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A Design of Fluorescent Probes for Superoxide Based on a Nonredox Mechanism

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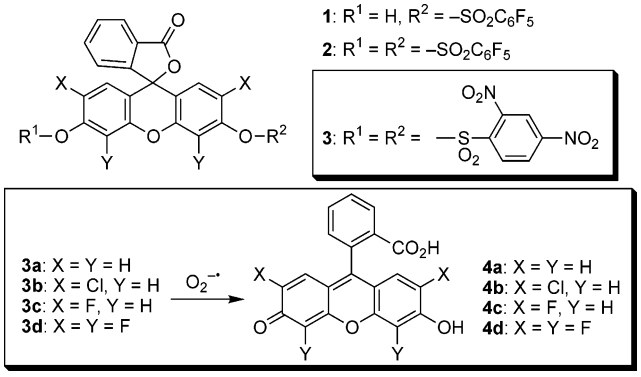
Reactive oxygen species (ROS) such as superoxide ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO•) are important mediators for the pathological conditions of various diseases.[1] Chemiluminescence- and fluorescence-based assays have been widely used to measure cell-derived $O_2^{-\bullet}$.[2] Although less sensitive than chemiluminescent methods, detecting $O_2^{-\bullet}$ using fluorescent probes takes full advantage of benefits provided by fluorescence microscopy, a microplate reader, and a cell sorter. Hydroethidine (HE) has been frequently used as a fluorescent probe.[2,3] The major drawback of HE is its poor selectivity toward $O_2^{-\bullet}$,[3b,d,4] which stems from its oxidative fluorescing mechanism. Although $O_2^{-\bullet}$ also works as a reductant, no fluorescent probe for $O_2^{-\bullet}$ based on a reductive fluorescing mechanism has been reported. This is attributed to the setback experienced in using spectrophotometric probes such as cytochrome *c* and nitroblue tetrazolium. It is well-known that these probes respond not only to $O_2^{-\bullet}$ but also to reductases.[2b,c,5] Thus, the focus in developing a fluorometric detection method of cell-derived $O_2^{-\bullet}$ is to design a fluorescing mechanism for probes without the recourse of a redox reaction. The resolution of this issue is of great importance, to impart a high degree of specificity to fluorescent probes for detecting $O_2^{-\bullet}$ in complex biological systems.

Recently it was found that H_2O_2 induced deprotection of monopentafluorobenzenesulfonyl fluoresceins (**1**) (Scheme 1), but the corresponding bis-protected derivatives **2** did not react with H_2O_2 at all. Thus, we demonstrated that **1** work as novel H_2O_2 fluorescent probes and that acetylated **1** permeate the cells of green algae and are transformed by cellular esterases into **1**, which then detect intracellular formation of H_2O_2.[6] On the basis of these findings, we further investigated what kinds of benzenesulfonyl groups would be protection groups of choice on designing sulfonylated fluoresceins as H_2O_2 probes. For this purpose, fluoresceins doubly protected with various types of benzenesulfonyl groups were chosen instead of the corresponding mono-protected ones. This is not only because the former is much more easily synthesized than the latter but also because, after screening protective groups of choice, changing their protection mode from bis- to monosulfonylated types will provide an access to sensitive fluorescent probes. When reactivity of several bis-sulfonylated fluoresceins toward $O_2^{-\bullet}$ generated by a xanthine oxidase (XO)/hypoxanthine (HPX) system in the presence or absence of superoxide dismutase (SOD) as well as H_2O_2, we found that bis(2,4-dinitrobenzenesulfonyl) fluoresceins (**3**) (Scheme 1) function as fluorescent probes not for H_2O_2 but for $O_2^{-\bullet}$. This paper proposes a novel fluorescing reaction to design fluorescent probes for specific detection of $O_2^{-\bullet}$.

Enzymatic reaction of **3**, XO, and HPX was traced fluorometrically for 10 min, yielding progressive curves (see Supporting Information). Among these probes, **3d** provided the largest fluorescent response to the enzymatic reaction. A 96-well microplate assay with this probe provided a highly sensitive method for measuring $O_2^{-\bullet}$ generated by the enzymatic reaction of HPX and XO at 37 °C for 10 min. The detection limit was 1.0 pmol (relative standard deviation, $n = 8$; 2.9%); up to 2.0 nmol of HPX, a good linear calibration curve was obtained with a correlation coefficient and slope of 0.9983 and 0.80 au/pmol, respectively.

Scheme 1



The absorption spectra observed after reaction of **3** either in the HPX/XO system or with KO_2 coincided with those of the corresponding fluoresceins **4**. Compound **3d** (50 μmol) was subjected to reaction with KO_2 (4 equiv) in DMSO−HEPES buffer (1:1) at room temperature. After 10 min, **3d** was completely consumed, and 2,4-dinitrobenzenesulfonic acid (**6**) was obtained as the sole byproduct in 191% HPLC yield. Product analysis with HPLC was also performed on a reaction mixture of **3d** (0.25 μmol) in the HPX (3 μmol)/XO (65 mU) system. After the enzymatic reaction was performed in HEPES at 37 °C for 5 h, almost no **3d** remained (about 80% of **3d** was consumed in 1 h), and two byproducts were noted on a chromatogram. One of them was **6** formed in 149% yield, and the other has not yet been identified. These results suggested that **3** is deprotected by $O_2^{-\bullet}$, leading to the formation of **4** and 2 equiv of **6**. It appeared that either electron-transfer reaction or nucleophilic substitution by $O_2^{-\bullet}$ was responsible for this transformation of **3**. On voltammetry in CH_3CN, **3a**−**3d** exhibited irreversible cathodic waves at −0.60, −0.63, −0.63, and −0.65 V vs Ag/AgCl, respectively. This small difference in the reduction potentials was unlikely to provide reasonable explanation on the observed big difference in their reactivity toward $O_2^{-\bullet}$. Thus, it was proposed that **3** is transformed to **4** by reaction with $O_2^{-\bullet}$ as a nucleophile rather than a reducing agent, which requires at least two molecules of $O_2^{-\bullet}$ for the formation of one molecule of **4** (Scheme 2).

The reactivity of **3** toward $O_2^{-\bullet}$, other ROS, reducing compounds, reductases, glutathione (GSH), and esterase was compared. GSH resulted in large fluorescent augmentation from **3a**, **3b**, and **3c** via nucleophilic aromatic substitution[7] (see Supporting Information), while **3d** provided relatively small response toward GSH in comparison with that for $O_2^{-\bullet}$ (Table 1). These results suggested

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Scheme 2. Proposed Mechanism for Deprotection of **3** to **4** by $O_2^{-\bullet}$

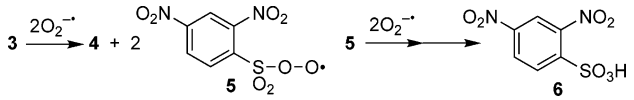

Table 1. Relative Fluorescent Intensity (RFI) Observed upon Reaction of **3d** with ROS and Other Biological Compounds

compounds	RFI[a]	compounds	RFI[a]
blank	10	NO• (NOC-5)[c]	13
$O_2^{-\bullet}$ (XO/HPX)	554	$ONOO^-$ (SIN-1)[d]	13
$O_2^{-\bullet}$ (XO/HPX)/SOD	60	ascorbic acid	11
H_2O_2	11	1,4-hydroquinone	11
t-BuOOH	10	CyP450 reductase/NADPH	104
NaOCl	12		
$^1O_2$ (H_2O_2 + NaOCl)[b]	16	diaphorase/NADH	36
HO• (H_2O_2 + Fe^{2+})	56	GSH	60
Fe^{2+}	91	esterase	15

[a] Estimated by setting the observed blank response for each probe as 10. [b−d] See refs 9, 10, and 11, respectively.

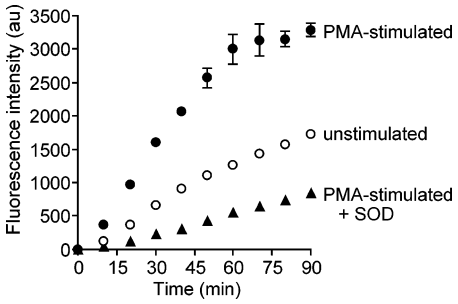

Figure 1. Time course for the change in fluorescence intensity observed with **3d** for PMA-stimulated or unstimulated human neutrophils (1×10^5 cells/well). The data points are the mean ± standard deviation ($n = 8$).

that **3a**, **3b**, and **3c** would be useless as $O_2^{-\bullet}$ probes. This is thought to be because GSH generally exists at a relatively high concentration in cells. As summarized in Table 1, **3d** provided highly specific fluorescent response toward $O_2^{-\bullet}$ over other ROS. The response observed in a Fenton system is not ascribed to HO• because Fe^{2+} itself caused deprotection of **3d**. Ascorbic acid, 1,4-dihydroquinone, and esterase induced only negligible response from **3d**. Fluorescent augmentation was also observed with the reduced forms of XO, cytochrome P450 (CyP450) reductase and diaphorase, as did GSH and Fe^{2+}. Among these reducing systems, CyP450 reductase-NADPH induced the largest response from **3d**, 19% of that observed for $O_2^{-\bullet}$. However, half of this response was thought to be a result of the generation of $O_2^{-\bullet}$ in the enzyme system, since SOD reduced the fluorescence augmentation observed for CyP450 reductase/NADPH by 50%. Thus, among the present probes, **3d** is the best fluorescent probe for detecting $O_2^{-\bullet}$ with relatively high specificity.

The applicability of **3d** as a probe for a fluorescence-based assay of cell-derived $O_2^{-\bullet}$ was demonstrated by experiments using neutrophils stimulated with phorbol myristate acetate (PMA). A cell suspension (1.0×10^6 cells/mL, 100 μL) was incubated at 37 °C with **3d** (25 μM, 50 μL) in the presence or absence of PMA (0.64 μM, 50 μL). As shown in Figure 1, the assay with **3d** provided much larger fluorescence augmentation at each measurement point for PMA-stimulated neutrophils than unstimulated cells. The responses observed for the stimulated cells were effectively reduced by addition of SOD (1000 U/mL, 10 μL). This result clearly indicated that the fluorescent responses observed with **3d** on PMA-stimulated neutrophils arise from $O_2^{-\bullet}$ released by the cells. Incremental increases in fluorescence intensity were also observed for PMA-stimulated neutrophils in the presence of SOD. This was thought to be due to endogenous thiols and reductases. The fluorescent augmentation observed for unstimulated neutrophils was ascribed not only to similar effects by these biological compounds but also to neutrophil activation by the interaction of the cells with the surface of the used tissue culture plate, as previously reported.[8]

In summary, the present work proposes a novel methodology for designing fluorescent probes for $O_2^{-\bullet}$ based on a nonredox mechanism, allowing highly specific detection of $O_2^{-\bullet}$ over other ROS. The undesired reactivity of a prototype probe **3d** toward thiols and reductases is thought to come from the 2,4-dinitrobenzenesulfonyl functionality. Thus, we expect that more practical fluorescent probes than **3d** will be developed by a judicious choice between bis and mono protection modes as well as the identities of benzenesulfonyl groups. Further studies along these lines are currently under way in our laboratory.

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Supporting Information Available: Detailed experimental procedures, spectral data of **3**, and additional data of fluorometric measurements with **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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