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Design of a Practical Fluorescent Probe for Superoxide Based on Protection–Deprotection Chemistry of Fluoresceins with Benzenesulfonyl Protecting Groups

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Abstract: A strategy for designing probes based on protection–deprotection chemistry involving fluoresceins and their benzenesulfonyl (BES) derivatives has led to the development of a much more practical superoxide ($O_2^{-\bullet}$) probe than the previously reported bis(2,4-dinitro-BES) tetrafluorofluorescein (**6a**). Examination of various BES derivatives, developed from the starting point of the prototype probe **6a**, yielded 4,5-dimethoxy-2-nitro-BES tetrafluorofluorescein (BESSo; **7j**) as the optimal reagent. A microtiter plate assay with BESSo showed a tenfold improved detection limit for $O_2^{-\bullet}$ compared with such an assay based on **6a**. BESSo showed markedly better specificity for $O_2^{-\bullet}$ than for GSH or other reactive oxygen species, and this specificity was significantly higher than that of Fe^{2+} and some reducing enzymes. These features have resulted in the development of an assay based on BESSo that is capable of providing more unambiguous results for $O_2^{-\bullet}$ release from neutrophils, with or without stimulation by phorbol myristate acetate, as compared with an assay based on **6a**. Intracellular generation of $O_2^{-\bullet}$ in human Jurkat T cells stimulated by butyric acid has been measured by using flow cytometry and fluorescence microscopy utilizing the acetoxymethyl derivative of BESSo.

Keywords: fluoresceins · fluorescence spectroscopy · fluorescent probes · molecular design · superoxide

Introduction

Reactive oxygen species (ROS) such as superoxide ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^\bullet$) are important mediators in pathological conditions caused by some 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, by detecting $O_2^{-\bullet}$ with fluorescent probes one may advantageously exploit the benefits of fluorescence microscopy, microplate readers, and cell sorters, which can supply spatial, temporal, or quantitative information. Hydroethidine (dihydroethidine, HE) has been widely used as a fluorescent probe.[2–4] The major drawback of HE is its poor selectivity for $O_2^{-\bullet}$. Oxidation of HE to fluorescent ethidium is also induced by peroxynitrite ($ONOO^-$), $HO^\bullet$, *t*BuOOH,[3d] H_2O_2 in the presence of peroxidase,[3b] and cytochrome c.[4] In addition, HE appears to catalyze the dismutation of $O_2^{-\bullet}$.[4] These reactions limit the applicability of HE as a probe for the quantitative measurement of $O_2^{-\bullet}$, which is mainly achieved through an oxidation-based fluorescing mechanism. $O_2^{-\bullet}$ acts not only as an oxidant but also as a reductant, and such a dual ability is not observed with other ROS. This suggests that a reduction-based mechanism could offer higher specificity in fluorescent probes for the detection of $O_2^{-\bullet}$ as compared with an oxidation-based mechanism. However, no fluorescent probe for detecting cell-derived $O_2^{-\bullet}$ has been developed due to difficulties encountered when using spectrophotometric probes such as cytochrome c and nitro blue tetrazolium (NBT), the absorbances of which are bathochromically shifted as a result of reduction by ascorbic acid, glutathione (GSH), and various reduc-

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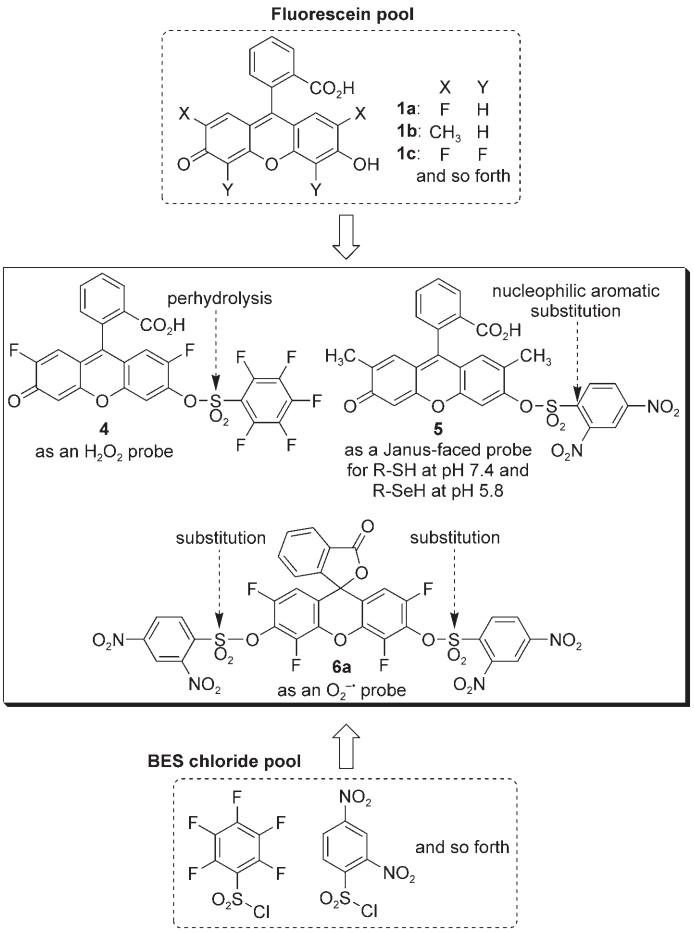
Supporting information for this article, including details of the experimental procedures, is available on the WWW under http://www.chemeurj.org/ or from the author.

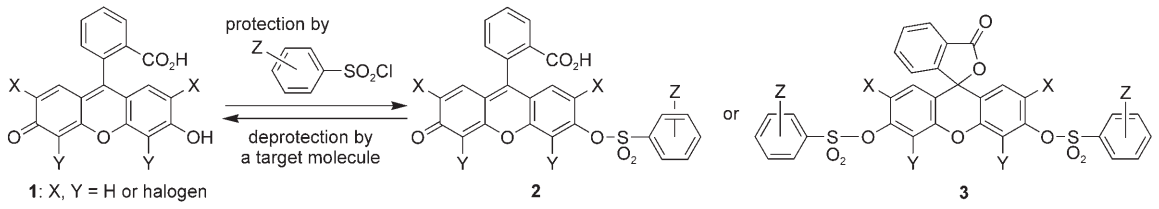
tases such as cytochrome P450 (CYP) reductase/NADPH as well as $O_2^{-\bullet}$.[2b,c,5] Thus, the focus in developing a fluorometric detection method for cell-derived $O_2^{-\bullet}$ is to design a fluorescing mechanism for probes that is not reliant on a redox reaction. The solution of this issue is of great importance. A suitable system would be expected to impart a high degree of specificity to fluorescent probes for detecting $O_2^{-\bullet}$ in complex biological systems.

Recently, we proposed a novel strategy for the design of fluorescent probes based on protection–deprotection chemistry involving fluoresceins (**1**) and their benzenesulfonyl (BES) derivatives (Scheme 1). Our strategy has stemmed from the following observations and suppositions. Compound **1** is highly fluorescent in aqueous solution, whereas **2** and **3** (protected forms of **1** with BES groups) exhibit little and no fluorescence, respectively. Thus, deprotection of **2** or **3** to yield **1**, induced by a chemical reaction highly characteristic of a certain target molecule, would allow the BES derivatives to function as fluorescent probes for the target molecule. Our strategy would thus have scope for modification of the specificity and sensitivity of the BES derivatives to be used as fluorescent probes based on a deprotection mechanism through rational molecular design. In the context of this strategy, the combination of **1** and the BES groups is likely to be the most important factor in the design of fluorescent probes, primarily because **1** and the BES groups function as leaving and electrophilic groups, respectively, upon deprotection of **2** or **3**. A matched pair of leaving and electrophilic groups is required in order to achieve specific and effective deprotection by a target molecule (i.e., to impart a high degree of specificity and sensitivity to **2** or **3** as probes for the target molecule). As general methods for the synthesis of **1**[6,7] and BES chlorides[8] have been established, a pool of compounds with diverse reactivities is available for the design of a wide variety of analogues of **2** or **3**. Our strategy to develop and tune **2** or **3** as a fluorescent probe for a target molecule takes full advantage of this fact. The choice of protection mode, that is, whether **2** or **3** is used, influences the sensitivity of the BES derivatives as fluorescent probes because mono-protected compound **2** is more susceptible to deprotection than the corresponding bis-protected compound **3**. In addition, **2** is preferred to **3** with regard to the miscibility of probe solutions in organic solvents with aqueous systems, because the mono-protected compound is less hydrophobic.

This strategy has been successfully applied in the design of novel fluorescent probes for H_2O_2,[9] thiols,[10] and selenols[11] merely by selecting the appropriate **1** and BES chlorides from pools of compounds with a wide variety of reactivities (Scheme 2). Perhydrolysis smoothly induced deprotection of **4** prepared from 2′,7′-difluorofluorescein (**1a**) and pentafluoro-BES chloride. HOO^- behaves as a stronger nucleophile than HO^- (the so-called α effect), to an extent that depends on the type of electrophile.[12–15] Among the BES derivatives examined, **4** proved to be the best substrate for perhydrolysis, although the reason for this is not well un-



Scheme 2. Fluorescent probes for hydrogen peroxide, thiols, selenols, and superoxide developed by the described optimization strategy, with indications of the initial reactions responsible for the fluorescing mechanism.



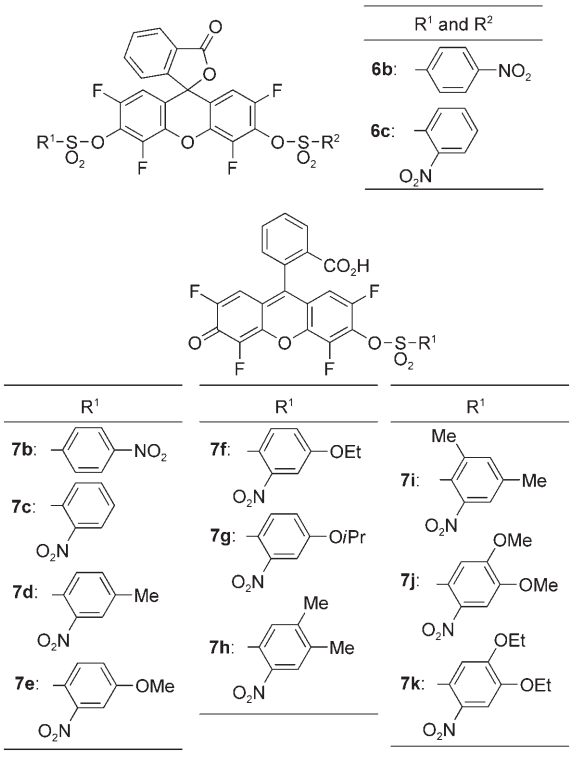
Scheme 1. Proposed strategy for the design of fluorescent probes based on protection–deprotection chemistry involving fluoresceins (**1**) and their BES derivatives (**2** or **3**).

derstood. By exploiting perhydrolysis rather than oxidation as a fluorescing reaction, **4** is capable of functioning as a fluorescent probe for H_2O_2, showing relatively high specificity and sensitivity.[9] Our strategy was also applied to the design of a red fluorescent probe for H_2O_2, namely bis(4-methyl-BES) naphthofluorescein.[16] As an alternative nonoxidative fluorescing mechanism to perhydrolysis of BES groups, it has recently been demonstrated that deprotective hydroxylation of boronates is useful for the design of H_2O_2 fluorescent probes.[17,18]

When a combination of 2′,7′-dimethylfluorescein (**1b**) and a 2,4-dinitro-BES group was selected, nucleophilic aromatic substitution[19,20] by thiols predominated over other reactions in the deprotection of the corresponding BES derivative **5**.[10] Compared with existing fluorescent[20] or chemiluminescent[21] thiol probes, the miscibility of an organic solution of **5** with an aqueous system was much better; hence **5** proved useful as a thiol probe in thiol quantification-based homogeneous assays for cholinesterase at pH 7.4. The assay with **5** worked well as a simple method for determining the inhibitory constants of inhibitors such as donepezil towards acetyl- and butyrylcholinesterases. For the design of a thiol probe based on this strategy, **1b** was preferred to fluoresceins bearing electron-withdrawing groups, such as **1a**. This is consistent with the trend in nucleophilic aromatic substitution that reaction proceeds more effectively with decreased acidity of the leaving group.[19,20] The lower acidity of **1b** relative to **1a** also helped to prevent the corresponding 2,4-dinitro-BES derivative from undergoing hydrolysis (i.e., providing large blank responses).

Interestingly, **5** has Janus-faced ability to probe selenols as well as thiols. These dual abilities of **5** can be selected merely by changing the pH of the medium. Compound **5** functions as a selenol probe at pH 5.8, facilitating not only the assay of selenocysteine, generated through in situ reduction of selenocystine by dithiothreitol, but also the measurement of selenocysteine residues in selenoproteins such as glutathione peroxidase and mammalian thioredoxin reductase after denaturing with guanidine.[11]

A fluorescent probe for $O_2^{-\bullet}$ has also been developed based on this strategy. It was found that a combination of 2′,4′,5′,7′-tetrafluorofluorescein (**1c**) and 2,4-dinitro-BES groups could be favorably employed for the deprotection of BES derivatives according to a nonredox mechanism based on nucleophilic substitution by $O_2^{-\bullet}$. In this case, bis(2,4-dinitro-BES) tetrafluorofluorescein (**6a**) showed better probe performance than the corresponding mono-protected derivative, because the latter was prone to hydrolysis, resulting in significantly high blank responses. The transformation of **6a** into **1c** represents a fluorescing reaction that is highly specific and sensitive towards $O_2^{-\bullet}$ as opposed to other ROS (Scheme 2).[23] As a result of this high specificity, **6a** can serve as a fluorescent probe for measuring extracellular $O_2^{-\bullet}$ released from neutrophils stimulated by phorbol myristate acetate (PMA). However, the specificity of this prototype probe had yet to be optimized. Fluorescence augmentation with **6a** also occurred through reaction with GSH, Fe^{2+}, or enzyme systems such as xanthine oxidase (XO)/hypoxanthine (HPX) in the presence of superoxide dismutase (SOD), CYP reductase/NADPH, and diaphorase/NADH. The extent of these reactions was 6–18% of that observed for $O_2^{-\bullet}$. These results probably preclude the use of **6a** in the specific measurement of $O_2^{-\bullet}$ in biological systems more complex than the experimental cell system. The fluorescent response towards GSH at a non-negligible level is likely to compromise the high specificity of **6a** for $O_2^{-\bullet}$ among other ROS in intracellular measurement of $O_2^{-\bullet}$ using this probe, because GSH is ubiquitous in cells at mM levels.[24] Thus, further tuning of the probe performance of **6a** through structural modification of its BES group has been conducted with a view to developing a practical $O_2^{-\bullet}$ probe that not only shows high specificity over GSH as well as other ROS, but also shows improved specificity over Fe^{2+}, CYP reductase/NADPH, and diaphorase/NADH. Based on our strategy presented herein, various BES derivatives have been designed in a stepwise manner from **6a**, and their performances as $O_2^{-\bullet}$ fluorescent probes have been examined. Our strategy based on protection–deprotection chemistry has again worked well for the rational design of fluorescent probes, and a more practical $O_2^{-\bullet}$ probe than the prototype probe **6a**, namely 2-nitro-4,5-dimethoxy-BES tetrafluorofluorescein (**7j**; BESSo), has been obtained through screening of these carefully designed candidates (Scheme 3).



Scheme 3. Probe candidates examined in this study.

Results and Discussion

2,4-Dinitro-BES versus 2- or 4-nitro-BES derivatives: In order to solve the selectivity problem of **6a**, a synthetic study employing 2,4-dinitro-, 2-nitro-, and 4-nitro-BES functionalities as protecting groups for amines was informative,[25–27] as it suggested that bis-protected derivatives of **1c** with a 2- or 4-nitro-BES group would exhibit a higher specificity for $O_2^{-\bullet}$ over GSH. To confirm this, fluorescence intensities after reaction of **6b** or **6c** with $O_2^{-\bullet}$ and GSH (final concentration 50 μM) at 37 °C for 10 min were compared with the intensity in the case of **6a**. In addition, the effect of SOD (final concentration 50 U mL^{-1}) on the fluorescence intensities after reaction with $O_2^{-\bullet}$ was also examined. All measurements were made on solutions in 96-well microtiter plates using a fluorescent plate reader, with excitation and emission wavelengths set at 505 and 544 nm, respectively. A working probe solution was prepared by diluting each probe solution (5 mM in DMSO) 200 times with pH 7.4 HEPES buffer. For the generation of $O_2^{-\bullet}$, an XO (final concentration 13 mU mL^{-1})/HPX (final concentration 50 μM) system was used. The results are summarized in Table 1, in which all fluorescent responses are shown as relative fluorescent intensities (RFI), that is, relative to the control, rather than as absolute values (FI). The control responses were obtained after merely incubating each BES derivative (final concentration 21.3 μM) in pH 7.4 HEPES buffer at 37 °C for 10 min. Quantum efficiencies (Φ_{fl}) estimated relative to the Φ_{fl} (0.85) of fluorescein in 0.1 M NaOH as a standard[28] are also included in the table. Compared with **6a**, each of the reactions led only to a small increase in fluorescence from **6b** and **6c** (entries 1–3). Although the response levels were too low to evaluate unequivocally, the results implied the following: 1) 2- and 4-nitro-BES groups are deprotected not by GSH, but by $O_2^{-\bullet}$. 2) A 2-nitro-BES group is less susceptible to deprotection by reaction with the reduced form of XO than is a 4-nitro-BES group. These implications were confirmed by similar experiments using the mono-protected compounds **7b** and **7c**. These compounds were examined to provide higher fluorescence responses toward $O_2^{-\bullet}$ than the bis-protected compounds **6b** and **6c**. Both of the mono-protected compounds did indeed show increased fluorescence upon reaction with $O_2^{-\bullet}$ relative to **6b** and **6c**. In addition, **7b** and **7c** provided FI values against $O_2^{-\bullet}$ more than five-fold greater than in the case of **6a**, although the RFI was lower than that of **6a** (entries 4 and 5). However, **7c** was superior to **7b** as a fluorescent probe for $O_2^{-\bullet}$. Compared with **7b**, **7c** exhibited higher sensitivity toward $O_2^{-\bullet}$, less susceptibility to deprotection by GSH as well as by the reduced form of XO, and a lower control response. In fact, **7c** showed no response to GSH, while providing a satisfactorily high response to $O_2^{-\bullet}$. This observation gives a strong indication of the potential utility of a 2-nitro-BES group as a protecting group in designing a practical $O_2^{-\bullet}$ probe. However, problems were encountered with **7c** compared with prototype probe **6b**: 1) a relatively higher control response, 2) a lower RFI on reaction with $O_2^{-\bullet}$, and 3) a higher response to $O_2^{-\bullet}$ in the presence of SOD.

Table 1. Relative quantum efficiencies of **6a**–**6c** and **7b**–**7k** and relative fluorescent intensities (RFI) upon reaction with $O_2^{-\bullet}$ with and without SOD and GSH.

Entry	Compound	Φ_{fl}	FI (au) for control[a]	RFI (times control)		
				$O_2^{-\bullet}$	$O_2^{-\bullet}$+SOD	GSH
1	**6a**	0	112	73.7	8.3	12.8
2	**6b**	0	51	1.8	1.6	1.0
3	**6c**	0	41	1.8	1.0	1.0
4	**7b**	0.0015	2652	15.8	13.5	1.4
5	**7c**	0.0004	1104	47.9	8.7	1.0
6	**7d**	0.0002	629	65.3	14.5	1.0
7	**7e**	0.0003	172	144.6	23.7	1.1
8	**7f**	0.0003	157	73.0	12.6	1.1
9	**7g**	0.0003	229	53.9	6.6	1.1
10	**7h**	0.0002	330	33.3	5.5	1.1
11	**7i**	0.0002	230	9.7	4.8	1.2
12	**7j**	0.0006	239	103.4	9.0	1.1
13	**7k**	0.0003	297	38.0	2.7	1.1

[a] Fluorescent intensity (FI) (control responses) observed on incubating each compound alone in pH 7.4 HEPES buffer.

2-Nitro-BES versus 4-methyl- or 4-alkoxy-2-nitro-BES derivatives: Incubating **7b** and **7c** in pH 7.4 HEPES buffer at 37 °C for 60 min resulted in 9% and 6% decomposition, respectively, to **1c**. Under similar conditions, **6a** underwent only 0.5% decomposition. These results indicate that the high control responses observed for **7b** and **7c** can be attributed to their hydrolysis to form **1c**. In general, the susceptibility of benzenesulfonates to alkaline hydrolysis decreases with increasing electron density of the BES benzene rings.[29] Therefore, modification of the 2-nitro-BES group with alkyl or alkoxy groups should prevent the high control response of **7c**. This modification can also be expected to reduce the response of **7c** to $O_2^{-\bullet}$ in the presence of SOD, because fluorescent responses of the BES derivatives to the reduced form of XO probably involve electron transfer between the BES groups and the enzyme. Such electron-transfer reactions to the BES groups will become unfavorable with an increase in the electron density of the BES benzene rings. The molecular design for imparting resistance to hydrolysis and electron transfer reactions may also decrease the fluorescent responses of BES derivatives toward $O_2^{-\bullet}$. However, achieving the highest RFI with the lowest blank response rather than the highest FI with a relatively high blank response was believed to be a more important criterion for developing a practical probe.

Methyl, methoxy, ethoxy, and isopropyloxy groups were chosen as electron-donating groups and introduced at the 4-position of the 2-nitro-BES group. The performances of **7d**–**7g** as $O_2^{-\bullet}$ probes were then evaluated as above. The results are also included in Table 1. As expected, these compounds proved to be more stable to simple hydrolysis, providing smaller control responses as compared with **7c** (entries 6–9). The alkoxy groups caused greater attenuation of the blank responses as compared with the methyl group. Compound

7e exhibited the best RFI values toward $O_2^{-\bullet}$ (entry 7). Comparing the probe performance using **7e** with that in the case of **7c**, the methoxy group on the 2-nitro-BES group reduced the blank response by 84%, while reducing the absolute FI value toward $O_2^{-\bullet}$ by only 47%. The effect of the methoxy group on FI toward $O_2^{-\bullet}$ was smaller compared with that of any other substitution, and the overall effect of the substituents on **7e** resulted in a favorable RFI value toward $O_2^{-\bullet}$ that was threefold greater than in the case of **7c**. The reactivities of **7d**–**7g** toward the reduced form of XO were not attenuated as much as the control responses. The ratios of the RFI values from reactions of **7d**–**7g** with an XO/HPX system in the presence and absence of SOD were similar to that observed for **7c**.

4-Methoxy- versus 4,5-dimethoxy-2-nitro-BES derivatives: The problems encountered with **7c** regarding its control response and the relatively low RFI on reaction with $O_2^{-\bullet}$ were solved by introducing a 4-methoxy substituent on the 2-nitro-BES group. A further attempt to attenuate the relatively high fluorescence augmentation observed on reaction of **7e** with $O_2^{-\bullet}$ in the presence of SOD was made by introducing another methoxy substituent on the 4-methoxy-2-nitro-BES group. Considering practical access to BES chlorides, a favorable position to introduce the second methoxy group was identified by comparing probe performances of dimethyl-2-nitro-BES derivatives **7h** and **7i**. As also shown in Table 1, the performance of **7i** was markedly poorer than that of **7h** (entries 10 and 11). It was considered likely that the presence of another methyl group at the 6-position of the 4-methyl-2-nitro-BES group would result in steric hindrance of the reaction with $O_2^{-\bullet}$, and that this effect would outweigh the electron-donating effect disfavoring reaction with the reduced form of XO. In contrast to the 6-position, additional introduction of a methyl group at the 5-position would reduce the response on reaction with the XO/HPX system in the presence of SOD. The response of **7h** to $O_2^{-\bullet}$ was decreased both in the absence and in the presence of SOD. However, **7h** provided a higher ratio between the RFI values for reaction with $O_2^{-\bullet}$ in the absence and presence of SOD as compared with **7d** (entries 6 and 10). These results suggest that the 4,5-positions are preferable to the 4,6-positions for the introduction of two methoxy substituents on the 2-nitro-BES group of **7c**. Accordingly, the probe performance of **7j** with a 4,5-dimethoxy-2-nitro-BES protecting group was evaluated. Its diethoxy counterpart **7k** was subjected to a similar evaluation, to confirm that a methoxy group was preferable to an ethoxy group for probe performance of 4,5-disubstituted 2-nitro-BES derivatives, as in the case of 4-substituted 2-nitro-BES compounds. Indeed, 4,5-dimethoxy substitution imparted a greater degree of probe performance compared with 4,5-diethoxy substitution. Compound **7j** showed a higher RFI toward $O_2^{-\bullet}$ than did **7k**, while these compounds produced similar ratios between the RFI values toward $O_2^{-\bullet}$ in the absence and presence of SOD (entries 12 and 13). In addition, modification of **7e** by a further methoxy substituent partially alleviated the problem encountered with **7e** caused by reactivity toward the reduced form of XO, allowing **7j** to provide superior probe performance. The $O_2^{-\bullet}$-induced RFI with **7j** was approximately 70% of that with **7e**, while **7j** allowed SOD to scavenge $O_2^{-\bullet}$ twice as effectively as **7e**. It should be mentioned here that incubating **7j** in pH 7.4 HEPES buffer at 37°C for 1 h led to only 2% decomposition to **1c**. We concluded that **7j** (BESSo) represents the optimal fluorescent probe for $O_2^{-\bullet}$ based on protection–deprotection chemistry.

Detailed probe performance of BESSo: A 96-well microtiter plate assay with BESSo (final concentration 21.3 μM) provided a highly sensitive method for measuring $O_2^{-\bullet}$ generated by an XO (final concentration 26 mU mL^{-1})/HPX system in pH 7.4 HEPES buffer at 37°C for 10 min. The detection limit corresponded to the amount of $O_2^{-\bullet}$ generated from HPX at 0.1 pmol/well (relative standard deviation (RSD), $n=8$; 3.5%), estimated as the lowest concentration affording fluorescent augmentation threefold greater than the standard deviation of blank responses. The use of BESSo instead of **6a** improved the detection limit of the fluorescent assay for $O_2^{-\bullet}$ by a factor of ten. A linear calibration curve for $O_2^{-\bullet}$ was obtained over the range from 1.0 to 1000 pmol HPX/well, with a correlation coefficient of 0.997 and a slope of 2.69 au pmol^{-1}. Although the linear concentration range produced by BESSo was similar to that obtained with **6a**, the sensitivity of BESSo toward $O_2^{-\bullet}$, as expressed by the slope of the calibration curve, was approximately threefold greater than that of **6a**.

Next, we compared the reactivities of BESSo (final concentration 21.3 μM) toward $O_2^{-\bullet}$, other ROS (final concentrations 50 μM), GSH (final concentration 50 μM), Fe^{2+} (250 μM), and reductases such as CYP reductase (final concentration 68 mU mL^{-1})/NADPH (final concentration 50 μM) and diaphorase (65 mU mL^{-1})/NADH (final concentration 50 μM) in pH 7.4 HEPES buffer. $HO^{\bullet}$, $^1O_2$, $NO^{\bullet}$, and $ONOO^-$ were generated in situ by reaction of H_2O_2 (final concentration 50 μM) and $Fe(ClO_4)_2$ (final concentration 250 μM), H_2O_2 (final concentration 50 μM) and NaOCl (final concentration 50 μM),[30] 3-(3-aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5; final concentration 50 μM),[31] and 3-morpholinosydnonimine (SIN-1; final concentration 50 μM),[32] respectively. The results are summarized in Figure 1, in which the observed fluorescent responses are shown as percentages of the fluorescence intensity in response to $O_2^{-\bullet}$ produced by BESSo (24700 au). Note that a value of 1% indicates no difference in fluorescent intensity from the blank response. The previously reported reactivities[23] of **6a** toward these compounds and enzyme systems, similarly expressed with respect to the fluorescence intensity (9000 au) toward $O_2^{-\bullet}$, are also shown in Figure 1. It can be seen that BESSo exhibited a highly specific response to $O_2^{-\bullet}$, not only over GSH, but also over H_2O_2, *t*BuOOH, NaOCl, $^1O_2$, $NO^{\bullet}$, and $ONOO^-$. The specificity of BESSo toward these ROS was even better than that of **6a**. Our previous work raised the possibility that Fe^{2+}, CYP reductase/NADPH, and diaphorase/NADH might affect the highly

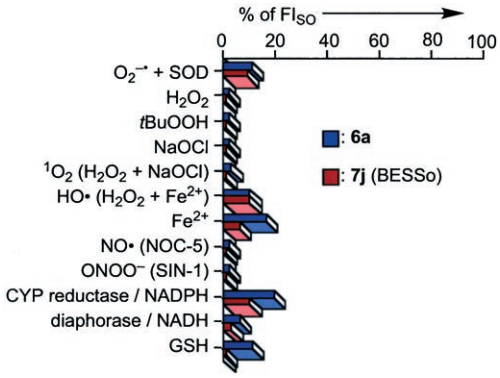


Figure 1. Comparison of fluorescence augmentations observed on reaction of BESSo or **6a** with ROS, reductases, and GSH. All data are shown as percentages of the fluorescence intensity (FI_{SO}) produced by BESSo or **6a** in response to $O_2^{-\bullet}$.

specific determination of $O_2^{-\bullet}$ by **6a**.[23] In particular, **6a** showed fluorescence augmentation in response to Fe^{2+} and CYP reductase/NADPH amounting to more than 15% of the augmentation observed in response to $O_2^{-\bullet}$. The problems associated with this reducing reagent and these enzymes were alleviated by the use of BESSo instead of **6a**. The fluorescence responses of BESSo to Fe^{2+}, CYP reductase/NADPH, and diaphorase/NADH were less than half of those seen with **6a**. The levels of fluorescence augmentation for the reactions of BESSo and **6a** with a Fenton (H_2O_2/Fe^{2+}) system were similar. The response of **6a** to a Fenton system was attributed to reaction with Fe^{2+} rather than $HO^{\bullet}$, because Fe^{2+} alone induced greater fluorescent augmentation than the Fenton system. In contrast, BESSo provided greater fluorescent intensity in response to a Fenton system than to Fe^{2+}, suggesting that in this case $HO^{\bullet}$ is responsible for the fluorescence augmentation. These results demonstrate that BESSo is a more practical $O_2^{-\bullet}$ probe than the prototype probe **6a** in terms of specificity and sensitivity. In addition, the solubility of BESSo in buffer is higher than that of **6a**: when each of the probe solutions in DMSO was diluted 200 times with pH 7.4 HEPES buffer, **6a** was almost saturated at a final concentration of around 25 μM, while the maximum final concentration of BESSo was found to be 200 μM. Although we cannot rule out the possibility that the coexistence of $HO^{\bullet}$, Fe^{2+}, and CYP reductase/NADPH might impair the specific determination of $O_2^{-\bullet}$ by BESSo, this possibility is believed to be relatively low and should not limit the application of BESSo as an $O_2^{-\bullet}$ probe. These detrimental compounds are not always present where $O_2^{-\bullet}$ is generated and the advantages of BESSo are greater than those of HE.

Analysis of mechanism and kinetics: BESSo was subjected to reaction with KO_2 (2.0 equiv) in pH 7.4 HEPES buffer. Immediately after initiation of the reaction at room temperature, the mixture turned fluorescent green and BESSo was completely consumed, as shown by HPLC analysis of the reaction mixture (Figure 2). The reaction yielded **1c** and 4,5-

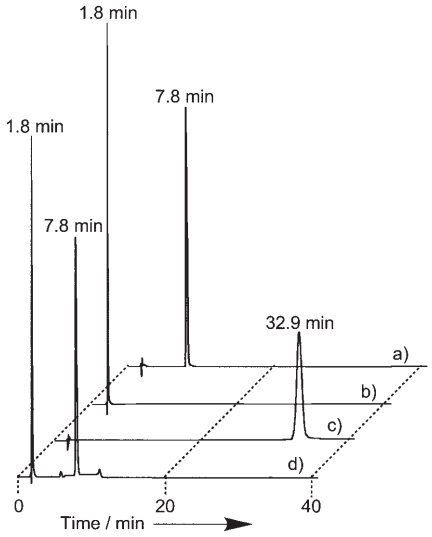
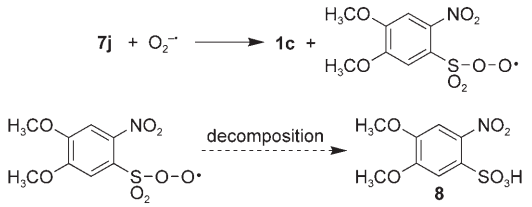


Figure 2. HPLC chromatograms of **1c** (25.2 μM) (a), **8** (24.9 μM) (b), BESSo (25.6 μM) (c), and products generated by the reaction of BESSo (25.0 μM) with KO_2 (d).

dimethoxy-2-nitrobenzenesulfonic acid (**8**) in HPLC yields of 91% and 96%, respectively. This result suggests that BESSo is deprotected through nucleophilic substitution by $O_2^{-\bullet}$, leading to the formation of **1c** and **8**, as in the case of **6a** (Scheme 4). Thus, BESSo is transformed to **1c** by reac-



Scheme 4. Proposed mechanism of deprotection of BESSo by $O_2^{-\bullet}$ to yield **1c**.

tion with $O_2^{-\bullet}$ as a nucleophile rather than as a reducing agent, which requires one equivalent of $O_2^{-\bullet}$ for the formation of one molecule of **1c**. Transformation of **6a** to **1c** requires two equivalents of $O_2^{-\bullet}$ based on a similar mechanism. Thus, BESSo is also preferred to **6a** as an $O_2^{-\bullet}$ probe with regard to stoichiometry.

The rate constant, k_{obsd}, for the conversion of BESSo to **1c** by $O_2^{-\bullet}$ was estimated by competitive kinetic analyses,[33–35] as are generally applied to determine rate constants for $O_2^{-\bullet}$ probes or scavengers. As a competitor to BESSo, SOD was used. A saturated final concentration of BESSo of 0.3 mM was used. The deployment of BESSo at this high concentration allowed negligible dismutation of $O_2^{-\bullet}$, and produced a rate of **1c** formation equivalent to that of $O_2^{-\bullet}$ formation in the absence of SOD. Based on such competitive kinetic analyses, a steady-state approximation under this assumption allowed definition of the ratio of the rates of formation of **1c** from reaction of BESSo and $O_2^{-\bullet}$ in the

absence and the presence of SOD (V_0 and V, respectively) as shown in Equation (1):

$$\frac{V_0}{V} = 1 + \frac{k_{SOD}[SOD]}{k_{obsd}[BESSo]} \quad (1)$$

in which k_{SOD} is the rate constant for the reaction of SOD with $O_2^{-\bullet}$, and [SOD] and [BESSo] are the concentrations of SOD and BESSo, respectively. According to this equation, k_{obsd} was estimated from the slope of a linear plot of V_0/V versus [SOD]. Thus, reactions of BESSo and $O_2^{-\bullet}$ in the presence of SOD at various concentrations were followed fluorometrically, affording V_0 and V values. A typical plot of V_0/V versus [SOD] is shown in Figure 3. When the reported value ($3.1\times10^9\,M^{-1}s^{-1}$)[36] of k_{SOD} was used, k_{obsd} was estimated to be $4.0\pm0.2\times10^3\,M^{-1}s^{-1}$. The rate constant for HE has been reported to be $2.6\pm0.6\times10^5\,M^{-1}s^{-1}$.[34] Although the rate constant for BESSo was thus 65 times smaller than that for HE, the specificity and sensitivity of BESSo as an $O_2^{-\bullet}$ probe are better than those of HE.

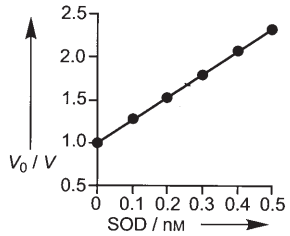


Figure 3. A typical plot of V_0/V versus concentration of SOD for estimating k_{obsd}.

Detection of extracellular $O_2^{-\bullet}$: The usefulness of BESSo as a probe for fluorescence-based assays of extracellular $O_2^{-\bullet}$ was compared with that of **6a** in experiments using neutrophils stimulated with PMA. A cell suspension (1.0×10^5 cells per well) was incubated at 37 °C with BESSo or **6a** in the presence or absence of PMA. As shown in Figure 4, an assay with **6a** or BESSo produced greater fluorescence in response to PMA-stimulated neutrophils than in response to unstimulated cells 10 min after incubation. The responses observed to the stimulated cells with both probes were reduced upon addition of SOD. These results indicate that the fluorescence responses observed with **6a** and BESSo to PMA-stimulated neutrophils result from $O_2^{-\bullet}$ release. However, the differences between fluorescence augmentation from stimulated and unstimulated cells, and from stimulated cells in the absence and presence of SOD, were larger with BESSo than with **6a**. Thus, BESSo showed improved specificity and sensitivity and hence represents a more practical probe than **6a** for fluorescent assays of extracellular $O_2^{-\bullet}$.

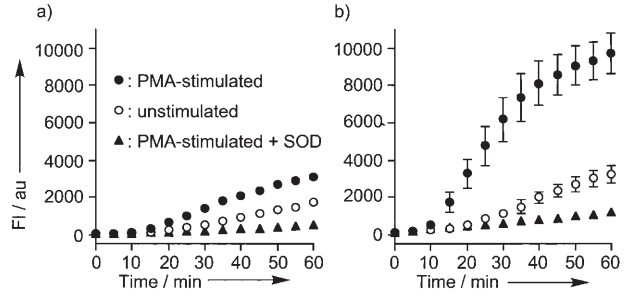
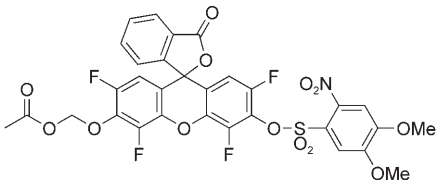


Figure 4. Temporal changes in fluorescence intensities (FI) observed with **6a** (a) and BESSo (b) for PMA-stimulated or unstimulated human neutrophils (1×10^5 cells per well). Data are expressed as mean ± standard deviations ($n=8$).

Detection of intracellular $O_2^{-\bullet}$: We also applied BESSo in the detection of intracellular $O_2^{-\bullet}$ generation. For this purpose, its acetoxymethyl derivative (BESSo-AM) was synthesized, which, it was envisaged, would be better able to permeate into cells than BESSo itself, wherein it would be transformed into BESSo by the action of intracellular esterase. The maximum final concentration of a BESSo-AM working solution was about 50 μM when prepared by diluting a stock solution in DMSO 150 or 200 times with buffer. Jurkat T cells were shown to undergo apoptosis upon treatment with butyric acid, which induces the production of ROS as well as ceramide in the cytosol.[37] Flow cytometry and fluorescence microscopy with BESSo-AM clearly indicated that the intracellular $O_2^{-\bullet}$ levels in Jurkat T cells became high when the cells were stimulated with butyric acid. Jurkat T cells (2×10^6 cells mL^{-1}, 500 μL) were incubated with BESSo-AM (final concentration 33 μM) at 37 °C for 1 h. The probe-loaded cells were further incubated in the presence or absence of butyric acid (final concentration 5 mM) at 37 °C for 1 h, and were then subjected to the fluorescence-based analysis for $O_2^{-\bullet}$. Flow cytometric measurements yielded 793 and 331 au as the mean fluorescence intensity values observed for stimulated and unstimulated cells, respectively. When cells were loaded with 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron, a cell-permeable $O_2^{-\bullet}$ scavenger) as well as BESSo-AM, and then subjected to stimulation with butyric acid, the mean fluorescence intensity value was reduced to 482 au. The phenomena observed by flow cytometry of Jurkat T cells could be clearly visualized by means of fluorescence microscopy. Representative phase contrast and fluorescence images obtained are shown in Figure 5. Stimulation with butyric acid increased the number of cells stained by the fluorescent product **1c**.



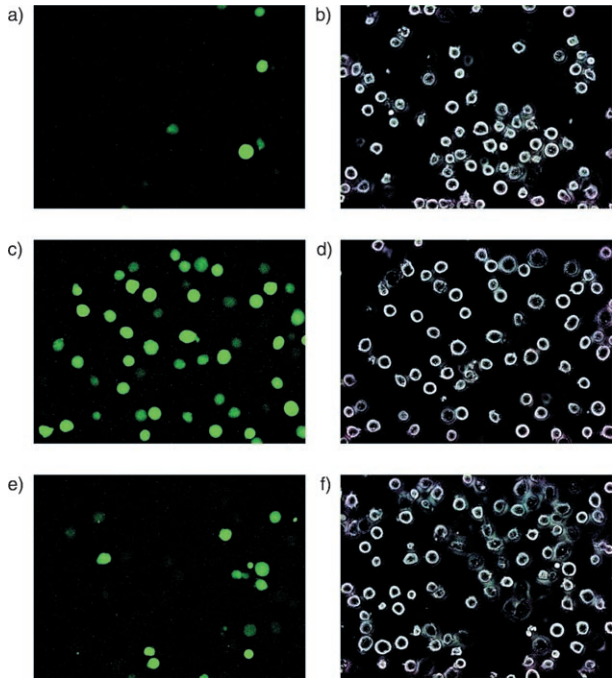


Figure 5. Phase contrast (b, d, f) and fluorescence images (a, c, e) obtained after incubating human Jurkat T cells loaded with BESSo-AM at 37 °C for 1 h in the absence (a, b) or the presence (c–f) of 5 mM butyric acid. (e, f) Cells loaded with Tiron as well as BESSo-AM were used.

Tiron markedly inhibited cell staining induced by stimulation with butyric acid. Since Tiron clearly functioned as an intracellular scavenger of $O_2^{-\bullet}$ in these experiments, the results demonstrate that BESSo-AM is capable of serving as a fluorescent probe for the detection of intracellular $O_2^{-\bullet}$. This intracellular assay with BESSo-AM thus revealed that ROS production in the cytosol of Jurkat T cells originates from the generation of $O_2^{-\bullet}$.

Conclusion

We have tested bis- and mono-protected derivatives of **1c** with a number of BES groups, which were selected not only to eliminate or significantly reduce undesired reactivity of the prototype $O_2^{-\bullet}$ probe **6a**, but also to validate our strategy based on protection–deprotection chemistry as a novel concept for probe design. Of the BES derivatives of **1c** that were examined, the 4,5-dimethoxy-2-nitro-BES derivative (BESSo) proved to be the best $O_2^{-\bullet}$ probe. Thus, compared with **6a**, BESSo allowed the measurement of $O_2^{-\bullet}$ with greater sensitivity, and exhibited greater specificity toward $O_2^{-\bullet}$ relative to GSH and to ROS such as H_2O_2, NaOCl, *t*BuOOH, $^1O_2$, NO$^\bullet$, and $ONOO^-$. The use of BESSo also significantly improved specificity toward $O_2^{-\bullet}$ over Fe^{2+} as well as over the reduced forms of CYP reductase and diaphorase. In particular, BESSo exhibited no fluorescent response to GSH, which is ubiquitous in cells at mM levels. These features allow BESSo and BESSo-AM to serve as fluorescent probes useful for extra- and intracellularly generated $O_2^{-\bullet}$, respectively.

This study has also confirmed the utility of a strategy based on protection–deprotection chemistry for the design of fluorescent probes, which would be difficult to achieve by other available methodologies. This strategy can provide novel fluorescent probes with tunable sensitivities and specificities by the appropriate selection of fluoresceins and BES chlorides, rather than by using totally different chemical structures. The pools of available fluoresceins and BES chlorides from which one may choose are diverse. This raises the possibility that this strategy may be more generally applied for the design of fluorescent probes for target molecules that show specific reactivity in inducing the deprotection of BES derivatives. Additional studies can be expected to result in the development of novel fluorescent probes for other target molecules.

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