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Novel bisbenzimide-nitroxides for nuclear redox imaging in living cells

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ABSTRACT

Nuclear oxidative stress damages genomic DNA and may lead to cell death, leading to aging and aging-related disorders. Though it is important to measure the nuclear oxidative stress separately, there are still little examples that applicable to living cells. We have designed and synthesized three bisbenzimide-nitroxides as probes to selectively visualize nuclear redox changes in terms of fluorescence. Compound **3**, containing two radical moieties, showed the largest reduction-induced fluorescence change, with good localization in nuclei. RAW264.7 murine macrophage cells were loaded with compound **3** and then treated with 100 μM hydrogen peroxide for 5 min to show the fluorescence increase. This fluorescence increase was inhibited by pretreatment of 1 mM ascorbic acid. These results show that compound **3** was suitable for nuclear-specific redox imaging in murine macrophages.

Oxidative stress is usually caused by overproduction or accumulation of reactive oxygen species (ROS). Although ROS plays a variety of beneficial roles in biological processes, including signaling and immune response,[1–4] an inappropriate concentration or location of ROS may result in oxidative modification of biomolecules such as membrane components,[5] proteins[6] and DNA.[7] Accumulation of such oxidative damage is considered to be associated with aging[8] and aging-related disorders, including carcinogenesis,[9,10] ischemia[11] and neurological disorders.[12–15] Therefore, determination of subcellular oxidative stress levels has potential value for the diagnosis and treatment of such disorders.

In particular, oxidative stress in nuclei is critical, because it may induce DNA damage.[16] The relationship between genomic DNA damage and ROS-mediated disorders has usually been studied by extracting and quantitating oxidatively modified nucleic acids from cells, serum or urine.[17–19] However, oxidized nucleic acids are also produced from the cytoplasmic nucleotide pool,[20,21] so it is difficult to selectively estimate nuclear oxidative stress in the living body.

Recently, several groups have developed functionalized nuclear-localizing molecules[22–24] by utilizing a nuclear localization signal peptide or genetic engineering, but there are still few examples of theoretically designed small molecular organelle-specific probes.

We have recently reported organelle-specific electron spin resonance (ESR) probes localizing to membrane[25] or mitochondria[26] or nuclei.[27] These probes are nitroxide derivatives bearing a localizing moiety and a fluorescent tag allows visualization of each probe's distribution in cells by microscopic imaging. Those ESR probes can be used to estimate the organelle-specific redox state in living cells by observing the ESR signal decay rate. As a next step, we aimed to use a similar strategy to develop fluorophore-nitroxide probes.

Fluorophores lose their fluorescence upon interaction with an adjacent radical species[28] via electron transfer from the excited state of the fluorophore to the radical moiety. On the other hand, nitroxide is gradually reduced by reductants such as ascorbic acid in biological systems, forming hydroxylamine, and this results in loss of the fluorescence-quenching effect of the radical.

Based on these phenomena, we designed novel bisbenzimide-nitroxide derivatives **1**–**3** (Fig. 1) as candidate nuclear-localizing redox probes. Bisbenzimide is the fundamental structure of the widely used nuclear stain Hoechst 33342, which has both DNA-binding ability and blue fluorescence.[29]

Syntheses of these compounds are summarized in Schemes 1 and 2. Compound **1** was prepared by repeated cyclization of aldehyde with phenylenediamine derivatives, followed by coupling with 4-aminoTEMPO. Compounds **2** and **3** were prepared by heterocyclization of the common intermediate **13** with each phenylenediamine.

As these bisbenzimide-nitroxides contain a radical moiety, we were not able to obtain ^{1}H or ^{13}C NMR spectra due to shortening of relaxation time. Therefore, nitroxide-containing compounds were characterized by NMR after addition of phenylhydrazine as a reductant.

We evaluated the spectral characteristics and responses of 10 μM bisbenzimide-nitroxides to nitroxide reduction by 100 μM

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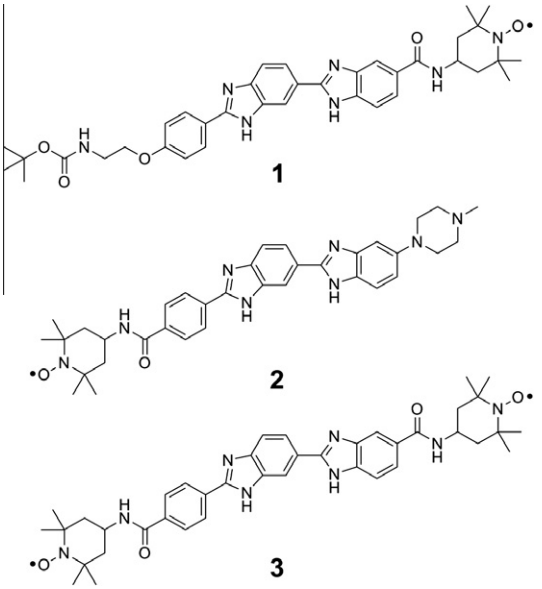


Figure 1. Structures of novel bisbenzimide-nitroxides **1**, **2** and **3**.

ascorbic acid in DMSO/D-PBS (9/1) solution. The fluorescence characteristics of these compounds were similar to those of Hoechst 33342 (λ_{ex} = 352 nm, λ_{em} = 451 nm; **1**: λ_{ex} = 350 nm, λ_{em} = 378 nm; **2**: λ_{ex} = 320 nm, λ_{em} = 390 nm; **3**: λ_{ex} = 350 nm, λ_{em} = 425 nm). Disappearance of the radical was confirmed by ESR observation. The ESR signal intensity of these compounds decreased with time, becoming constant within 15 min. The intensity was decreased to 6–14% of the initial level in presence of 100 μM ascorbic acid.

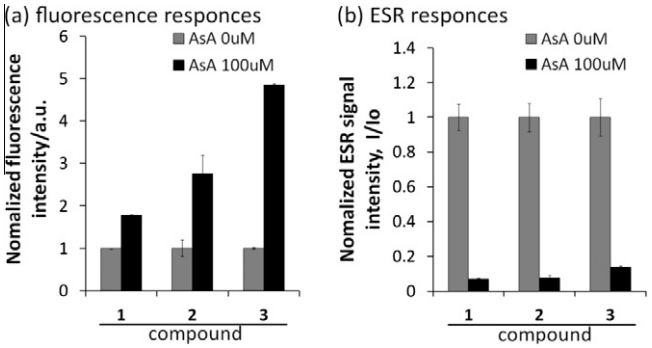


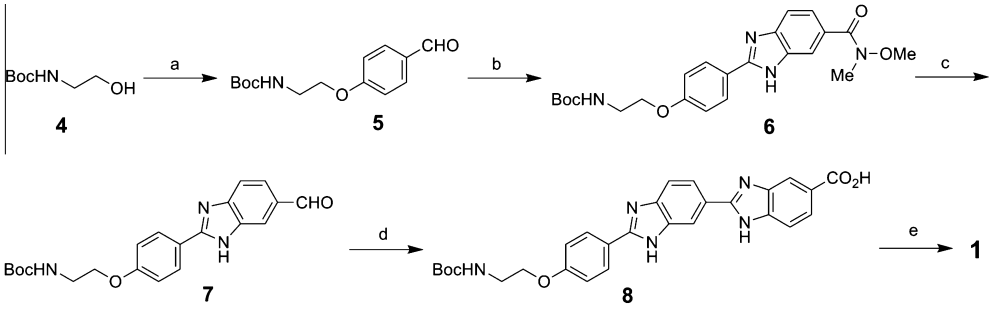
Figure 2. Fluorescence and ESR responses of 10 μM bisbenzimide-nitroxides to 100 μM ascorbic acid (AsA) in DMSO/D-PBS (9/1) solution. Values are presented as the means ± SD of three experiments.

Subsequent reduction induced a fluorescence recovery of 180–490% (Fig. 2).

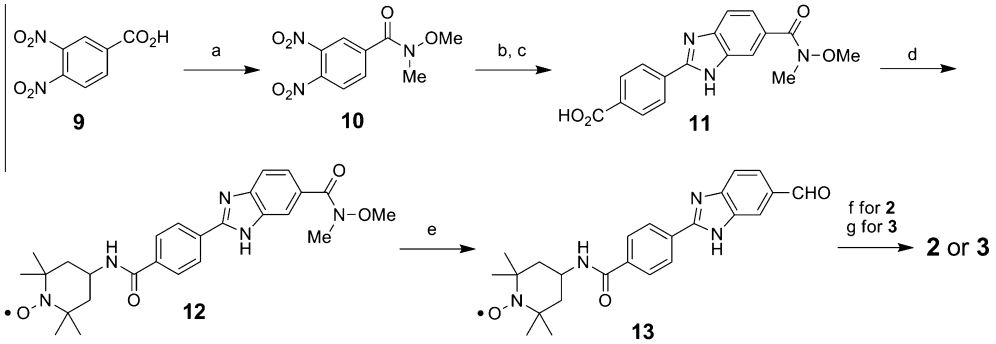
The double-nitroxide compound **3** showed the largest fluorescence recovery, despite having the smallest reduction range in ESR. It is possible that the multiple nitroxide structures work synergistically to induce a stronger fluorescence-quenching effect.

The nuclear stain Hoechst 33342 shows an increase of fluorescence on binding to DNA. Thus, our bisbenzimide-nitroxides, which are based on the same structure, may also exhibit changes of fluorescence intensity in response to factors other than reduction of nitroxide. Therefore, we investigated the influence of DNA on bisbenzimide-nitroxide fluorescence.

A buffered solution of **3** (10 μM in TNE-buffered solution containing 0.1% DMSO) was subjected to fluorescence spectroscopy in presence of 0–1000 μM ascorbic acid or 1 μg/mL DNA (Fig. 3).



Scheme 1. Synthesis of **1**. Reagents and conditions: (a) 2-(Boc-amino)ethanol, DEAD, PPh_3, THF, rt, 12 h, 52%; (b) 3,4-diaminobenzoic acid *N*,*O*-dimethylhydroxamide, $Na_2S_2O_5$, EtOH, reflux, 2 h, 95%; (c) DIBAL, THF, −40 to 0 °C, 69%; (d) 3,4-diaminobenzoic acid, $Na_2S_2O_5$, EtOH, reflux, 12 h, 99%; (e) 4-amino-TEMPO, EDCI, HOBt, DMF, rt, 30 h, 64%.



Scheme 2. Syntheses of **2** and **3**. Reagents and conditions: (a) oxalyl chloride, cat. DMF, CH_2Cl_2, rt, 1 h, then *N*,*O*-dimethylhydroxylamine, Et_3N, CH_2Cl_2, rt, 30 min, 98%; (b) H_2, 5% Pd/C, MeOH, rt, 40 h; (c) terephthalaldehydic acid, $Na_2S_2O_5$, EtOH, reflux, 6 h, 81%; (d) 4-amino-TEMPO, EDCI, HOBt, DMF, rt, 5 h, 95%; (e) $LiAlH_4$, THF, −78 °C to rt, 19 h, 54%; (f) 4-(4-methylpiperazin-1-yl)-1,2-phenylenediamine, EtOH, reflux, 38 h, 18%; (g) 4-(3,4-diaminobenzoylamino)-TEMPO, EtOH, reflux, 6.5 h, 48%.

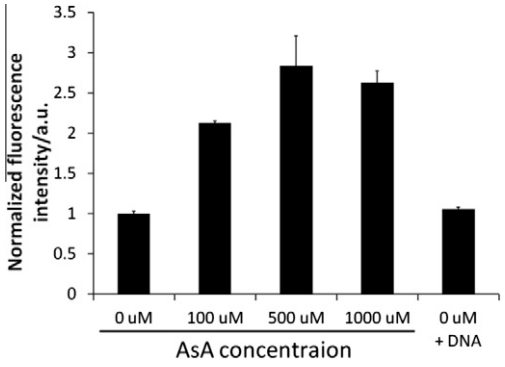


Figure 3. Fluorescence responses of 10 μM compound **3** to various concentrations of ascorbic acid or 1 μg/mL DNA in TNE-buffered solution. Values are presented as the means ± SD of three experiments.

The fluorescence intensity of **3** showed a concentration-dependent increase of up to 2.8-fold in the range of 0–500 μM ascorbic acid, while the fluorescence change induced by DNA was negligible, indicating that existence of DNA does not contribute to the fluorescence increase of **3**.

Next, we examined the suitability of compound **3** for nuclear-specific redox imaging in living cells. Cells of the RAW264.7 murine macrophage cell line were treated with 50 μM compound **3** for 24 h, and then observed by confocal microscopy. All images were created by merging of images of the same area in different focal planes. The cells may not move only horizontally but also change its shape vertically during the observation. To avoid the fluorescence changes due to such vertical movement of the cells, we used total fluorescence intensity from the stacked images of different focus planes.

Cell nuclei were counterstained with 5 μM SYTO Green 11 Nucleic Acid Stain. Compound **3** was reduced by intracellular ascorbic acid, resulting in an increase of the fluorescence level, which allowed distribution of the dye to be observed. Blue fluorescence of compound **3** was colocalized with green fluorescence of SYTO Green 11 (Fig. 4). It was shown from the results in Figure 3 that the fluorescence of compound **3** did not affected by DNA. This means that the compound **3** shows DNA-independent fluorescence although Hoechst 33342 shows DNA-dependent fluorescence. We only observed nuclear fluorescence staining with compound **3**, meaning that the compound **3** is distributed predominantly in nuclei in Figure 4.

To observe the fluorescence change in living cells, the cells were treated with 50 μM compound **3** for 3 h, then were exposed to ROS by treatment with 100 μM hydrogen peroxide (H_2O_2). Blue fluorescence of compound **3** was increased after 5 min exposure to H_2O_2 (Fig. 5a) and this increase was suppressed by pretreatment with 1 mM ascorbic acid (Fig. 5b). The frequency–brightness relationship shows that the number of bright cells increased with a corresponding decrease of dark cells (Fig. 5c). The median brightness of H_2O_2 treated cells were raised from 76.7 to 106.6. On the

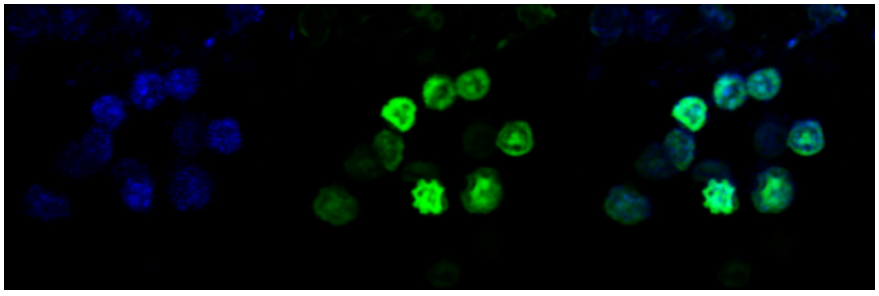


Figure 4. Distribution of 50 μM compound **3** in RAW 264.7 cells (left). The cell nuclei were stained with 5 μM SYTO Green 11 Nucleic Acid Stain (center).

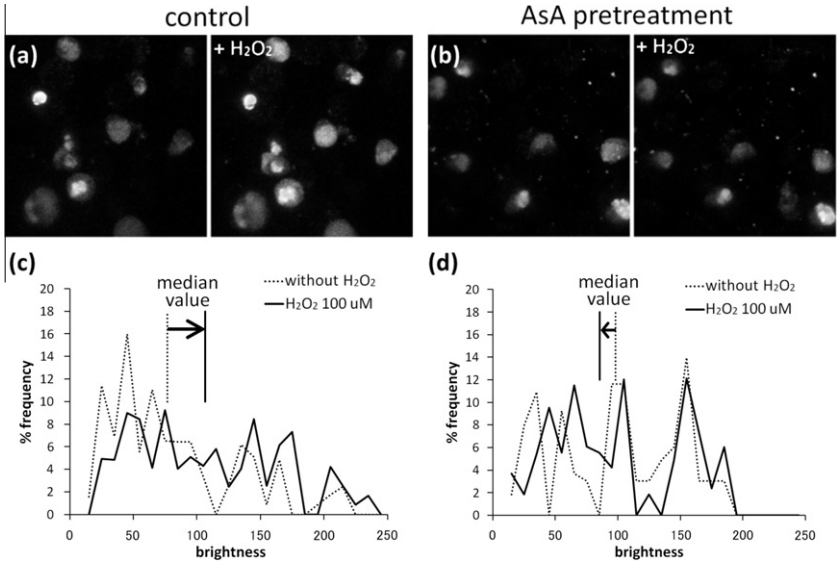


Figure 5. (a) Fluorescence changes of 50 μM compound **3** before (left) and after (right) addition of 100 μM H_2O_2 in RAW 264.7 cells. (b) Same as (a) except for pretreatment with 1 mM AsA. (c) Relationship between fluorescence brightness of the cell and frequency. (d) Same as (c) except for pretreatment with 1 mM AsA. The median brightness was shown as vertical solid/dashed line in (c) and (d). Frequency was calculated from cell number in arbitrary areas of three dishes.

other hand, ascorbic acid pretreated cells did not show significant fluorescence increase (Fig. 5b). The difference of frequency-brightness relationship before and after addition of H_2O_2 was not noticeable, showing the slight decrease of the median brightness from 98.2 to 85.6 by addition of H_2O_2.

H_2O_2 is known to generate hydroxyl radical by Fenton reaction with biological Fe(II) ion.[30] Nitroxide can be oxidized by hydroxyl radical, forming oxoammonium.[31] This oxoammonium form is easily reduced by two-electron reductants. Compound **3** was considered to have been oxidized by hydroxyl radical generated from H_2O_2, and subsequent two-electron reduction results in loss of the paramagnetic property. In the case of pretreatment with ascorbic acid, H_2O_2 or other ROS are trapped by the excess amount of ascorbic acid, so that the redox level does not change. Even though TEMPO can also lose its paramagnetic property by the reduction with cellular reductant such as ascorbic acid directly, TEMPO is relatively rapidly reduced by such reductants and reached its redox equilibrium status. In the experiments, the ESR signal intensity of compound **3** was found to become stable before the oxidant treatment. It was considered that the effect of the initial reduction by intracellular ascorbic acid and other reductants was canceled in our experimental conditions.

In conclusion, compound **3** provided the largest fluorescence recovery among the three novel bisbenzimide-nitroxides we synthesized. This fluorescence increase was ascorbic acid concentration-dependent and was not affected by the presence of DNA. Compound **3** was localized in cell nuclei and showed s substantial fluorescence increase upon exposure to 100 μM H_2O_2. Our results indicate that compound **3** can be used as a probe to visualize the redox state in nuclei of living cells.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2012.01.042.

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