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Synthesis and properties of fluorescence probe for detection of peroxides in mitochondria

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ABSTRACT

In this study, a new type of fluorescence probe, diphenylpyrenylphosphine-conjugated alkyltriphenylphosphonium iodide which was accumulated in mitochondria, has been synthesized. This probe was detected peroxide in living cell. Comparison of the reactivity toward several peroxide indicated that the probe was existed in mitochondrial membrane. Using this probe, generation of peroxide in mitochondria of living cell was successfully visualized.

Mitochondria are functionally important subcellular organelles; they are a major source of reactive oxygen species (ROS) in mammalian cells and are major targets of oxidative damage. Such damage to mitochondria has serious consequences such as cell death caused by the termination of energy generation. A large number of studies have been conducted on this topic.[1] Primarily, the mitochondrial respiratory chain produces superoxide anions ($O_2^{\cdot-}$), which in turn react to form further reactive oxidants. Types of damages caused by ROS to mitochondrial components include lipid peroxidation, protein oxidation, and mitochondrial DNA mutation.[2,3] The lipid peroxidation of polyunsaturated fatty acids (PUFAs) incorporated in the mitochondrial membrane is one of the most dangerous oxidative stresses, which induces a mitochondrial permeability transition.[4–6] In order to detect this stress, the development of visualizable tools for microscopy is necessary. Although a rhodamine-based fluorescence probe has been reported for the selective detection of highly reactive oxygen species, to the best of our knowledge, no studies have been conducted for the detection of peroxides in mitochondria.[7,8] Triarylphosphines are highly reactive with peroxides but not with $O_2^{\cdot-}$. Diphenylpyrenylphosphine (DPPP) has potential utility in the detection of peroxides because phosphine is easily oxidized to yield phosphine oxide, which has high fluorescence intensity.[9] However, as these compounds do not significantly accumulate within mitochondria, their effectiveness remains limited. It has been reported that compounds that conjugate to a triphenylphosphonium cation are preferentially taken up by mitochondria. Lipophilic cations penetrated through the lipid bilayer; this is because the positive charge is dispersed over a large surface area and the potential gradient enables their accumulation into the matrix.[10–12] For the detection of lipid peroxides in mitochondria, we prepare a diarylpyrenylphosphine-conjugated alkyltriphenylphosphonium moiety as a functional group for binding the moiety to the mitochondrial membrane. Here, we discuss the localization of a fluorescence probe to mitochondria by employing fluorescence microscopy and reactivity; this probe is used for the detection of several peroxides in the mitochondrial matrix.

The fluorescence probe [3-(4-phenoxyphenylpyrenylphosphino)propyl]triphenylphosphonium iodide (MitoDPPP) was synthesized through the six steps. [(4-Methoxy)phenyl]phenyl pyrenylphosphine (**1**) was prepared from dichlorophenylphosphine and anysyl magnesium bromide. The phosphine **1** was oxidized to give corresponding phosphine oxide **2** in 97% yield. The phosphine oxide **2** was converted to [4-(hydroxyl)phenyl]phenylpyrenylphosphine borane (**4**) via reduction of phosphine oxide **3** and hydroboration in 75% yield. The phosphine borane was conjugated to 3-iodopropyltriphenylphosphonium iodide (**6**) followed by deprotection with Et_2NH to give MitoDPPP in 18% yield (Scheme 1). The fluorescence intensity of MitoDPPP is low due to the intramolecular quenching of the phosphinyl moiety.

The oxidation of MitoDPPP with methyl linoleate hydroperoxide (MeLOOH), cumene hydroperoxide (CumOOH), *tert*-butyl hydroperoxide (*t*-BHP) and H_2O_2 proceeded in methanol to give phosphine oxide (MitoDPPPO), whose fluorescence (λ_{ex} 350 nm, λ_{em} 380 nm) was 35 times higher than that of MitoDPPP (Scheme 2).

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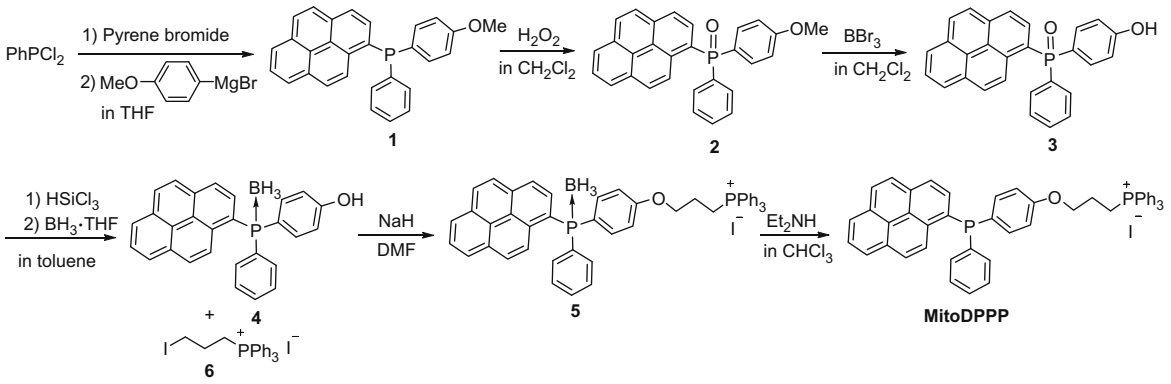


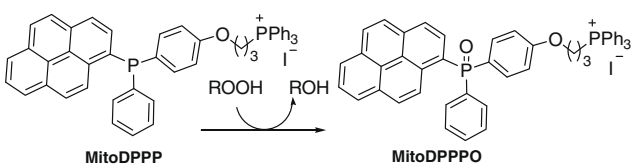
Scheme 1. Synthesis of MitoDPPP.

MitoDPPP is oxidized by peroxide but not by free radicals such as hydroxyl radicals (OH·) or $O_2^{\cdot-}$.[13] The reactivity of MitoDPPP was similar to that of diphenylpyrenylphosphine (DPPP).[14] The reactivity of DPPP toward hydroperoxides in homogeneous solution have been reported to depend on the steric factor around the hydroperoxyl group. Nevertheless CumOOH has sterically crowded group, the reactivity was higher than that of *t*-BHP. Thus the reactivity of peroxides in homogeneous solution was not only dominated by steric factor of the peroxide but also polarity and solubility (Fig. 1).

On the other hand, in liposome suspension, MitoDPPP is supposed to localize within lipid membranes because of preferential reaction with lipophilic hydroperoxides such as MeLOOH and CumOOH. Due to poor lipophilicity of H_2O_2, the reactivity was lower than that in methanol. The oxidation of MitoDPPP with H_2O_2 was moderately increased by addition of Fe^{2+} as a catalyst for Fenton reaction. The hydroxyl radical will be able to oxidize unsaturated olefin in liposome. The result indicates that the oxidation of liposome was slight in this condition (Fig. 2).

The localization of MitoDPPP was confirmed by comparing the fluorescent microscopy results of human hepatoma HepG2 cells obtained by staining MitoRed, a rhodamine-based mitochondrial dye, with those of the HepG2 cells obtained by staining MitoDPPPO. The cells were plated on a glass cover slip that was set in Dulbecco's modified Eagle's medium (DMEM) supplemented with serum and maintained at 37 °C for 24 h, as described in a previous study.[15] The cells were treated with 1.6 μM of MitoDPPP and 10 nM of MitoRed for 15 min under dark conditions, followed by washing twice with a DPBS buffer and treatment with 1.0 mM of *t*-BHP. The two dyes merged well, which indicated that MitoDPPP was selectively localized in mitochondria (Fig. 3).

Next, we attempted the detection of peroxides in the mitochondria of living cells by employing fluorescence microscopy. The HepG2 cells were treated with 1.6 μM of MitoDPPP for 15 min under dark conditions, followed by washing twice with the PBS buffer. The cells were subsequently treated with 1.0 mM of peroxides or 10 μM of MeLOOH for 15 min. A comparison of the brightness of the cells subjected to peroxide treatment with that of the untreated cells, where the brightness of both these types



Scheme 2. Oxidation of MitoDPPP with peroxide.

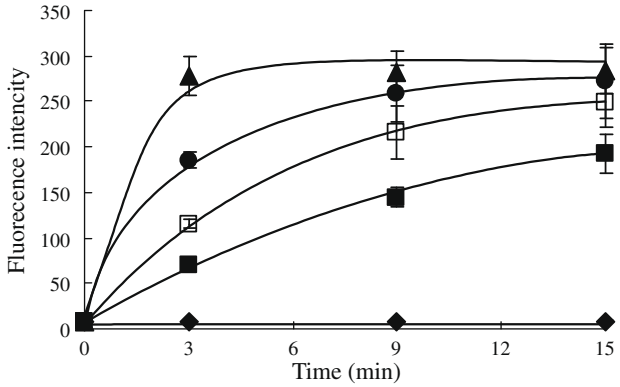


Figure 1. Reaction of ROOH with MitoDPPP in methanol; ● CumOOH 1.0 mM, ■ *t*-BHP 1.0 mM, □ H_2O_2 1.0 mM, ▲ MeLOOH 1.0 mM, ♦ control.

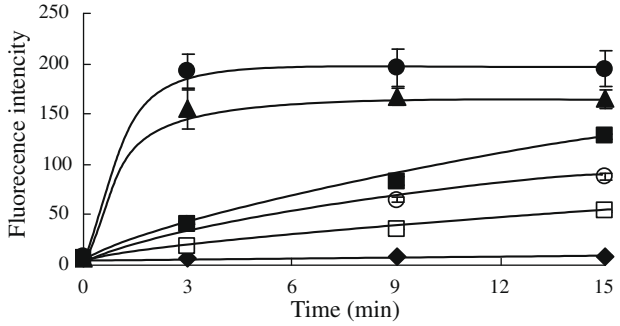


Figure 2. Reaction of ROOH with MitoDPPP in liposome; ● CumOOH 1.0 mM, ■ t-BHP 1.0 mM, □ H_2O_2 1.0 mM, ○ H_2O_2 1.0 mM + Fe^{2+} 10 μM, ▲ MeLOOH 10 μM, ♦ control.

of cells was obtained by fluorescence microscopy, revealed that after 15 min following peroxide treatment, there was an increase in the fluorescence intensity of MitoDPPP loaded into the mitochondria in the cells. The result revealed the ability of MitoDPPP to detect peroxides in mitochondria. On the other hand, in the case of HepG2 cells loaded with MitoDPPP, the fluorescence intensity did not increase after 15 min. The fluorescence intensities of MitoDPPPO, resulting from the oxidation of MitoDPPP loaded into intact HepG2 cells, were also measured by fluorescence spectroscopy for evaluating the effectiveness of MeLOOH, CumOOH, *t*-BHP, and H_2O_2 as oxidants. The oxidations of MitoDPPP proceeded by MeLOOH, CumOOH, and *t*-BHP, on the other hand, the oxidation by H_2O_2 was slow in comparison with lipophilic peroxide (Fig. 4).

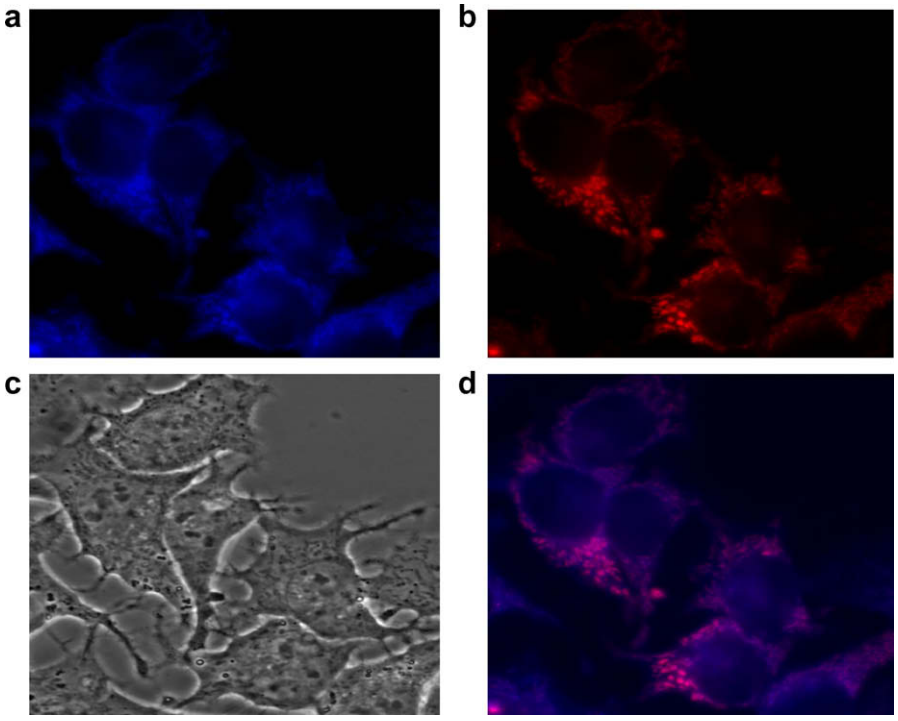


Figure 3. Localization of MitoDPPP in mitochondria in HepG2 cells. MitoDPPP and MitoRed were loaded into HepG2 cells. (a) Fluorescent image of MitoDPPPO formed by oxidation of MitoDPPP with *t*-BHP; (b) fluorescent image of MitoRed; (c) brightfield image; (d) merged image.

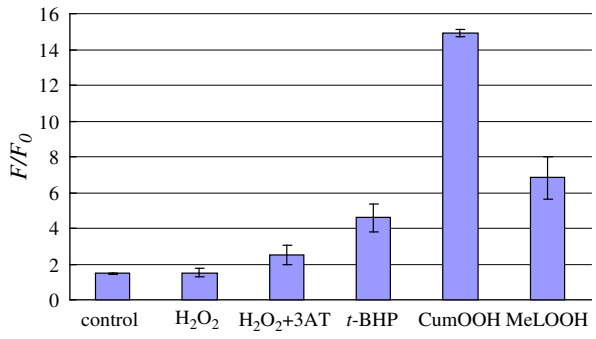


Figure 4. Reactivity of MitoDPPP with various peroxides in HepG2 cells at 37 °C. HepG2 cells loaded MitoDPPP was treated 1.0 mM of peroxide (MeLOOH: 10 μM). The figure shows relative fluorescence intensity (F/F_0 measured at 380 nm with excitation at 350 nm), after the reaction with peroxides for 15 min.

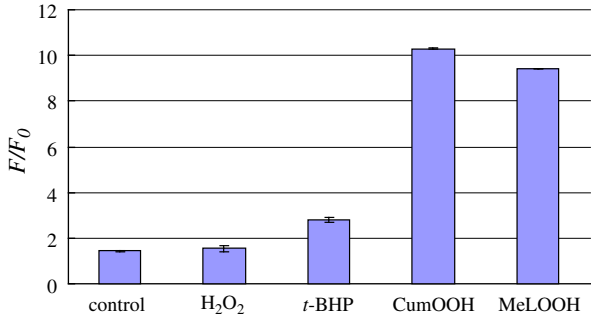


Figure 5. Reactivity of MitoDPPP with various peroxides in isolated mitochondria at 37 °C. Isolated mitochondria from 2.0×10^5 cells loaded MitoDPPP was treated 10 μM of peroxide (MeLOOH: 1.0 μM). The figure shows relative fluorescence intensity (F/F_0 measured at 380 nm with excitation at 350 nm), after the reaction with peroxides for 15 min.

The tendency of the reactivity is similar to the reaction in liposome solution. Cells have quenching systems for H_2O_2. Catalase is one of enzyme in the system. In order to judging whether catalase would be acted as a scavenger for H_2O_2, the oxidation of HepG2 cells loading MitoDPPP was carried out by H_2O_2 in the presence of 3-aminotriazol (3-AT) as an inhibitor of catalase. The fluorescence intensity was not so increased by addition of 3-AT (10 mM). The difference between their efficiencies as oxidants was caused by the difference in their membrane permeability. It was reported that *t*-BHP was a membrane-permeable reagent for inducing cell death through apoptosis and necrosis.[16,17] These phenomena are thought to be mediated by the generation of free radicals and by lipid peroxidation. The difference in the present results of the oxidation efficiencies of H_2O_2 and other peroxide originated from the difference in their membrane permeability. The efficiencies of these peroxides in isolated mitochondria[18] were similar to those of intact cell and liposome solution (Fig. 5). These results suggest that MitoDPPP accumulates in mitochondrial membrane.

It has been reported that lipophilic triarylphosphines that accumulate in a cell membrane do not detect H_2O_2.[19] When MitoDPPP loaded HepG2 cells were washed with PBS buffer containing 5% DMSO solution, the fluorescence intensity of some cells were increased by the oxidation with H_2O_2 (Fig. 6). DMSO is a widely used agent in cell biology. It is well known as a cryoprotectant and a permeability enhancing agent.[20,21] On the basis of the difference in the reactivity for peroxide, at least, diphenylpyrenylphosphinoyl moiety of MitoDPPP is buried into the mitochondrial lipid bilayer, and the oxidation with H_2O_2 promotes by DMSO.

Finally, we attempted to detect lipid peroxide generating in cells. The MitoDPPP loaded cell was treated with glutamate (100 μM), which inhibit cystine transport leading to both reduction of intraceller cystine and GSH resulting that lipid peroxidation of membrane was caused by intraceller H_2O_2.[22] When the HepG2 cells were incubated with glutamate solution for 20 h, the fluorescence intensity was increased by oxidation with peroxide (Fig. 7b). After incubating with glutamate for 2 h, the increasing of intensity

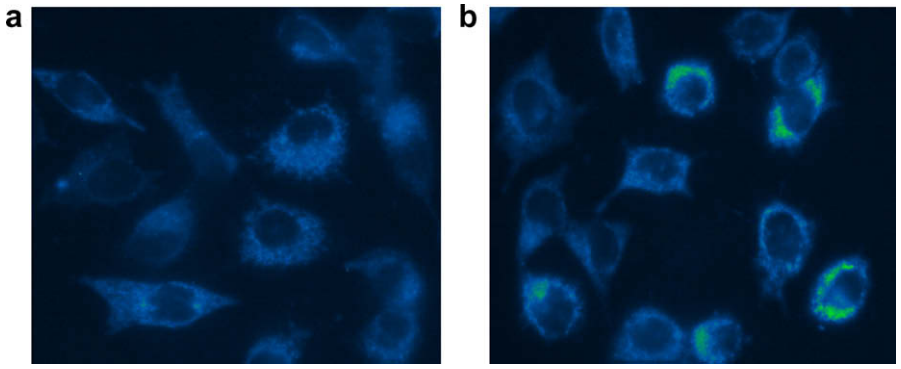


Figure 6. Distribution map of brightness of HepG2 cells loading MitoDPPP: (a) washing with DPBS buffer prior to treat with H_2O_2; (b) washing with DPBS buffer containing 5% DMSO prior to treat with H_2O_2.

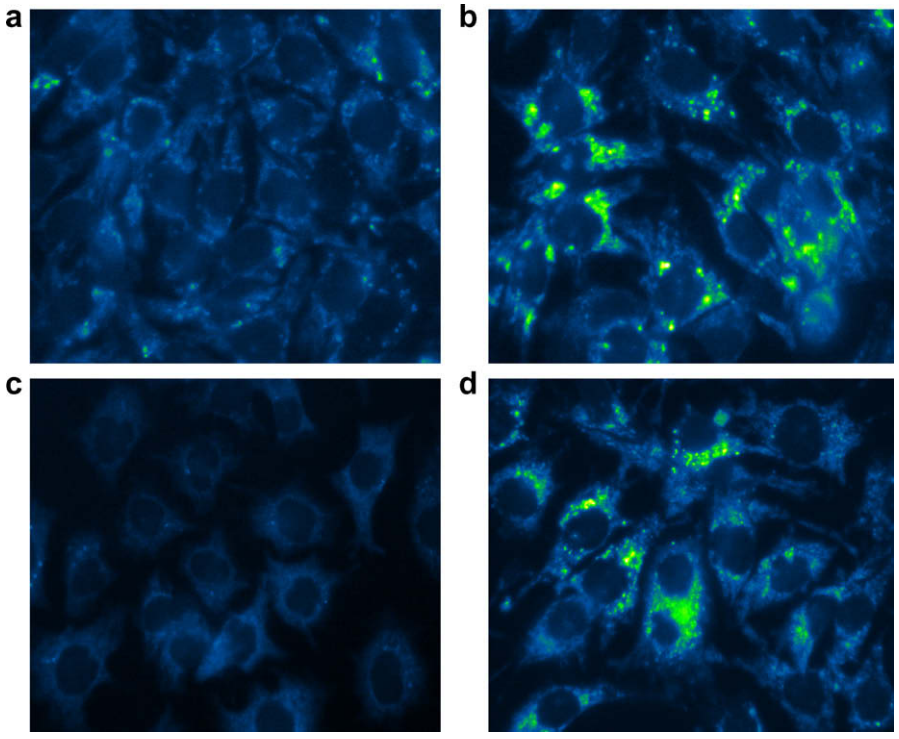


Figure 7. Distribution map of brightness of HepG2 cells loading MitoDPPP: (a) control (loading 1.6 μM of MitoDPPP for 15 min, washing twice with DPBS buffer, incubating for 20 h in dark); (b) treatment with glutamate (100 μM) for 20 h; (c) treatment with glutamate for 2 h with addition of 3-AT before measuring; (d) treatment with glutamate for 2 h with 5% DMSO washing before measuring.

was too small to detect by microscope. In the presence of 3-AT, the fluorescence intensity did not also increase (Fig. 7c). However, washing with PBS buffer containing 5% DMSO after glutamate treatment, the intensity was increased significantly (Fig. 7d). The difference suggests that MitoDPPP is difficult to contact with H_2O_2 produced in mitochondria without DMSO treatment. Thus, oxidation of MitoDPPP in mitochondrial membrane might be dominated by the formation of lipid peroxide of the membrane.

In summary, we have synthesized a fluorescent probe, localized to mitochondria, for the selective detection of peroxides that are permeable in membrane and cause cell death. Observations made using this probe have revealed that lipophilic peroxide effectively penetrates the mitochondrial lipid bilayer. H_2O_2 in mitochondria sensitive fluorescence probe (MitoPY1) has been repoted.[23] The probe has hydrophilic moiety as a functional group. In contrast, due to the high hydrophobicity of functional group in our probe, the affinity of the moiety to mitochondrial membrane is higher than that of MitoPY1. Since the present probe is inactive toward H_2O_2 in mitochondria, lipid peroxides can be distinguished from H_2O_2.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.05.017.

References and notes

1. *Oxidative stress, Disease and Cancer*; Singh, K. K., Ed.; Imperial College Press: London, 2006.
2. Douglas, C. W., Lott, M. T. In Stevnsner, T., Ed., *Molecular Biology of Aging*, 1999, pp 125–147.
3. Robinson, R. *Trends Biol. Sci.* **2000**, *25*, 502.
4. Boveris, A.; Oshino, N.; Chance, B. *Biochem. J.* **1972**, *128*, 617.

5. St-Pierre, J.; Buckingham, J. A.; Rocbuck, S. J.; Brand, M. D. *J. Biol. Chem.* **2002**, *277*, 44784.
6. Brookes, P. S.; Yoon, Y.; Robotham, J. L.; Anders, M. W.; Sheu, S. S. *Am. J. Physiol. Cell Physiol.* **2004**, *287*, C817.
7. Koide, Y.; Urano, Y.; Kenmoku, S.; Kojima, H.; Nagano, T. *J. Am. Chem. Soc.* **2007**, *129*, 10324.
8. Kenmoku, S.; Urano, Y.; Kojima, H.; Nagano, T. *J. Am. Chem. Soc.* **2007**, *129*, 7313.
9. Akasaka, K.; Ijichi, S.; Watanabe, K.; Ohrui, H.; Meguro, H. *J. Chromatogr.* **1992**, *596*, 197.
10. Kelso, G. F.; Porteous, C. M.; Hughes, G.; Ledgerwood, E. C.; Gane, A. M.; Smith, R. A. J.; Murphy, M. P. *Ann. N.Y. Acad. Sci.* **2002**, *959*, 263.
11. Murphy, M. P.; Smith, R. A. *Adv. Drug Delivery Rev.* **2000**, *41*, 235.
12. Jauslin, M. L.; Meier, T.; Smith, R. A.; Murphy, M. P. *FASEB J.* **2003**, *17*, 1972.
13. Matot, I.; Manevich, Y.; Al-Mehdi, A.-B.; Song, C.; Fisher, A. B. *Free Radical Biol. Med.* **2003**, *34*, 785.
14. Okimoto, Y.; Watanabe, A.; Niki, E.; Yamashita, T.; Noguchi, N. *FEBS Lett.* **2000**, *474*, 137.
15. Hasan, M. R.; Koikawa, S.; Kotani, S.; Miyamoto, S.; Nakagawa, H. *Exp. Cell Res.* **2006**, *312*, 1950.
16. Haidara, K.; Morel, I.; Abalea, V.; Gascon, B. M.; Denizeau, F. *Biochim. Biophys. Acta* **2002**, *1542*, 173.
17. Piret, J. P.; Arnould, T.; Fuks, B.; Chatelain, P.; Remacle, J.; Michaels, C. *Biochem. Pharmacol.* **2004**, *67*, 611.
18. Liu, X.; Kim, C. N.; Yang, J.; Jemmerson, R.; Wang, X. *Cell* **1996**, *86*, 147.
19. Soh, N.; Ariyoshi, T.; Fukaminato, T.; Nakajima, H.; Nakano, K.; Imato, T. *Org. Biomol. Chem.* **2007**, *5*, 3762.
20. Williams, A. C.; Barry, B. W. *Adv. Drug Delivery Rev.* **2004**, *56*, 603.
21. Yu, Z. W.; Quinn, P. J. *Mol. Membr. Biol.* **1998**, *15*, 59.
22. Higuchi, Y. *J. Cell. Mol. Med.* **2004**, *8*, 455.
23. Dickson, B. C.; Chang, C. J. *J. Am. Chem. Soc.* **2008**, *130*, 9638.