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# Novel Fluorescent Probe for Analysis of Hydroperoxides Based on Boron Dipyrromethane Fluorophore

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**A new reagent, *N*-[2-(diphenylphosphino)ethyl]-4-(1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene-8-yl)benzamide (DPPEA-BODIPY), was designed and synthesized for analysis of hydroperoxides. DPPEA-BODIPY fluoresces at low levels in the visible range ($\lambda_{ex}/\lambda_{em}$ = 502 nm/515 nm) and reacts with hydroperoxides to produce DPPEA-BODIPY oxide, which fluoresces at high levels. The fluorescence intensity of the reaction mixture was observed to be linearly related to the methyl linoleate hydroperoxide (MeLOOH) concentration.**

**Key words:** BODIPY; hydroperoxide; lipid hydroperoxide; reagent

Recently, reactive oxygen has attracted attention for its association with disease. In particular, lipid hydroperoxides have been connected to arteriosclerosis, hyperlipidemia, cancer, and aging.[1–5] Several analytical methods have been proposed for lipid hydroperoxides and lipid peroxidation.[6]

Diphenyl-1-pyrenylphophine (DPPP) is used as a fluorescence probe for the imaging of lipid peroxidation and the flow injection analysis of lipid hydroperoxide. However, the excitation and emission wavelengths of DPPP are in such a UV range ($\lambda_{ex}/\lambda_{em}$ = 352 nm/380 nm)[7–10] that they damage the biological sample and suffer interference by autofluorescence from the biological sample.[11] Therefore, a novel reagent that has the excitation and emission wavelengths in the visible region is desired.

BODIPY fluorophores, which show high quantum yields, hydrophobicity, and large extinction coefficients, and fluoresce at around 550 nm, are widely used to measure and to label the constituents of biological samples.

In this study, for the analysis of hydroperoxides, we designed and synthesized a new BODIPY reagent (DPPEA-BODIPY) which utilized a photo-induced electron transfer mechanism containing a diphenyl phosphine.

*Synthesis of DPPEA-BODIPY.* As depicted in Scheme 1, 2,4-dimethylpyrrole (5 g, 52.55 mol) and methyl 4-formylbenzoate (4.31 g, 26.25 mmol) were dissolved in anhydrous $CH_2Cl_2$ (100 ml), and this mixture was degassed by bubbling a nitrogen gas through it for 30 min. TFA (one drop) was added to the solution, which was stirred for 15 h at room temperature under a nitrogen atomosphere. DDQ (5.97 g, 26.26 mmol), dissolved in a mixed solvent of anhydrous THF (25 ml) and anhydrous $CH_2Cl_2$ (25 ml), was added dropwise over a period of 15 min to the mixture, which was stirred for 4 h at room temperature. A saturated aqueous $NaHCO_3$ was added, and the mixture was extracted with $CH_2Cl_2$. The organic layer was washed with water and brine, dried over $Na_2SO_4$, and concentrated *in vacuo*. The crude product was purified by aminopropyl silica gel column chromatography using hexane-ethyl acetate to afford **3** (1.71 g, yield 19.5%), NMR $\delta_H$ (400 MHz, TMS, $CDCl_3$): 1.27 (6H, s, $CH_3$), 2.34 (6H, s, $CH_3$), 3.96 (3H, s, $CH_3$), 5.89 (2H, s, CH), 7.40–7.42 (2H, d, $J$ = 7.9 Hz, benzene), 8.11–8.13 (2H, d, $J$ = 8.0 Hz, benzene), MS $m/z$: calcd. for $C_{21}H_{23}N_2O_2$ $[M + H]^+$: 335.18, found: 335.20. **3** (1.6 g, 4.78 mmol) was dissolved in anhydrous toluene (65 ml), triethylamine (9.2 ml) and $BF_3 \cdot Et_2O$ (9.2 ml) were added and the mixture was stirred for 3 h at room temperature. A saturated aqueous $NaHCO_3$ was added and the mixture was extracted with $CH_2Cl_2$. The organic layer was washed with brine, dried over $Na_2SO_4$, and concentrated *in vacuo*. The crude product was purified by aminopropyl silica gel column chromatography using hexane-ethyl acetate to afford **4** (1.76 g, yield 96.2%). NMR $\delta_H$ (400 MHz, $CDCl_3$): 1.36 (6H, s, $CH_3$), 2.56 (6H, s, $CH_3$), 3.97 (3H, s, $CH_3$), 5.99 (2H, s, CH), 7.40–7.42 (2H, d, $J$ = 8.0 Hz, benzene), 8.17–8.19 (2H, d, $J$ = 8.3 Hz, benzene), MS $m/z$: calcd. for $C_{21}H_{21}BF_2N_2O_2Na$ $[M + Na]^+$: 405.16, found: 405.21. **4** (0.5 g, 1.31 mmol) and potassium carbonate (0.3 g, 2.17 mmol) in THF-water (60 ml–60 ml) was stirred for 10 h at 50 °C for 14 h at room temperature. After cooling, the mixture was washed with diethyl ether, and the water phase was extracted with $CH_2Cl_2$, dried over $Na_2SO_4$, and concentrated *in vacuo*. This crude product **5** was used without further purification. MS $m/z$: calcd. for $C_{20}H_{18}BF_2N_2O_2$ $[M - H]^-$: 367.14, found: 367.23. **5** (0.48 g), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.89 g, 4.64 mmol), *N*,*N*-dimethyl-

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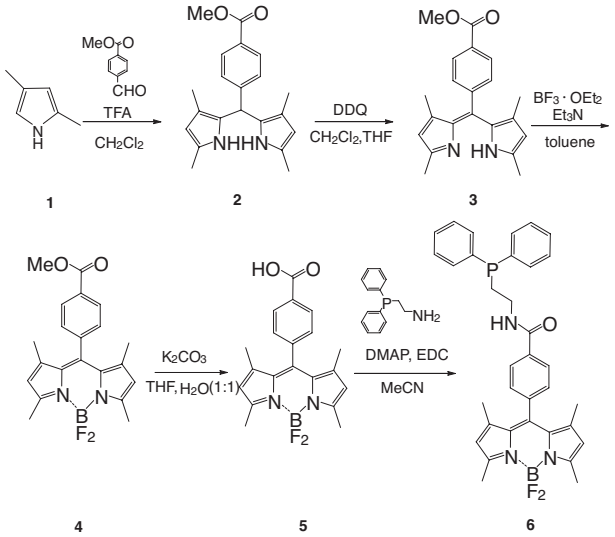


**Scheme 1.** Synthesis Scheme for DPPEA-BODIPY.

4-aminopyridine (0.31 g, 2.54 mmol) and 2-(diphenylphosphino)ethylamine (1 g, 4.36 mmol) were disolved in anhydrous MeCN (75 ml), which was stirred under a nitrogen atomosphere in the dark for 30 min at 0 °C and for an additional 4 d at room temperature. Distilled water was added, and the mixture was extracted with $CH_2Cl_2$. The organic layer was washed with distilled water and brine, dried over $Na_2SO_4$, and concentrated *in vacuo*. The crude product was purified in the dark by silica gel column chromatography (chloroform) and subsequent recrystallization under an argon atmosphere from diethyl ether to afford **6** (160.26 mg, yield 21.1%, from compound 4). NMR $\delta_H$ (400 MHz, $CDCl_3$): 1.34 (s, 6H, $CH_3$), 2.46–2.50 (2H, t, $CH_2$), 2.56 (6H, s, $CH_3$), 3.67–3.75 (2H, m, $CH_2$), 5.98 (2H, s, CH), 6.30 (1H, s, NH), 7.32–7.38 (8H, m, benzene), 7.47–7.52 (4H, m, benzene), 7.70–7.72 (2H, d, $J = 8.3$ Hz, benzene), MS *m/z*: calcd. for $C_{34}H_{33}BF_2N_3OPNa$ $[M + Na]^+$: 602.23, found: 602.11.

The yield of DPPEA-BODIPY was 4%, and the structure of DPPEA-BODIPY was characterized by mass spectrometry (Waters ZQ, Nihon Waters, Tokyo) and FT-NMR (Bruker AV400M Spectrometer, Bruker BioSpin, Tsukuba, Japan). To prevent air oxidation, DPPEA-BODIPY was stored at −20 °C in the dark, and was stable for two months.

DPPEA-BODIPY oxide was synthesized in the oxidation manner in the literature.[10] DPPEA-BODIPY oxide: NMR $\delta_H$ (400 MHz, $CDCl_3$): 1.35 (6H, s, $CH_3$), 2.56 (6H, s, $CH_3$), 2.63–2.68 (2H, m, $CH_2$), 3.87–3.95 (2H, m, $CH_2$), 5.98 (2H, s, CH), 7.35–7.37 (2H, d, $J =$ 8.0 Hz, benzene), 7.48–7.57 (6H, m, benzene), 7.75–7.80 (4H, m, benzene), 7.93–7.95 (2H, d, $J = 8.0$ Hz, benzene), 8.12 (1H, s, NH), MS *m/z*: calcd. for $C_{34}H_{33}BF_2N_3O_2PNa$$[M + Na]^+$: 618.23, found: 618.15.

The reaction of DPPEA-BODIPY with lipid hydroperoxides and its excitation and emission spectra are shown in Fig. 1. DPPEA-BODIPY was estimated to fluoresce at low levels for the spacer between a phosphine and fluorophore. The oxidant was expected to fluoresce at high levels due to the hindrance of photo-induced electron transfer like other phosphine reagents.[12]

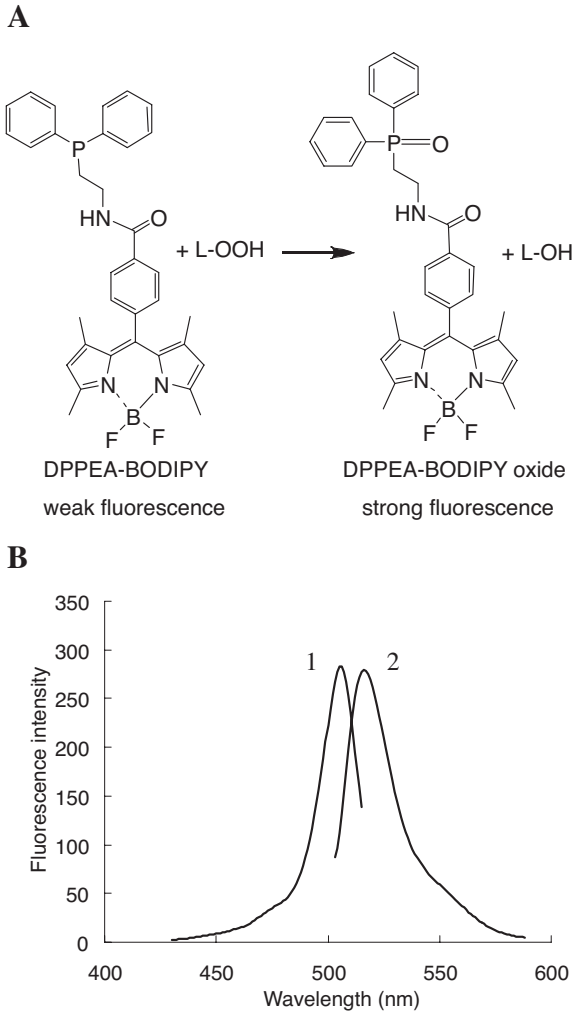


**Fig. 1.** Reaction of DPPEA-BODIPY with Hydroperoxide (A), Excitation Spectrum (1) and Emission Spectrum (2) of DPPEA-BODIPY in Chloroform (B).

The excitation fluorescence spectrum of DPPEA-BODIPY was measured in chloroform. The excitation and emission wavelengths of both DPPEA-BODIPY and its oxidant produced by the reaction between DPPEA-BODIPY and hydrogen peroxide were approximately 500 nm ($\lambda_{ex}/\lambda_{em} = 502$ nm/515 nm, Fig. 1), the same as the other BODIPY reagents. The concentration of quenching was also measured with DPPEA-BODIPY and DPPEA-BODIPY oxide.

*Lipid hydroperoxide analysis.* The lipid hydroperoxide of the hydroperoxide sample was synthesized by air oxidation of methyl linoleate. Its concentration was quantified by the DPPP method,[7] which was used to evaluate the validity of lipid hydroperoxide analysis of DPPEA-BODIPY. 2,6-Di-*tert*-butyl-*p*-cresol (BHT) was dissolved at chloroform in 3 mg per 10 ml, and this was used for the stock solution and the reaction solvent. The DPPP method was modified with DPPPEA-BODIPY, which was applied in MeLOOH concentration analysis. In test tubes, 50 µl of 55.23 µM (2.76 nmol) DPPEA-BODIPY was added to 100 µl of various concentrations (0–61.76 µM) of MeLOOH. The test tubes were sealed tightly with screw caps, protected from light, and reacted at 37 °C for 60 min. The reaction mixture was cooled in an ice bath, and 3 ml of chloroform was added

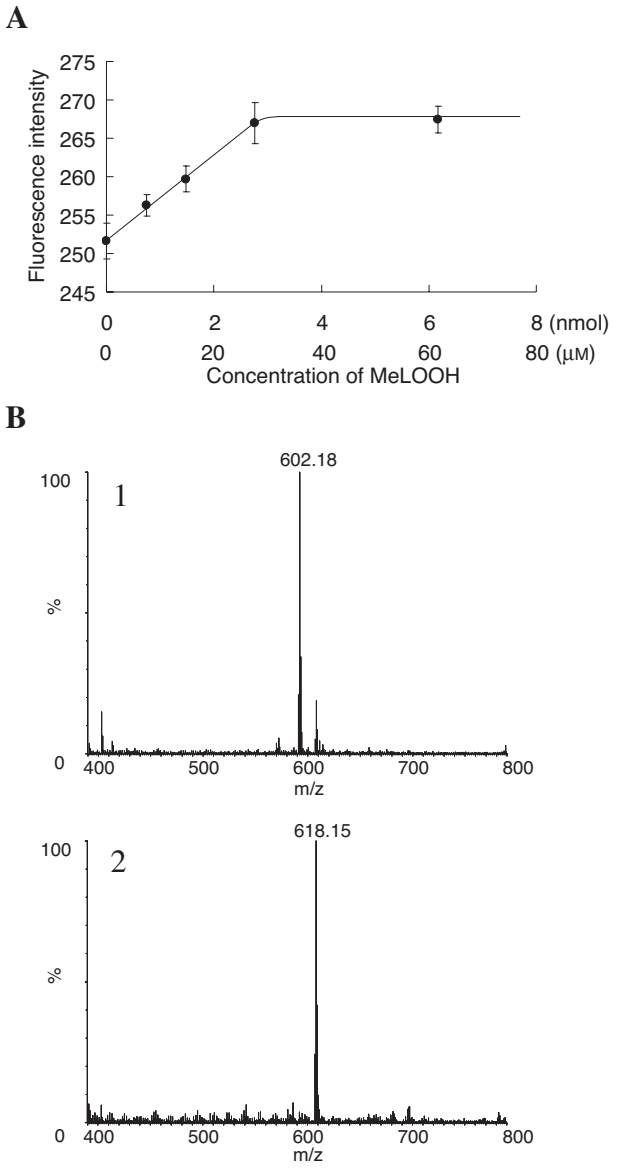


**Fig. 2.** Relationship between the Concentration of Methyl Linoleate Hydroperoxide and Fluorescence Intensity (A), ESI Mass Spectra of DPPEA-BODIPY (2.65 nmol) after Reaction with 0 nmol Methyl Linoleate Hydroperoxide (1) and DPPEA-BODIPY (2.65 nmol) after Reaction with 2.76 nmol Methyl Linoleate Hydroperoxide (2) (B).

to the mixture. The fluorescence intensity at 515 nm (ex 502 nm) was measured.

Figure 2A shows the relation between the intensity of fluorescence and the quantity of hydroperoxide. The fluorescence intensity of the reaction mixture showed a linear relationship with the MeLOOH concentration (0–2.76 nmol:0–27.6 μM).

The reaction of the MeLOOH and DPPEA-BODIPY was examined by mass spectrometry to confirm that the high fluorescence was produced by DPPEA-BODIPY oxide. Mass spectrometric results were obtained for two reaction mixtures where 52.9 μM DPPEA-BODIPY (2.65 nmol) reacted with MeLOOH (0 μM and 27.6 μM (2.76 nmol)). Then they were reacted at 37 °C for 60 min by the DPPP modified method. In the 0 μM reaction mixture, DPPEA-BODIPY oxide was not produced. On the other hand, in the 27.6 μM reaction mixture, DPPEA-BODIPY disappeared completely, and DPPEA-BODIPY oxide was produced (Fig. 2B).

These results indicate that DPPEA-BODIPY reacts with hydroperoxides quantitatively to produce DPPEA-BODIPY oxide. This oxide fluoresces in the visible range, which was able to use for the reagent of the hydroperoxide determination.

Recently, the sensor has been studied for the analytical methods. In the sensor, an optical chemical sensor such as a sensing film is a relatively simple analytical method that has several advantages, such as rapidity and ease of preparation and procedure.[13] The hydrophobic probe is suitable for the sensing film sensor, and DPPEA-BODIPY is a hydrophobic probe based on BODIPY.

On the other hand, the sensor fabricated on the microelectromechanical system (MEMS) has been studied for the compact analytical system,[14,15] and the probe of long excitation and emission wavelength needs for the down-sized analytical instrument.

Hence this new fluorescent molecular probe will be used to investigate the quantity of hydroperoxides, and it is expected to be useful in the development of analytical methods such as sensors, which is in progress.

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