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Photophysical properties and application in live cell imaging of B,B-fluoro-perfluoroalkyl BODIPYs†

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The photophysical properties of newly identified B,B-fluoro-perfluoroalkyl BODIPYs (**2** and **3**), which possess a fluoro group and a trifluoromethyl or pentafluoroethyl group at the boron center, were investigated. B,B-Fluoro-perfluoroalkyl BODIPYs **2** and **3** exhibited better photophysical/chemical properties than B,B-difluoro-BODIPY **1**, as follows: (1) higher photostability both in methanol solvent and in a live cell environment, (2) higher stability against acid degradation, and (3) improved fluorescence signal-to-noise ratios in a cell system. These favorable properties of B,B-fluoro-perfluoroalkyl BODIPYs are likely due to the highly electron-withdrawing nature of the perfluoroalkyl groups on the boron atom, which reduces the reactivity to $^1\text{O}_2$ and strengthens the complexation of the dipyrromethene ligands to the boron atom. Thus, B,B-fluoro perfluoroalkyl BODIPYs may be useful functional molecules for various applications.

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1. Introduction

Boron-dipyrromethenes (BODIPYs) are among the most important photochemical functional molecules for applications such as fluorescence imaging and photodynamic therapy.¹ BODIPYs exhibit large molar extinction coefficients, a high and environment-independent fluorescence quantum yield, and sharp absorption and emission spectra.

Numerous types of BODIPYs have been reported, most of which possess two fluorine atoms at the boron center (BF_2 -BODIPY). B,B-Fluoro-organo BODIPYs (BFR-BODIPYs) possessing an organo group (R) instead of a fluorine atom at the boron center, however, have unique properties.² We recently disclosed a short and convergent synthesis of BFR-BODIPYs.³ Because this method is operative under mild conditions, a variety of functionalized carbon substituents, whose introduction was difficult or unfeasible by previous methods, can be installed at the boron center. Specifically, B,B-fluoro trifluoromethyl BODIPY (**2**, Fig. 1) exhibits the most prominent photostability among various BFR-BODIPYs and BF_2 -BODIPYs.^{3,4} Therefore, we studied the photophysical

properties of B,B-fluoro perfluoroalkyl BODIPYs (**2** and **3**) and their potential usefulness in biologic research.

2. Results and discussion

2.1 Synthesis of B,B-fluoro-perfluoroalkyl BODIPYs

B,B-Fluoro-pentafluoroethyl BODIPY **3** was synthesized similarly to **2**.³ Thus, $\text{BF}_2\text{C}_2\text{F}_5$ was *in situ* generated by treating commercially available $\text{K}[\text{BF}_3\text{C}_2\text{F}_5]$ with trimethylsilyl triflate in CH_3CN and then added to a dichloromethane solution of tetramethyl dipyrromethene and ethyl diisopropyl amine (DIPEA). The coupling reaction was conducted at room temperature for 3 h, affording **3** in 23% isolated yield.

2.2 Three-dimensional structure of **2**

Single crystals of **2** were obtained from ethyl acetate, and the three-dimensional structure was determined by X-ray diffraction (Fig. 2). The boron center was in a tetrahedral geometry

Fig. 1 Structures of BODIPYs (**1**–**3**).

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with an N1–B–N2 angle of 107.15(14)°. In addition, the average bond length of N1–C1 and N2–C9 (1.355 Å) was shorter than that of N1–C4 and N2–C6 (1.399 Å), indicating the presence of the double-bond character at N1–C1/N2–C9. These observations are similar to those of other BODIPY structures. On the other hand, the B–N1 bond length of **2** (1.545(2) Å) was shorter than that of BF₂-BODIPY **1** (1.557 Å⁵). The shorter B–N bond length is likely due to the stronger electron-withdrawing ability of a trifluoromethyl group than that of a fluorine atom, which enhances the B–N bond connectivity. The B–F1 bond length of **2** (1.389(2) Å) was also slightly shortened compared with that of **1** (1.395 Å⁵). Interestingly, the boron center of **2** was out-of-plane from the dipyrromethene ligand toward the trifluoromethyl group (the dihedral angle at B–N2–C6–C5 = 12.2(2)°, while the boron center of **1** was in the in-plane position (the corresponding dihedral angle = 0.2°).⁵ Relative to the previously reported BFR-BODIPYs (R = Ph: 3.4° (ref. 6); R = C₆F₅: 0.2–3.6° (ref. 7); R = CH₂Br: 3.2° (ref. 3)), the distortion in **2** is strikingly large. The fluorine atom was nearly in-plane with the dipyrromethene ligand.

2.3 Photophysical properties and chemical stability

The maximum absorption wavelength (λ_{abs}), molar extinction coefficient ($\log \epsilon$), maximum fluorescence emission wavelength (λ_{em}), and fluorescence quantum yield (Φ_{f}) of **2** and **3** were similar to those of **1** (Table 1 and S1†). Introducing the perfluoroalkyl groups at the boron center, however, remarkably increased the photostability compared with **1**. Previously, we observed higher photostability of **2** than that of **1**, which we verified by absorption spectrometry.³ In the present study, the improved photostability of fluoro-perfluoroalkyl BODIPYs was confirmed using a photobleaching assay. Thus, when 1–3 dissolved in methanol were irradiated with a 500 nm light, the degradation rates of the fluorescence intensities of **2** and **3** were much slower than that of **1**; 67%/47%/24% of **2** and 75%/59%/37% of **3** remained after irradiation for 15 min/30

min/60 min, respectively, while only 34%/11%/2% of **1** remained (Fig. 3a). Also, when solutions of 1–3 were incubated under acidic conditions (0.5 M HCl in water: methanol = 1:11), the fluorescence intensities of **2** and **3** were maintained at significantly higher levels (>80%) compared to that of **1** (22%) after 10 h (Fig. 3b). Accordingly, the photo- and chemical stabilities of the BODIPYs were notably enhanced through the introduction of the perfluoroalkyl group at the boron center.

2.4 Application to cell systems

The fluorescence imaging abilities of 1–3 in live cells were examined. BODIPYs are taken up by cells, stained membranes and intracellular organelles such as the endoplasmic reticulum and Golgi apparatus.⁸ Both **2** and **3** exhibited no significant cytotoxicity up to 10 μM , like **1** (Fig. 4a). Interestingly, when the cells were treated with 1–3, B,B-fluoro-perfluoroalkyl BODIPYs **2** and **3** exhibited higher fluorescence signal-to-noise ratios than **1** (Fig. 4b, upper panels). Thus, **2** and **3** showed clear fluorescence images at a concentration of only 0.3 μM , while 10 μM **1** was necessary to obtain a similar amount of fluorescence. It is possible that the trifluoromethyl and pentafluoroethyl groups in **2** and **3**, respectively, enhanced the cellular uptake of the BODIPYs due to their higher hydrophobicity than the fluorine atom in **1**. In addition, when cells stained with 1–3 were irradiated with a light for the same time period, the fluorescence intensities of **2** and **3** were higher than that of **1** (Fig. 4b, lower panels). The time-course plots of the fluorescence intensities in cells indicated that the photobleaching rates of **2** and **3** were significantly lower than **1** (Fig. 4c). Specifically, the fluorescence intensity of **3** was the most prolonged, consistent with the result in methanol (Fig. 3a). Therefore, the superior photostability of perfluoroalkyl BODIPYs was maintained in the live cell environment.

2.5 Mechanistic insights into photo-degradation

When degassed methanol was used as a solvent, the photodecomposition of 1–3, verified by fluorescence intensity, was predominantly suppressed (Fig. 5a). This result is consistent with the fact that the degradation of BODIPY is mainly mediated by molecular oxygen.⁹ Thus, the self-produced singlet oxygen (¹O₂), via energy transfer from the BODIPYs in an excited triplet state to the molecular oxygen (³O₂), decomposes the fluorophore of the BODIPYs. The emission level of ¹O₂, verified by the amount of luminescence at 1280 nm, in 1–3 upon photo-irradiation with 523 nm was similar (~5500 counts, Fig. 5b). On the other hand, the sensitivity to photo-degradation was in the order of 1 > 2 > 3 (Fig. 3a). The HOMO energy levels of **2** and **3** (−5.826 eV and −5.861 eV, respectively) were significantly lower than that of **1** (−5.680 eV) based on density functional theory calculations (Table 1 and Fig. S2†). The lowered HOMO energy levels of **2** and **3**, promoted by introducing highly electron-withdrawing perfluoroalkylboron groups, should decrease the efficiency of

Fig. 2 ORTEP view of **2** with thermal ellipsoids at 50% probability. All hydrogen atoms are omitted for clarity. Bottom left: Front view; bottom right: side view.

Table 1 Photophysical/electrochemical properties of BODIPYs 1–3

	R	$\lambda_{\text{abs}}^{a,b}$ (nm)	$\log \epsilon^a$	$\lambda_{\text{em}}^{a,b}$ (nm)	Φ_{f}^a	HOMO ^d (eV)	LUMO ^d (eV)
1	F	504	4.91	509	0.80 ^c	−5.680	−2.755
2	CF ₃	502	4.95	509	0.83	−5.826	−2.876
3	C ₂ F ₅	503	4.94	509	0.82	−5.861	−2.919

^a Taken in methanol. ^b Fig. S1. ^c Ref. 14. ^d Density functional theory calculations with B3LYP/LAV3P*+.

the orbital overlap with the LUMO of ¹O₂, leading to increased tolerance to oxidative degradation. Similarly, the higher stability of 3 than that of 2 can be attributed to the lower HOMO level of 3. Consistently, the cyclic voltammogram indicates that the anodic peak potential (*E*_{pa}) of 2 and 3 (0.936 V and 0.937 V, respectively) was clearly higher than that of 1 (0.866 V) (Fig. S3†).

3. Conclusions

In this study, we investigated the photophysical properties of newly identified B,B-fluoro-perfluoroalkyl BODIPYs 2 and 3, possessing trifluoromethyl and pentafluoroethyl groups at the boron center, respectively. The fluoro-perfluoroalkyl BODIPYs 2 and 3 exhibited higher photostability and chemical stability than the corresponding BF₂-BODIPY 1. Interestingly, when 2 and 3 were applied to a live cell system, the fluorescence signal-to-noise ratios were markedly improved compared with 1. In addition, the higher photostability of 2 and 3 compared with that of 1 was retained in the live cell environment. Mechanistic studies suggest that the strongly electron-withdrawing perfluoroalkyl groups render BODIPYs 2 and 3 more electron-deficient, which reduces the reactivity to ¹O₂ and stabilizes the B–N bonds. The B,B-fluoro-perfluoroalkyl BODIPYs complement previous BODIPYs whose photostability is improved by other structural modifications.¹⁰ In addition, the B-perfluoroalkyl modification was accessible to the dipyrromethene containing an alkyne functionality at the *meso* position,¹¹ demonstrating potential applicability of the new BODIPY for the bioconjugation.¹² In our group, designing new photo-catalysts based on B,B-fluoro-perfluoroalkyl BODIPY structures, which reduces path-

ogenicity derived from protein aggregation *via* the ¹O₂-mediated oxygenation,^{4b,13} is ongoing.

4. Methods

4.1 General

Chemicals were purchased from commercial suppliers: Dojin Laboratories (Kumamoto, Japan), Kanto Chemical Co., Inc. (Tokyo, Japan), Nacalai Tesque, Inc. (Kyoto, Japan), Peptide Institute, Inc. (Osaka, Japan), Sigma-Aldrich, Inc. (St. Louis, MO, USA), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and Watanabe Chemical Industries, Ltd. (Hiroshima, Japan), and were used without further purification. Flash column chromatography was performed with silica gel Merck 60 (230–400 mesh ASTM; Merck, KGaA, Darmstadt, Germany). The water used in the assay was purified in advance using a Millipore Milli-Q water purification system (Merck, KGaA) to obtain filtered deionized water. The MS spectrum was recorded on a JEOL JMS-T100LC AccuTOF spectrometer (JEOL, Ltd., Tokyo, Japan). NMR spectra were recorded on a JEOL ECS400 (400 MHz for ¹H NMR, 100 MHz for ¹³C NMR, 370 MHz for ¹⁹F NMR, and 125.7 MHz for ¹¹B NMR) spectrometer (JEOL,

Fig. 3 a) Photostability of BODIPYs. Methanol containing 1–3 (10 μ M) was irradiated with a 500 nm light at rt. (*n* = 3, mean $\pm$ SD); b) chemical stability of BODIPYs under acidic conditions. 0.5 M HCl (water : methanol = 1 : 11) containing 1–3 (1 μ M) was incubated at rt. (*n* = 3, mean $\pm$ SD).

Fig. 4 a) Cytotoxicity of BODIPYs. PC12 cells were treated with 1–3 for 2 days (*n* = 3, mean $\pm$ SD); b) fluorescence images of PC12 cells using 1–3 before and after light irradiation; c) time-course plots of the fluorescence intensity of 1–3 in cells (*n* = 4, mean $\pm$ SD).

Fig. 5 a) Decomposition of 1–3 under non-degassed/degassed methanol; b) emission of singlet oxygen (luminescence at 1280 nm) produced from photoexcited 1–3 ($\lambda = 523$ nm).

Ltd.). Proton and carbon chemical shifts are reported relative to the solvent used as an internal reference. Fluorine and boron chemical shifts are reported relative to hexafluorobenzene ($\delta -164.9$ ppm) and boron trifluoride ($\delta 0.00$ ppm), respectively, as external references. Absorbance measurement was performed using a Shimadzu UV-1800 spectrometer (Shimadzu, Co.) with a rectangular quartz cell (5 mm path length). Fluorescence measurement was performed using a Shimadzu RF-5300PC spectrometer (Shimadzu, Co.) with a rectangular quartz cell (3 mm path length). For photobleaching and chemical stability assays of BODIPYs and CellTiter-Glo® luminescent cell viability assay, GloMax® explorer multimode detection system (Promega Co., WI, USA) was used.

4.2 Synthesis of 3

A similar procedure to that of 2 (ref. 3) using potassium trifluoro(pentafluoroethyl)borate afforded 3 as a red solid (23%). Purification was conducted by silica gel column chromatography (hexane/AcOEt = 20/1 $\rightarrow$ hexane/AcOEt = 10/1). ^1H NMR (CDCl_3 , 400 MHz): δ 2.25 (s, 6H), 2.51 (s, 6H), 6.07 (s, 2H), 7.07 (s, 1H); ^{13}C NMR (CDCl_3 , 100 MHz): δ 11.31, 15.08 (m), 119.77, 133.29, 141.81, 158.34; ^{19}F NMR (CDCl_3 , 370 MHz): δ -185.18 (m, 1F), -133.76 (s, 2F), -86.44 (s, 3F); ^{11}B NMR (CDCl_3 , 125.7 MHz): δ -0.61. HRMS (ESI^+): m/z calcd for $\text{C}_{15}\text{H}_{15}\text{BF}_6\text{N}_2\text{Na}^+$ [$\text{M} + \text{Na}$] $^+$: 371.1130. Found 371.1125.

4.3 Extinction coefficient

10, 7.5, 5.0, and 2.5 μM BODIPYs 1–3 in methanol were prepared, and absorption spectra of these solutions were measured. The absorbance at the maximum absorbance wavelength (A) and the concentration (C) were plotted on a graph (A versus C). The extinction coefficient of each compound was determined from the gradient of the graph.

4.4 Fluorescence quantum yield

2.5 μM BODIPYs 1–3 in methanol were prepared, and absorbance (A) at the excitation wavelength ($\lambda = 455$ nm) and the integrated fluorescence intensity (F) were recorded. Fluorescence quantum yields (Φ) were calculated according to the following equation:

$$\Phi = \Phi_{\text{Ref}} \frac{A_{\text{Ref}}}{A} \frac{F}{F_{\text{Ref}}}$$

where the subscript Ref denotes the reference fluorophore. $\text{BF}_2\text{-BODIPY}$ 1 was used as the reference ($\Phi = 0.80$).¹⁴

4.5 Photostability of BODIPYs

10 μM 1–3 in methanol were exposed to a 500 nm LED (10 mW). At each time point, an aliquot (10 μL) of the solution was diluted to 1 μM with methanol (90 μL) on a 96-well plate, and the fluorescence intensity was measured using a GloMax® explorer system ($\lambda_{\text{ex}} = 475$ nm, $\lambda_{\text{em}} = 500\text{--}550$ nm).

4.6 Stability of BODIPYs under acidic conditions

100 μM 1–3 in methanol were diluted to 1 μM with a mixed solvent of 6 M hydrochloric acid and methanol (1:11, 0.5 M hydrochloric acid) on a 96-well plate, and incubated at room temperature. At each time point, the fluorescence intensity was measured using a GloMax® explorer system ($\lambda_{\text{ex}} = 475$ nm, $\lambda_{\text{em}} = 500\text{--}550$ nm).

4.7 $^1\text{O}_2$ luminescence

100 μM 1–3 in methanol (1 mL) were irradiated with a laser (532 nm) and the $^1\text{O}_2$ luminescence around 1280 nm was measured, using a near-infrared emission spectrometer C8232TR3-45 equipped with a Nd-YAG laser (Hamamatsu Photonics K.K., Shizuoka, Japan).

4.8 Density functional theory calculations

Geometry optimization and energy calculation in the ground-state structures of BODIPYs 1–3 were carried out with the density functional theory (DFT). Becke's three parameter hybrid exchange function with the Lee–Yang–Parr gradient corrected correlated functional (B3LYP) was used in conjunction with the LAV3P* basis set¹⁵ as implemented in Maestro 9.3/Jaguar 7.9 (Schrödinger, LLC, New York, NY, 2012).

4.9 Cyclic voltammetry experiment

Cyclic voltammetry data were taken using a three-electrode system with a platinum wire auxiliary electrode. The working electrode was glassy carbon, and a Ag/AgCl electrode was used as the reference. A 0.1 M solution of tetrabutylammonium perchlorate in dry acetonitrile was used as the electrolyte solvent in which the compounds were dissolved at 1 mM. The scan rate was 100 mV s^{-1} .

4.10 Cytotoxicity of BODIPYs

PC12 cells (Riken BioResource Center, Ibaraki, Japan) were seeded at a density of 1×10^4 cells per 100 μL per well in DMEM (including 5% horse serum and 10% fetal bovine serum) on a poly-D-lysine-coated 96-well plate, and incubated at 37 $^\circ\text{C}$ under 5% CO_2 for 3 days. After removing the medium, the PC12 cells were washed with 150 μL of serum-free DMEM, and incubated at 37 $^\circ\text{C}$ in 100 μL of DMEM.

(including 0.1% horse serum) containing BODIPYs 1–3 (0.01–10 μ M, 1% methanol). After the incubation for 2 days, the cell viability was measured using the CellTiter-Glo® luminescent cell viability assay.

4.11 Live cell imaging

PC12 cells were seeded and incubated at 37 °C under 5% CO₂ for 3 days similarly to the above. After removing the medium, the PC12 cells were washed with 100 μ L of FluoroBrite DMEM (Thermo Fisher Scientific Inc., MA, USA) containing 0.1% horse serum, and incubated at 37 °C for 1 h in 100 μ L of FluoroBrite DMEM (including 0.1% horse serum) containing BODIPYs 1–3 (0.3 or 10 μ M, 1% methanol). After washing with 100 μ L of FluoroBrite DMEM (including 0.1% horse serum), another 100 μ L of FluoroBrite DMEM was added to the wells, and fluorescence images were taken on a Leica DMI8 (Leica microsystems GmbH, Wetzlar, Germany) with a 40 $\times$ objective lens. A FITC filter set was used for the imaging and the photobleaching assay. Individual cells were selected by ROI (region of interest) for calculation of fluorescence intensity ratios. The photobleaching assay was performed using the setting fluorescence intensity manager (FIM) with the maximum intensity.

Conflicts of interest

There are no conflicts to declare.

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