Electronic Supplementary Material (ESI) for Chemical Communications

Supplementary Information

Rational design of a boron dipyrromethene (BODIPY)-based photobleaching-resistant fluorophore applicable to protein dynamics study

Toru Komatsu[a], Daihi Oushiki[a], Aoi Takeda[a], Masaki Miyamura[c], Tasuku Ueno[a], Takuya Terai[a], Kenjiro Hanaoka[a], Yasuteru Urano[b], Tomoko Mineno[c], and Tetsuo Nagano[*a]

[a]*Graduate School of Pharmaceutical Sciences, the University of Tokyo,7-3-1 Hongo, Bunkyo-ku, Tokyo 113-003*

[b]*School of Medicine, the University of Tokyo,7-3-1 Hongo, Bunkyo-ku, Tokyo 113-003*

[c]*Faculty of Pharmacy, Takasaki University of Health and Welfare, 60 Nakaouri, Takasaki-shi, Gunma 370-0033*

Table of Contents

Methods

Chemical synthesis and characterization. General chemicals were of the best grade available, supplied by Tokyo Chemical Industries, Wako Pure Chemical, Aldrich Chemical Co., and Invitrogen, and were used without further purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on a JEOL JNM-LA300 instrument (300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR) or JEOL JNM-LA400 instrument (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR). δ values are in ppm relative to tetramethylsilane (TMS). Mass spectra (MS) were measured with a JEOL JMS-T100LC AccuTOF (ESI).

Photoirradiation study. For experiments in cuvettes, photoirradiation was performed with an SM-25 Hyper Monolight (single wavelength illumination (5 mW/cm^2) equipped with a 150 W xenon lamp, Bunkoukeiki) at the maximum absorbance wavelength of each fluorophore. Fluorophores were dissolved in PBS (100 mM, pH 7.4), then photoirradiation was performed for 40-120 min, and fluorescence spectra were measured at each time point. For experiments on a 384-well plate, a fiber optic illuminator MAX-301 (band path illumination (1-2 mW/ cm^2) equipped with a 300 W xenon lamp, Asahi Spectra) was used for photoirradiation; 3×3 wells were irradiated at the same time. A combination of blue filters (490 ± 10 nm) and green filters (520 ± 10 nm) was used for photoirradiation to cover the excitation wavelengths of all the BODIPY derivatives. Fluorescence of each well was measured using an EnVision 2103-020 plate reader (Perkin Elmer).

Reaction of BODIPY with singlet oxygen. Aliquots of 10 μL of fluorophores in PBS-MeOH 2:1 (0.1 μM) were prepared on a 384-well plate, and 10 μL of EP-1 in MeOH (200 μM) was added to each well. The plate was sealed and incubated for 12 hrs, and the fluorescence of each well was measured using an EnVision 2103-020 plate reader (Perkin Elmer). Experiments were performed three times, and values were averaged.

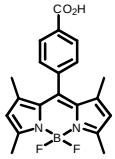
Formation of singlet oxygen. Solutions of fluorophores in MeOH (20 μM) were prepared, and the luminescence spectra around 1268 nm were measured with excitation at the absorbance maximum wavelength using a fluorescence spectrometer, Fluorog-3 (Horiba). In order to fix the value of the protonated/deprotonated ratio, 5% 0.05 N NaOH aq. was added to a solution of 2,6-diCO_2H-BDP, 2,6-diSO_3H-BDP, and fluorescein. The luminescence at 1268 nm was normalized to that of fluorescein.

Cell culture and transfection. COS-7 cells were cultured in DMEM-10% FBS. For imaging, cells were plated on PDL-coated glass-bottomed dish, and transfected with a plasmid of EGFP or (N) SNAP-EGFR, an N-terminal fusion of SNAP-tag with EGF receptor. FuGene-6 (Roche) was used as the transfection reagent, and imaging was performed two days after transfection.

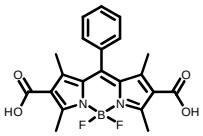
Photobleaching experiment. For labeling of SNAP-tag protein, COS-7 cells were incubated in 10 μM solution of BGFL or BG-diCO_2R-BDP for 30 minutes. After washout three times, cells were fixed with 4% formaldehyde solution to avoid the translocation of proteins during photobleaching, and washed with PBS 0.1% Triton X-100 three times. GFP-transfected cells were fixed in the same way. Fluorescence images were taken on a confocal microscope (TCS-SP5, Leica), and photoirradiation was performed 30 times using the FRAP module of the operating software; the photobleaching was done with the aid of a magnifying sight on the confocal microscope, so that only the limited cell area in the sight was exposed to the light source. Light at 488 nm was used for both excitation and photobleaching (0.04 mW/cm^2 for excitation and 0.2 mW/cm^2 for emission). Photobleaching value (PB %) is the average percentage reduction of fluorescence in the right side of the photobleached images (value ± S. E.).

Supplementary methods for chemical synthesis and characterization of compounds

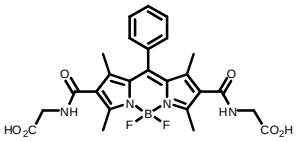
BODIPY library and references



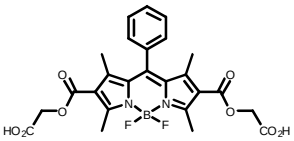
BODIPY



2,6-diCO_2H-BDP

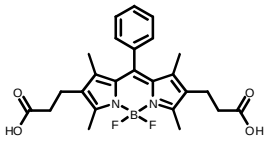


2,6-diCONHR-BDP



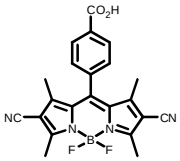
2,6-diCO_2R-BDP

Chem. Commun. 2009, 7015-7017



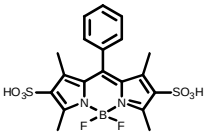
2,6-diP^H-BDP

J. Am. Chem. Soc. 2004, **126**, 3357-3367



2,6-diCN-BDP

J. Am. Chem. Soc. 2006, **128**, 10640-10641

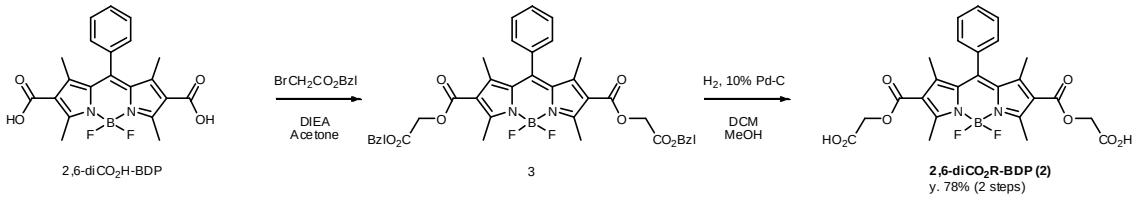


2,6-diSO_3H-BDP

J. Org. Chem. 2008, **73**, 1963-1970

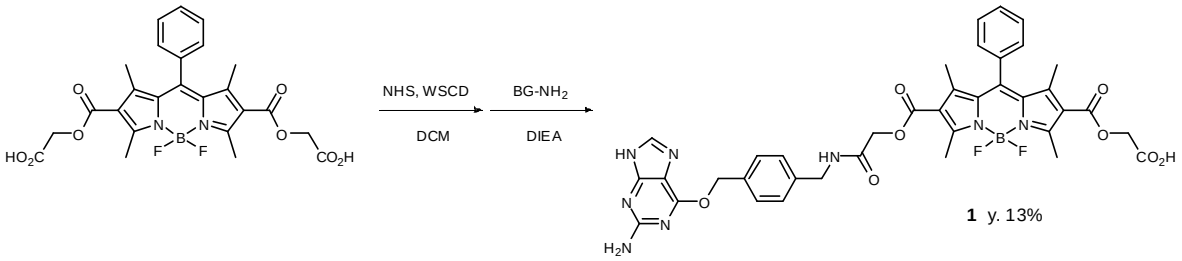
Preparation of BODIPY library

Synthesis and characterization of 2,6-carboxyl-substituted BODIPY derivatives (CO_2H, CONHR, and CO_2R) are described in reference 17. However, the relatively low yield of 2,6-diCO_2R-BDP was problematic, so we optimized the synthetic conditions as follows.



Scheme S2. Synthesis of 2,6-diCO_2R-BDP.

Preparation of 2,6-diCO_2R-BDP (2). 2,6-diCO_2H-BDP (80 mg, 0.19 mmol) was prepared according to reference 17 with some modifications as follows. 2,6-diCO_2H-BDP was dissolved in acetone (30 mL) and DIEA (2 mL). Benzyl bromoacetate (75 μL, 0.45 mmol) was added dropwise, and the solution was refluxed for 15 hr, until TLC monitoring (silica; dichloromethane (DCM)) showed complete formation of the product. Then 100 mL DCM was added. The organic layer was washed with phosphate buffer (pH ~ 7) twice and brine, then dried over Na_2SO_4, filtered, and evaporated. Since the ^{1}H NMR spectrum indicated that the product (**3**) was almost pure, benzyl deprotection was performed without further purification. Compound **3** was dissolved in 20 mL DCM and 10 mL MeOH. After addition of a small amount of 10% Pd-C, the reaction mixture was stirred under a H_2 atmosphere for 3 hr. When TLC monitoring (silica; DCM-2% MeOH-0.2% AcOH) showed that deprotection was complete, the Pd-C was filtered off, and the filtrate was evaporated. The crude product was purified by column chromatography (silica; DCM-2% MeOH-0.2% AcOH) to afford pure **2** as a red solid (78 mg, y. 78%).



Scheme S2. Synthesis of BG-diCO_2R-BDP.

Preparation of BG-diCO_2R-BDP (1). O^6-benzylguanine-NH_2 was prepared according to the reference 21. To a solution of **2** (20 mg, 0.038 mmol) in 20 mL DCM, *N*-hydroxysuccinimide (4.8 mg, 0.042 mmol) and WSCD-HCl (7.3 mg, 0.038 mmol) were added. The reaction mixture was stirred at 40°C for 2 hr, when TLC monitoring confirmed formation of the succinimidyl ester. The reaction mixture was added dropwise to a solution of O^6-benzylguanine-NH_2 (20 mg, 0.074 mmol) in 200 μL *N*,*N*-diisopropyl-*N*-ethylamine (DIEA) and 2 mL of acetonitrile. After the mixture had been stirred at r. t. for 2 hr, DCM was added, and the whole was washed with 0.1 N HCl (aq) and brine. The organic layer was dried over Na_2SO_4, filtered, and evaporated. The crude product was purified by column chromatography (silica; DCM-5% MeOH-0.5% AcOH), and further purified by preparative HPLC to afford **1** as a red solid (3.2 mg, yield 13%). ^{1}H NMR (300 MHz, acetone-d_6) δ 1.54 (s, 3H); 1.59 (s, 3H); 2.65 (s, 3H); 2.68 (s, 3H); 4.34 (d, 2H, *J* = 6.0 Hz); 4.69 (s, 2H); 4.72 (s, 2H); 5.36 (s, 2H); 7.23 (d, 2H *J* = 8.7 Hz); 7.34 (d, 2H, *J* = 8.7 Hz); 7.36 (m, 2H); 7.51 (m, 3H); 7.86 (s, 1H); 7.95 (t, 1H, *J* = 6.0 Hz). LRMS (ESI$^+$): *m/z* calcd. for (M - F)$^+$, 761.2700; found, 761.2660.

Supplementary experiments

Evaluation of electron deficiency of BODIPYs

Calculations of the HOMO/LUMO energy of 2,6-substituted BODIPY derivatives (with neutral substituents) were performed with B3LYP/6-31G(d) Gaussian 03W. Calculated HOMO/LUMO energy was plotted against the Hammett constant of the substituents (σ_p) and a good correlation was obtained (**Figure S1**). On this basis, we used the σ_p value to evaluate the order of electron deficiency of the 7 BODIPY derivatives.

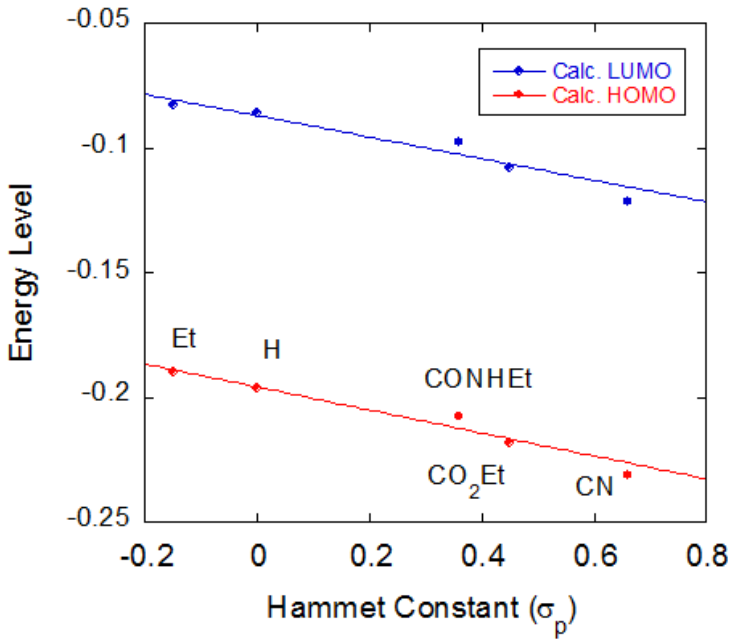


Figure S1. Relationship of calculated HOMO/LUMO energy level (B3LYP) of 2,6-substituted BODIPYs and Hammett constant (σ_p) of substituents.

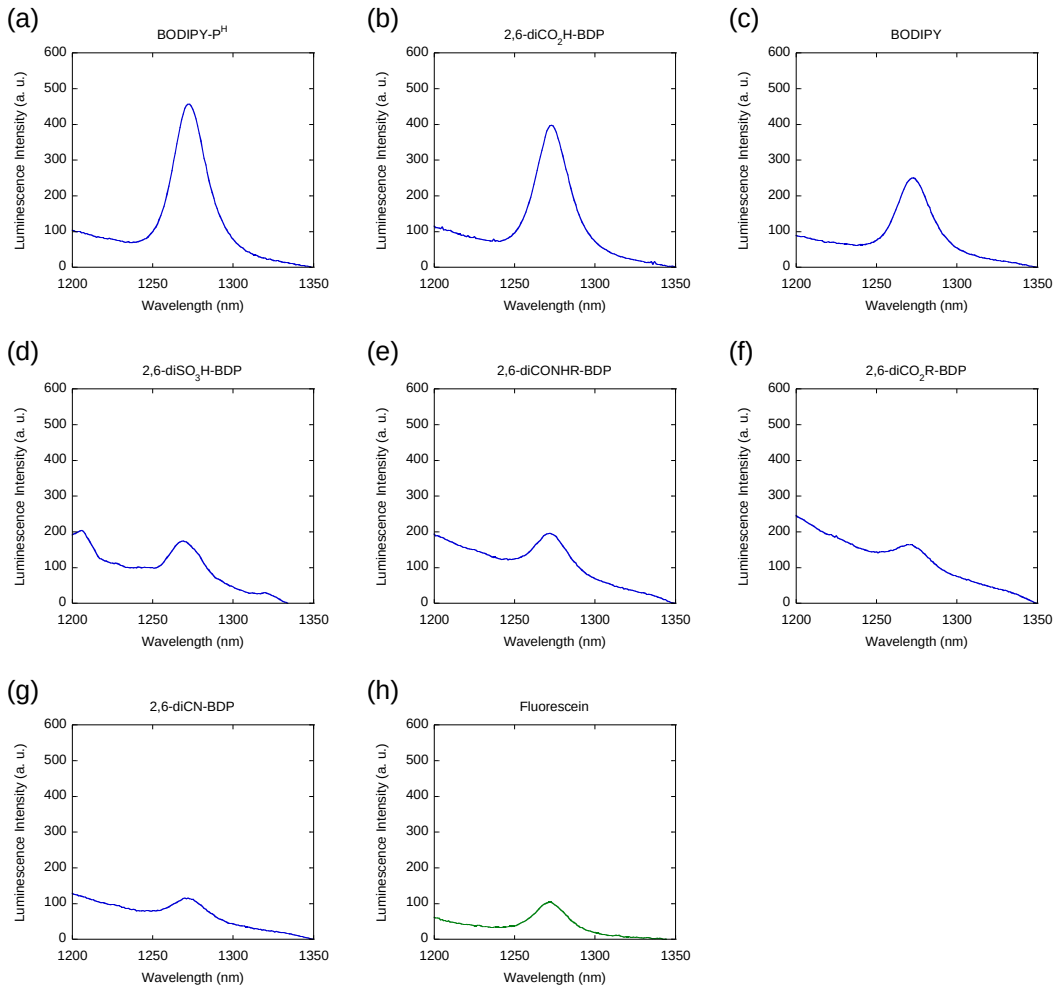


Figure S2. Luminescence spectra (around 1268 nm) of 2,6-substituted BODIPY derivatives in MeOH (20 μM) for determination of singlet oxygen formation upon photoirradiation.

Membrane permeability of 2,6-diSO_3H-BDP and BG-diCO_2R-BDP. 2,6-diSO_3H-BDP (5 μM) was added extracellularly to COS-7 cells, and after 30 min, fluorescence images were taken on a confocal microscope. No fluorescence was detected inside cells, suggesting that 2,6-diSO_3H-BDP was membrane-impermeable (**Figure S3**). A similar experiment was performed for BG-diCO_2R-BDP, and this compound was confirmed to enter the cells.

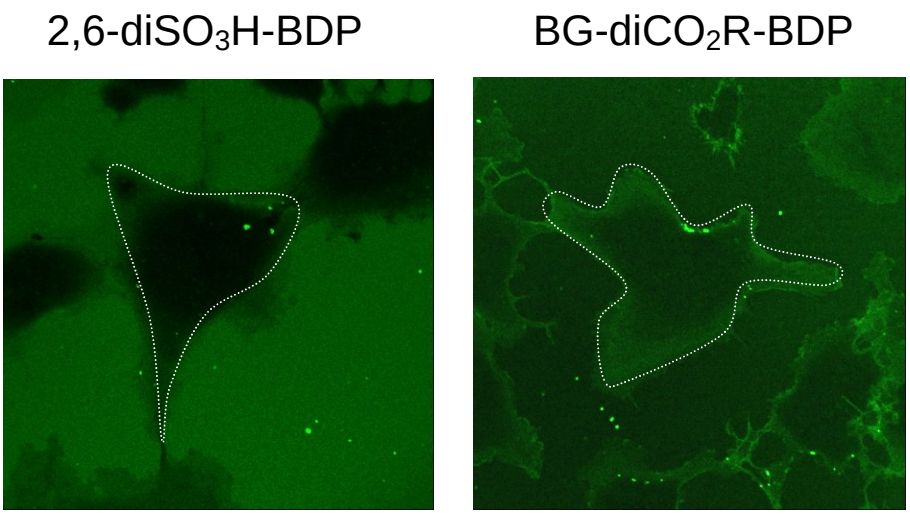


Figure S3. Membrane permeability of 2,6-diSO_3H-BDP and BG-diCO_2R-BDP. Fluorescence images of COS-7 cells incubated with 5 μM dye solution for 30 min are shown. The white dotted lines indicate the outline of a cell.

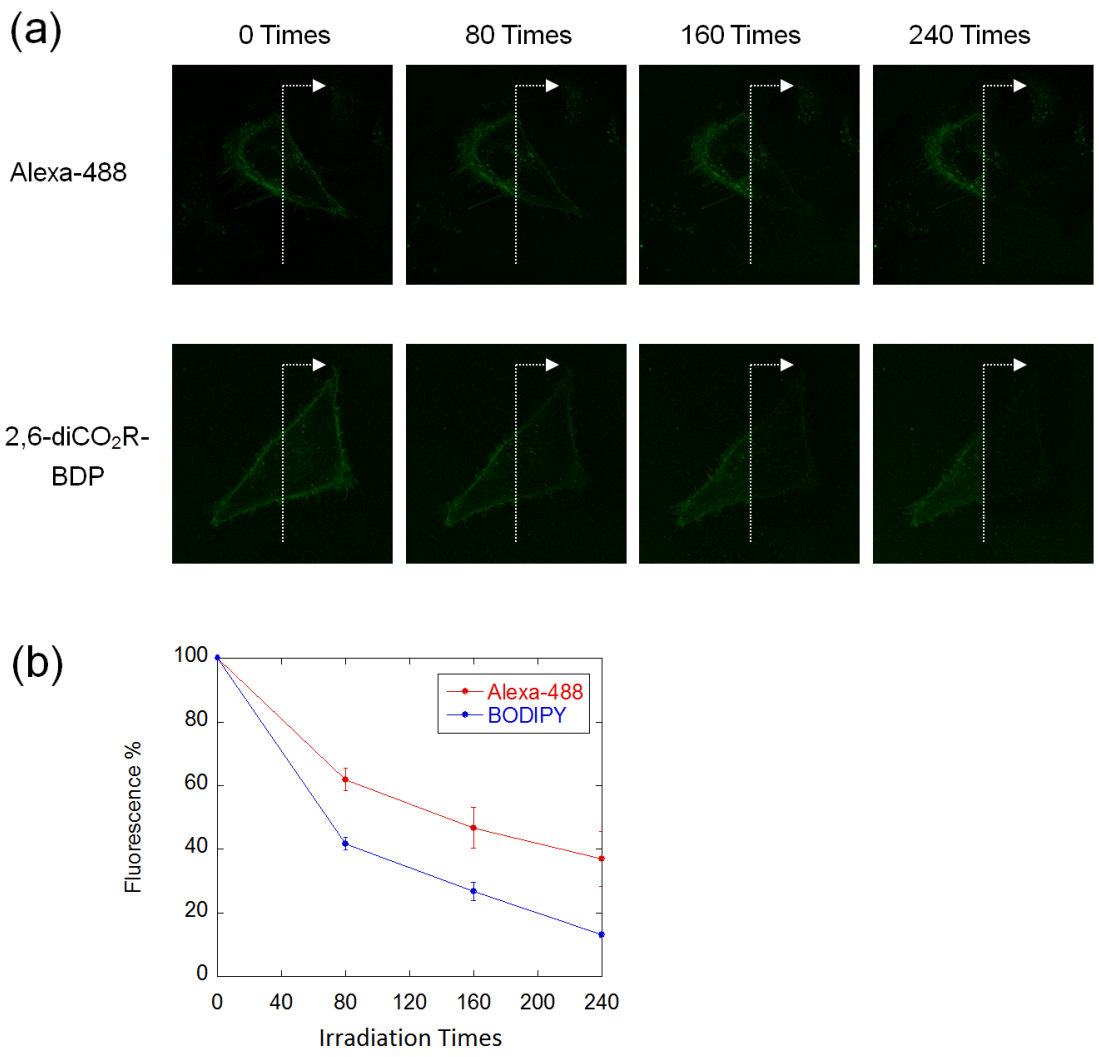


Figure S4. Photobleaching of Alexa-488 (DRBG-488; reference 23) or 2,6-diCO_2R-BDP-labeled SNAP-tagged EGF receptor. Cells were prepared in the same manner as described in the legend to **Figure 3**. Photoirradiation (488 nm; 0.25 mW/cm^2) was performed 80, 160, and 240 times on only the right side of cells, as described in Methods. (a) Fluorescence images of cells. (b) The percentage of photobleaching after the indicated times of photoirradiation. Values are average of three independent experiments for each condition.

Acknowledgements

This research was supported in part by a Grant-in-Aid for Scientific Research (Specially Promoted Research 22000006 to TN) by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Personal Acknowledgements

The first author would like to thank former and current members of the Nagano lab, especially Dr. Yoshimi Tomita, Dr. Daisuke Asanuma, Dr. Nobuhiro Umeda, Masahiro Abo, Tomoya Hirata, and Yukihiko Nakajima for valuable discussions during this research.