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Highly Sensitive Fluorescence Probes for Nitric Oxide Based on Boron Dipyrromethene Chromophore—Rational Design of Potentially Useful Bioimaging Fluorescence Probe

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Abstract: Boron dipyrromethene (BODIPY) is known to have a high quantum yield (ϕ) of fluorescence in aqueous solution but has not been utilized much for biological applications, compared to fluorescein. We developed 8-(3,4-diaminophenyl)-2,6-bis(2-carboxyethyl)-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene (DAMBO-P^H), based on the BODIPY chromophore, as a highly sensitive fluorescence probe for nitric oxide (NO). DAMBO-P^H had a low ϕ value of 0.002, whereas its triazole derivative (DAMBO-P^H-T), the product of the reaction of DAMBO-P^H with NO, fluoresced strongly ($\phi = 0.74$). The change of the fluorescence intensity was found to be controlled by an intramolecular photoinduced electron transfer (PeT) mechanism. The strategy for development of DAMBO-P^H was as follows: (1) in order to design a highly sensitive probe of NO, the reactivity of *o*-phenylenediamine derivatives as NO-reactive moieties was examined using 4,5-diaminofluorescein (DAF-2, a widely used NO fluorescence probe), (2) in order to avoid pH-dependency of the fluorescence intensity, the PeT process was controlled by modulating the spectroscopic and electrochemical properties of BODIPY chromophores according to the Rehm−Weller equation based on measurement of excitation energies of chromophores, ground-state reduction potentials of PeT acceptors (BODIPYs), and calculation of the HOMO energy level of the PeT donor (*o*-phenylenediamine moiety) at the B3LYP/6-31G* level, (3) in order to avoid quenching of fluorescence by stacking of the probes and to obtain probes suitable for biological applications, hydrophilic functional groups were introduced. This strategy should be applicable for the rational design of other novel and potentially useful bioimaging fluorescence probes.

Introduction

In recent years, fluorescence microscopic imaging has made rapid progress as a method for monitoring biomolecules in living cells. Fluorescence probes are essential tools for biological imaging, affording high sensitivity, real-time detection, and simple measurement.[1] A large number of fluorescence probes for detecting biomolecules have been developed, but most of them were obtained not rationally but empirically. We have found that the fluorescence properties of fluorescein derivatives can be controlled by intramolecular photoinduced electron transfer (PeT),[2] and we succeeded in designing and synthesizing novel fluorescence probes based on the PeT mechanism,[3] such as DMAX for singlet oxygen.[4] If such a PeT-dependent fluorescence off/on switching mechanism is applicable to other fluorophores as well as to fluorescein derivatives, it should allow the development of novel and useful fluorescence probes for various biomolecules.

Nitric oxide (NO) plays an important role in human physiology as an intra- and extracellular messenger molecule.[5] Since NO is unstable and is produced at low concentrations, it is quite difficult to detect NO sensitively in living cells. Several years ago, we developed fluorescent indicators for NO, diaminofluoresceins (DAFs).[6] DAFs themselves are nonfluorescent but are converted to the highly fluorescent triazole forms by reaction with NO in the presence of oxygen. Among the DAFs, 4,5-diaminofluorescein (DAF-2) is widely used for real-time biological imaging of NO.[7]

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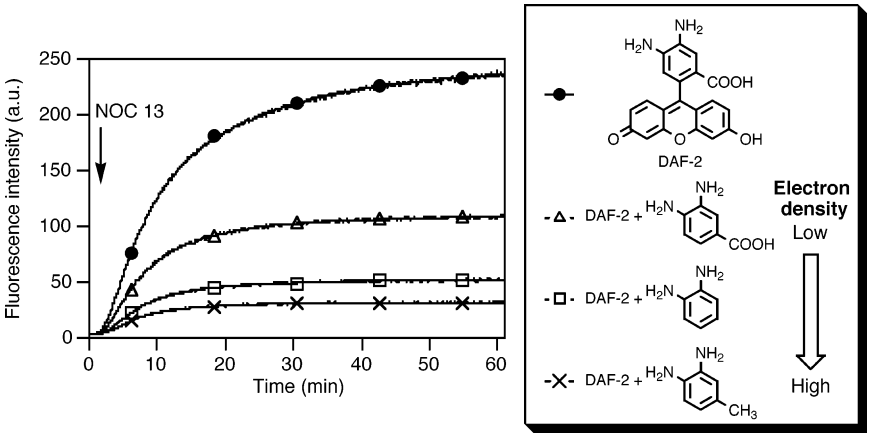


Figure 1. Inhibitory effect of *o*-phenylenediamine derivatives on the fluorescence increase in the reaction of DAF-2 with NO. The fluorescence intensity in the reaction of DAF-2 (5 μM) with NOC 13 (5 μM) was determined at 515 nm with excitation at 495 nm in the presence or in the absence of some *o*-phenylenediamine derivatives (5 μM) in 0.1 M sodium phosphate buffer (pH 7.4), containing 0.2% DMSO as a cosolvent. NOC 13 was added at the point indicated by the arrow.

However, in living cells NO is not only released at very low levels but also is lost through reactions with biomolecules such as thiols, so that there is a requirement for NO probes with higher sensitivity. More sensitive probes would need to have higher reactivity than DAF-2 with NO.

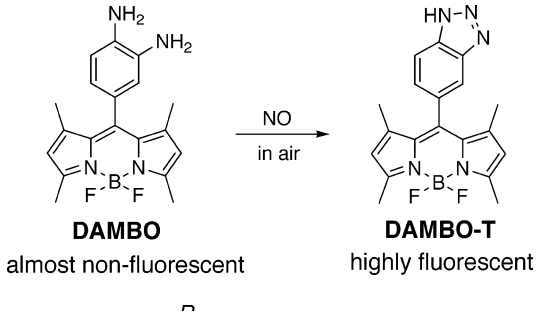
We report herein the design and synthesis of boron dipyrromethene (BODIPY)-based fluorescence NO probes that are more sensitive than DAFs. BODIPYs are of interest as chromophores due to their desirable photophysical properties.[8−10] It is also easy to modify BODIPY chemically for preparation of various derivatives. Daub et al. studied in detail the photophysical behavior of aza crown-substituted BODIPY and its analogue by means of steady-state and time-resolved fluorometry.[11] However, although fluorescein-based biological probes such as Fluo-3 (Ca^{2+} probe),[1b] DAF-2 (NO probe), and ZnAF-2 (Zn^{2+} probe)[3a] are commercially available, BODIPY-based functional probes are not yet available for biological use.

We report here the development of a pH-independent and highly sensitive fluorescence probe for NO based on the BODIPY structure.

Results and Discussion

Design of Highly Sensitive Fluorescence Probes for Nitric Oxide. DAF-2 is a commercially available NO probe, and its diester (DAF-2 DA) is useful for bioimaging of NO in living cells. First of all, we examined a way to improve DAF-2 from the viewpoint of sensitivity. The reactivity of DAFs with NO is considered to be dependent on the electron density of the *o*-phenylenediamine moiety. Figure 1 shows the degree of inhibition of the fluorescence increase due to the product (DAF-2 T) in the reaction of DAF-2 with an NO donor (NOC 13)[12] in the presence of various *o*-phenylenediamine derivatives.

Scheme 1. Reaction of DAMBO with NO



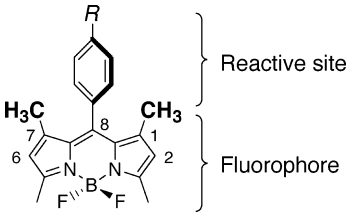


Figure 2. Structure of BODIPY with an aryl moiety. The aryl moiety (reactive site) and BODIPY (fluorophore) are twisted.

o-Phenylendiamine with electron-donating substituents caused greater inhibition of the fluorescence increase, which indicates that the reactivity toward NO is determined by the electron density of reactive sites in the *o*-phenylenediamine moiety. *o*-Phenylenediamine derivatives with high electron density are essential for improvement of the NO probe's sensitivity. DAF-2 possesses a serious disadvantage in this regard, as the *o*-phenylenediamine moiety of DAF-2 has an electron-withdrawing carboxyl functional group. However, chemical modification to remove the carboxyl functional group from the fluorescein structure of DAF-2 is impractical because 6-hydroxy-9-phenylfluorone has a low quantum yield of fluorescence (ϕ) of around 0.2.[13] Thus, a different fluorescence platform is required to design highly sensitive NO probes.

The BODIPY structure appears to be a fluorophore with potential for high sensitivity, since BODIPYs generally have high extinction coefficients and high quantum efficiencies in water as well as in organic solvents.[8] For example, 1,3,5,7-tetramethyl-8-phenyl-BODIPY is highly fluorescent.[11] It is easy to design and synthesize BODIPY-based fluorescence probes

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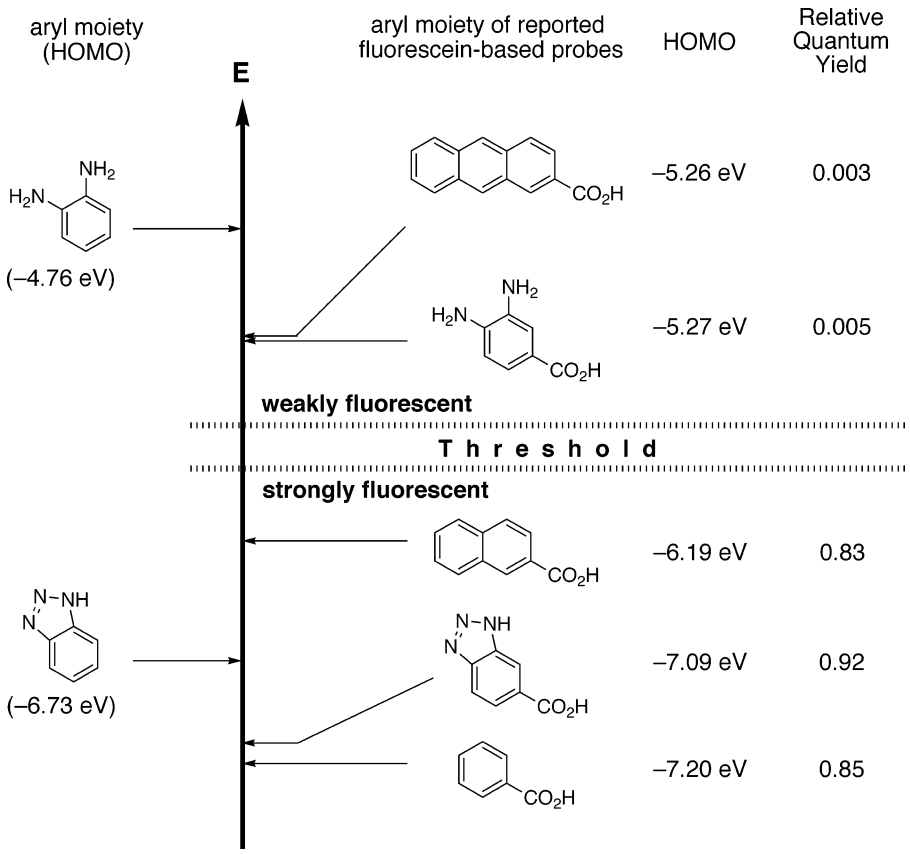
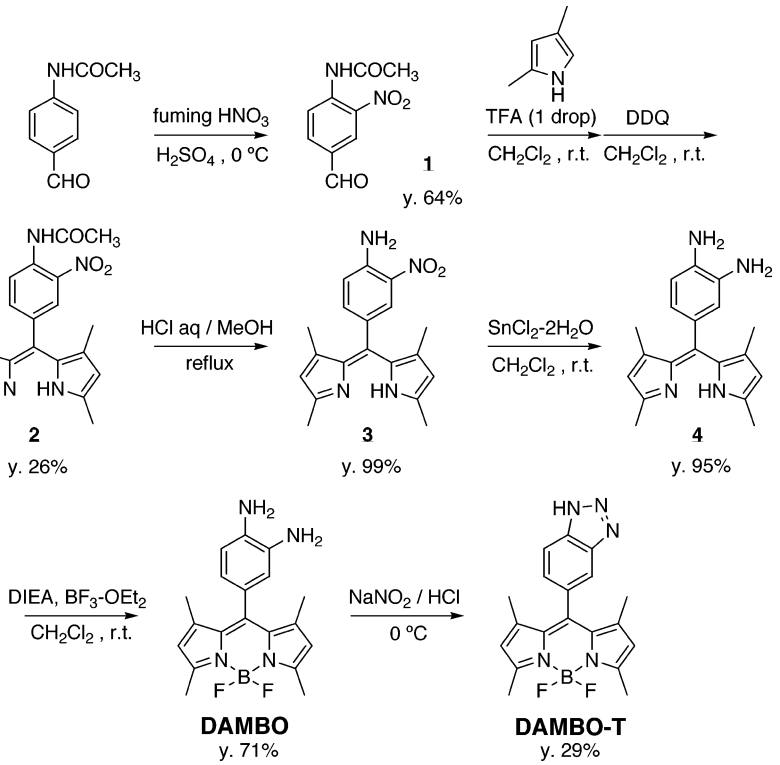


Figure 3. HOMO energy level of the aryl moiety. These values were obtained from B3LYP/6-31G* calculations.

Scheme 2. Synthetic Scheme of DAMBOs



with an electron-rich reactive site for NO. In addition, derivatives of BODIPY can emit fluorescence over a wide range from 500 to 700 nm.[14] Thus, BODIPY seems to be a promising platform for sensitive fluorescence probes.

We designed a diaminobenzene-BODIPY derivative (DAMBO, Scheme 1) which does not have any electron-withdrawing functional group on *o*-phenylenediamine. Owing to the presence of the methyl groups at the C-1 and C-7 positions, the diamino-

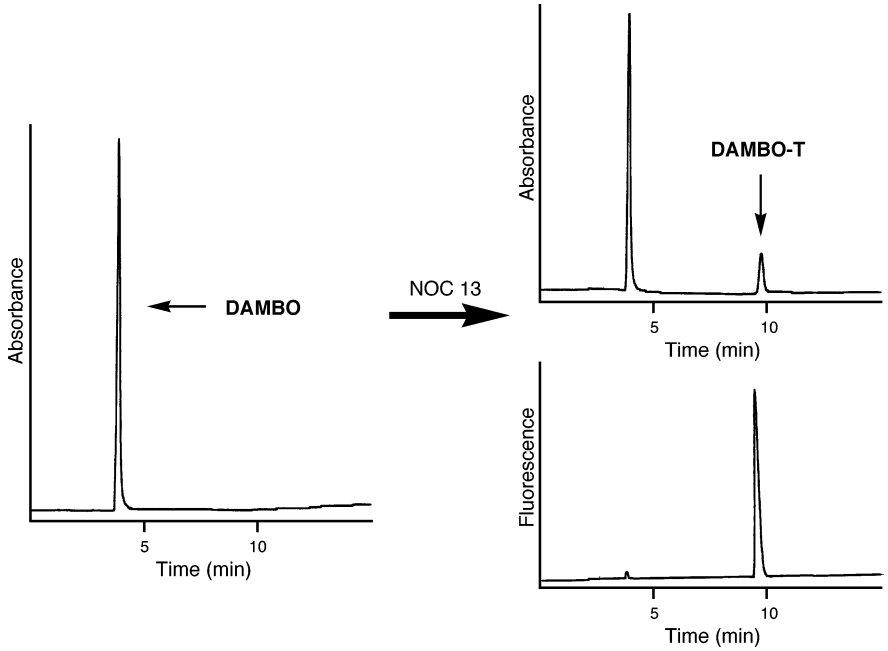


Figure 4. HPLC chromatogram in the reaction of DAMBO with NOC 13. The reaction of DAMBO (5 μM, 0.1% DMSO) with NOC 13 (20 μM) was carried out in 0.1 M sodium phosphate buffer (pH 7.4) at 37 °C for 1 h. The product was detected using HPLC: Eluent: CH_3CN/0.1% H_3PO_4 aq = 3/2; flow rate = 1.0 mL/min; detection wavelength = 495 nm (UV−Vis), 495/515 nm (fluorescence).

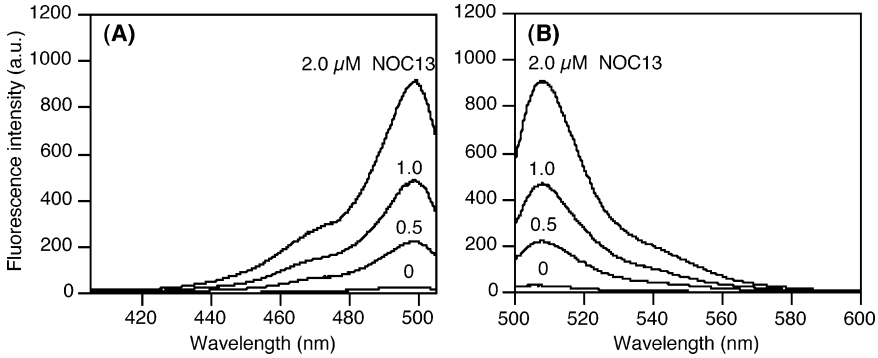


Figure 5. (A) Excitation spectra (emission at 510 nm) and (B) emission spectra (excitation at 495 nm) of DAMBO (5 μM, 0.1% DMSO) in 0.1 M sodium phosphate buffer (pH 7.4) 1 h after the reaction of various concentrations of NOC 13 ranging from 0 to 2.0 μM.

benzene moiety and BODIPY moiety are twisted and conjugatively uncoupled (Figure 2).[9] This steric structure is similar to that of fluorescein derivatives whose fluorescence properties could be controlled by the PeT mechanism. The free energy change of the PeT process can be described by the Rehm−Weller equation[15] where $E_{1/2}$ (D^+/D) and $E_{1/2}$ (A/A^-) are the

$$\Delta G_{PeT} = E_{1/2}(D^+/D) - E_{1/2}(A/A^-) - \Delta E_{00} - C$$

ground-state oxidation potential of the donor and the reduction potential of the acceptor, respectively, ΔE_{00} is the excitation energy, and C is the electrostatic interaction term.

Since the reduction potential and the excitation energy of 1,3,5,7-tetramethyl-BODIPY (BODIPY 505/515; absorbance maximum: 501 nm, reduction potential: −1.16 V vs SCE) were nearly the same as those of fluorescein,[16] the threshold of fluorescence off/on in BODIPY was expected to be similar to that in fluorescein derivatives. Thus, according to the calculation of the highest occupied molecular orbital (HOMO) energy level of the diaminobenzene moiety at the B3LYP/6-31G* level, *o*-phenylenediamine in DAMBO has a sufficiently high HOMO energy level (−4.76 eV) to make DAMBO nonfluorescent. On the other hand, triazolobenzene had a lower HOMO energy level (−6.73 eV), which suggests that DAMBO-T (triazole form), the reaction product with NO, should be highly fluorescent (Figure 3,[4] Scheme 1).

Synthesis, Fluorescence Properties, and Sensitivity of DAMBO. DAMBO was synthesized from 2,4-dimethylpyrrole and the appropriate aldehyde through four steps (Scheme 2). As expected, DAMBO reacted with NO to yield highly fluorescent DAMBO-T without any byproduct, as confirmed by HPLC (Figure 4), and the fluorescence intensity increased proportionally to the concentration of NOC 13 (Figure 5). The absorbance and fluorescence properties of DAMBO are sum-

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Table 1. Absorbance and Fluorescence Properties of DAMBOs at pH 7.4[a]

compound	absorbance max(nm)	extinction coefficient ($\times 10^4$ $M^{-1}cm^{-1}$)	emission max (nm)	relative quantum yield[b]
DAMBO	496	7.3	505	0.001
DAMBO-T	498	5.2	507	0.40
DAMBO-P^H	519	7.3	535	0.002
DAMBO-P^H-T	521	5.6	537	0.74

[a] All data were measured in 0.1 M sodium phosphate buffer (<0.2% DMSO as a cosolvent). [b] Quantum yield of fluorescence was determined using that of fluorescein (0.85) in 0.1 M NaOH aq. as a standard.

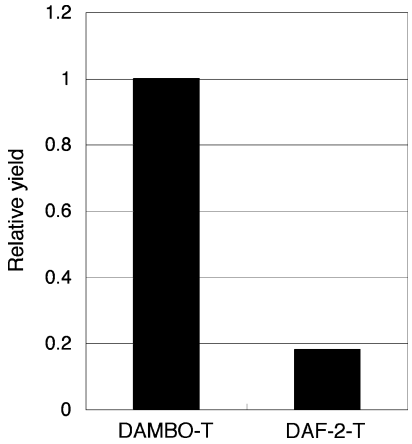


Figure 6. Relative yields of the corresponding triazoles in the competitive reaction of DAMBO and DAF-2 (5 μM, 0.1% DMSO) with NOC 13 (10 μM) in 0.1 M sodium phosphate buffer (pH 7.4). The triazoles were quantified by means of HPLC after reaction for 1 h. Eluent: CH_3CN/10 mM sodium phosphate buffer (pH 7.4) = 3/2 (DAMBOs), 6/94 (DAF-2s); flow rate = 1.0 mL/min; detection wavelength = 495 nm (UV−Vis), 495/515 nm (fluorescence).

marized in Table 1. Conversion of the diamino form to the triazole form by reaction with NO caused little change of the absorbance maxima but greatly increased the quantum efficiency, a characteristic feature of PeT probes.

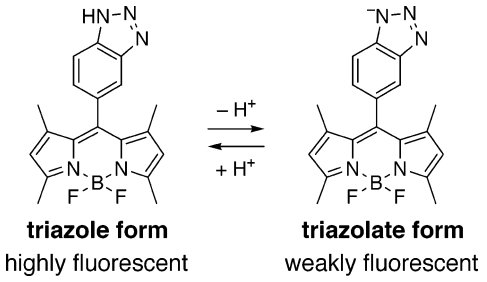
Figure 6 shows a comparison of sensitivity between DAF-2 and DAMBO. In a competitive reaction, the reaction efficiency of DAMBO with NO to afford the corresponding fluorescent triazole form was about 6 times higher than that of DAF-2. These results show that raising the electron density of the reaction site is effective as a strategy to acquire higher sensitivity to NO.

Table 2. Fluorescence Increase of DAMBO after Reaction with Several ROS, Nitrite, and Nitrate[a]

reactant	after 30 min reaction	NO was added after the reaction[b]
NO[b]	280	
·OH[c]	3	250
H_2O_2[d]	2	240
NO_2^- [e]	3	270
NO_3^- [f]	2	250

[a] DAMBO (final 5 μM, 0.1% DMSO) was added to 0.1 M sodium phosphate buffer (pH 7.4). The fluorescence intensity was determined at 512 nm with excitation at 495 nm. [b] NO solution (15 μL, final 9.5 μM) was added and stirred at 37 °C for 30 min. [c] Ferrous perchlorate (100 μM) and H_2O_2 (1 mM) were added, and the mixture was stirred at 37 °C for 30 min. [d] H_2O_2 (1 mM) was added and stirred at 37 °C for 30 min. [e] $NaNO_2$ (100 μM) was added and stirred at 37 °C for 30 min. [f] $NaNO_3$ (100 μM) was added and stirred at 37 °C for 30 min.

Scheme 3. Protonated and Deprotonated Forms of DAMBO-T



Though DAMBO had higher reactivity, its selectivity for NO over various reactive oxygen species remained high. Even hydroxyl radical, the most potent oxidant among reactive oxygen species, did not generate fluorescence, and DAMBO emitted fluorescence only when reacted with NO (Table 2).

As we reported previously,[17] *o*-phenylenediamine derivatives actually react not with NO radical but with NO^+ species to yield triazole forms. Thus, probes bearing *o*-phenylenediamine derivatives as a reactive site are NO^+ probes in the strict sense. However, we consider that a fluorescence increase with our DAFs or DAMBOs at least qualitatively reflects NO production, because NO^+ species are mostly produced through very rapid oxidation of NO radical. Of course, especially in biological applications, it is desirable to use specific nitric oxide synthase inhibitors to confirm that the fluorescence increase is dependent upon the production of NO radical.

As shown in Figure 7, DAMBO could be used as a probe even in acidic aqueous media, while most fluorescein-based

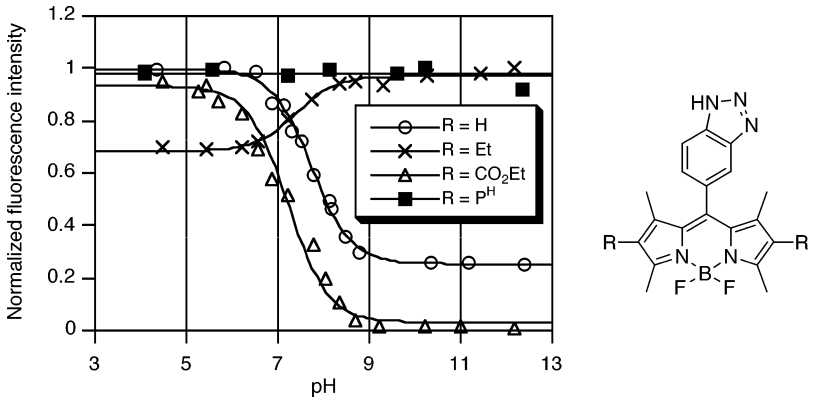


Figure 7. Effect of pH on the fluorescence intensity of DAMBO-R-Ts (R = H, P^H; 0.5 μM, 0.1% DMSO, R = Et, CO_2Et; 50 nM, 0.5% DMSO) in sodium phosphate buffer (0.1 M). The fluorescence intensities of DAMBO-T, DAMBO-Et-T, DAMBO-CO_2Et-T, and DAMBO-P^H-T were determined at 512, 535, 507, and 535 nm with excitation at 495, 520, 495, and 520 nm, respectively.

Scheme 4. Synthetic Scheme of BODIPY Derivatives

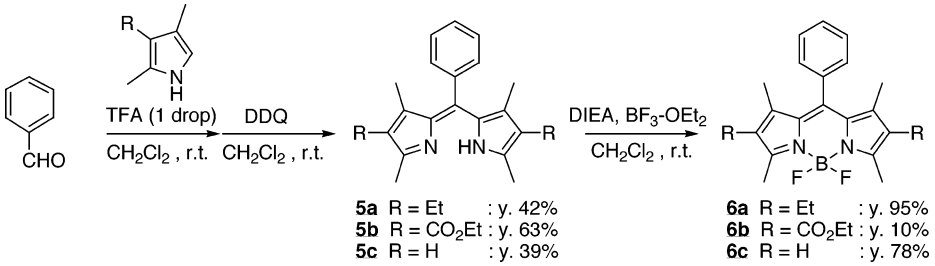


Table 3. Spectroscopic and Electrochemical Properties of BODIPY Derivatives[a]

compound	absorbance max (nm)	emission max (nm)	relative quantum yield[b]	$E_{1/2}$ (A/A$^-$) (V) vs SCE[c]	relative calcd ΔG_{PeT} (eV)[d]
6a	520	534	0.67	−1.27	+ 0.19
6b	496	507	0.41	−0.87	−0.33
6c	497	507	0.48	−1.19	

[a] Measured in acetonitrile (0.1% DMSO as a cosolvent). [b] Quantum yield of fluorescence was determined using that of fluorescein (0.85) in 0.1 M NaOH aq as a standard. [c] Measured in acetonitrile. Scan rate: 0.1 V s^{-1}. [d] Calculated relative ΔG_{PeT} values to that of **6c** as a standard.

probes lose fluorescence owing to lactonization reaction under acidic conditions.[18] However, Figure 7 shows that the fluorescence of DAMBO-T strikingly decreased at pH above 7. The triazolate (the deprotonated form of the triazole) of DAMBO-T was expected to be formed at pH above 7, because the pK_a value of benzotriazole is reported to be 8.27[19] and that of DAMBO-T was calculated to be 7.7 (Scheme 3). Change of fluorescence intensity around neutral pH is a serious problem and undesirable for biological imaging.

Design and Synthesis of pH-Independent NO Probe, DAMBO-P^H. The fluorescence quenching of DAMBO-T at pH above 7 can be explained by the following mechanism, i.e., the PeT process becomes accelerated because of the increased electron-donating ability of the triazolate functional group compared to the triazole functional group. The free energy change of the PeT process is determined not only by the electron-donating ability of reactive sites (benzotriazolate in this case), but also by the reduction potential and the excitation energy of the fluorophore according to the Rehm−Weller equation. Thus, to avoid the occurrence of PeT in the benzotriazolate form, we synthesized BODIPY derivatives with various substituents at the C-2 and C-6 positions to change the reduction potential and the excitation energy of the fluorophore (Scheme 4).

Introducing ethyl functional groups made the reduction potential more negative and the excitation energy smaller, while introducing ethoxycarbonyl functional groups provided the opposite results (Table 3). The fluorescence and electrochemical properties of BODIPY derivatives[9,20] were markedly changed by structural modification. The relative ΔG_{PeT} values of the two BODIPY derivatives were calculated with respect to the value for the BODIPY derivative nonsubstituted at the C-2 and C-6 positions (**6c**) as a standard. The changes of the relative ΔG_{PeT} due to the substituents at the C-2 and C-6 positions were calculated as +0.19 and −0.33 eV for **6a** and **6b**, respectively. Since the HOMO energy level of the triazolate form of DAMBO-T was located just on the threshold, the value of +0.19 eV was considered to be large enough to prevent the PeT process thermodynamically (Figure 8). In other words, the triazolate form of ethyl-substituted DAMBO-T should be highly fluorescent, while that of DAMBO-T is only weakly fluorescent.

DAMBO-T derivatives with ethyl or ethoxycarbonyl substituents at the C-2 and C-6 positions (DAMBO-R-Ts) were synthesized according to the route shown in Scheme 5. Surprisingly, the pH profiles of fluorescence of DAMBO-R-Ts show that DAMBO-Et-T was extremely fluorescent even under basic conditions (ϕ value = 0.59 at pH 7.4), independently of the pH at above 7 (Figure 7). On the contrary, the fluorescence of DAMBO-CO_2Et-T, with a more negative ΔG_{PeT} value, completely disappeared under basic conditions. As shown in Figure 8, benzotriazolate does not undergo an electron transfer to BODIPY with the Et functional group at R, while it does with BODIPY with the CO_2Et functional group at R. At pH above 7, fluorescence off/on switching could be controlled by the reaction with NO. However, there was another obstacle to the development of a novel and sensitive NO probe. The fluorescence of DAMBO-Et-T decreased slightly under acidic conditions at pH below 7, where most of DAMBO-Et-T is in the triazole form, not the triazolate form. The degree of the intensity decrease depended upon the concentration of DAMBO-Et-T (Figure 9), which suggests that the triazole form of DAMBO-Et-T was stacking in the aqueous solution, leading to the decrease of the fluorescence intensity. The BODIPY chromophore substituted with ethyl functional groups at the C-2 and C-6 positions is highly hydrophobic, while the triazolate form may not stack because of the rather hydrophilic monoanion. Thus, we designed and synthesized DAMBO-P^H with 2-carboxyethyl functional groups at the C-2 and C-6 positions (Scheme 6). Carboxyl functional groups should be effective for preventing stacking due to their higher hydrophilicity, and ethylene chains should serve to block the PeT process by changing the reduction potential and excitation energy.

The absorbance and fluorescence properties of DAMBO-P^H and DAMBO-P^H-T are shown in Table 1. The fluorescence of DAMBO-P^H-T was completely independent of pH from 3 to 13 (Figure 7), and the quantum efficiency was higher than that of DAMBO-T. Further, the Stokes shift was larger than that of DAMBO-T, which is very desirable for avoidance of light scattering and for reducing the self-absorption. Regarding sensitivity, the reaction efficiency of DAMBO-P^H with NO was higher than that of DAF-2 and the fluorescence increases of

(17) Kojima, H.; Sakurai, K.; Kikuchi, K.; Kawahara, S.; Kirino, Y.; Nagoshi, H.; Hirata, Y.; Akaike, T.; Maeda, H.; Nagano, T. *Biol. Pharm. Bull.* **1997**, *20*, 1229−1232.
(18) Chen, S.-C.; Nakamura, H.; Tamura, Z. *Chem. Pharm. Bull.* **1979**, *27*, 475−479.
(19) Wang, H.; Bruda, C.; Persy, G.; Wirz, J. *J. Am. Chem. Soc.* **2000**, *122*, 5849−5855.
(20) Kollmannsberger, M.; Rurack, K.; Resch-Genger, U.; Rettig, W.; Daub, J. *Chem. Phys. Lett.* **2000**, *329*, 363−369.

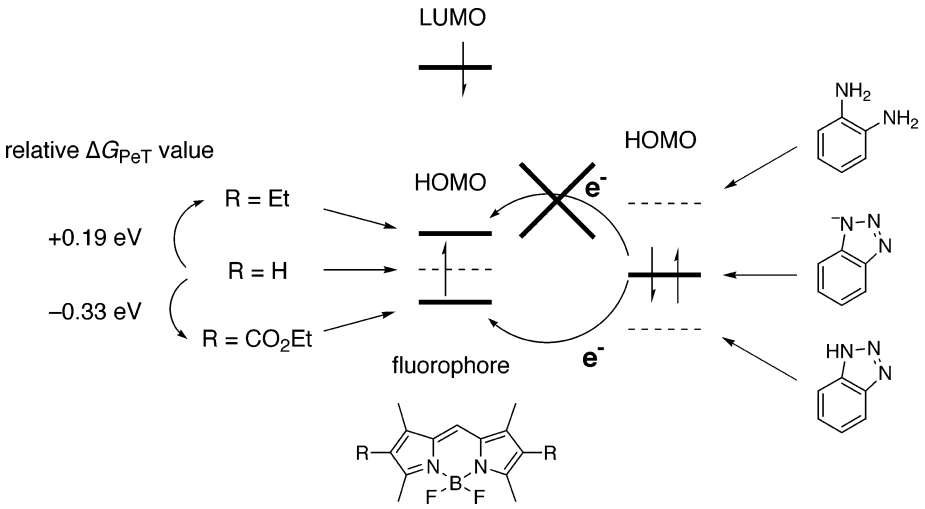
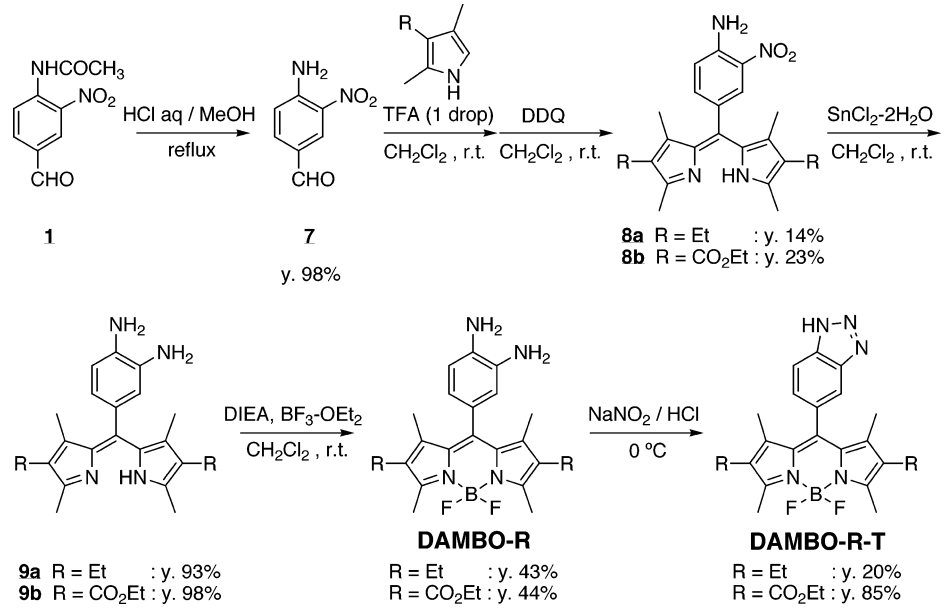


Figure 8. Schematic PeT process.

Scheme 5. Synthetic Scheme of DAMBO-Rs



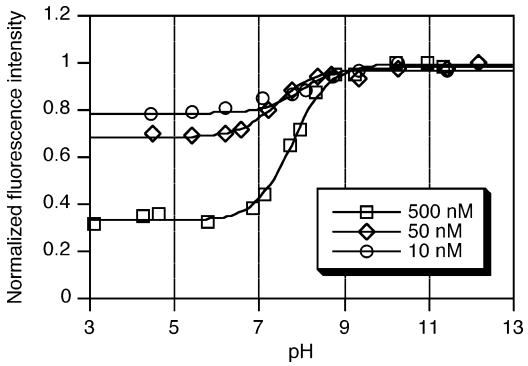


Figure 9. Effect of pH on the fluorescence intensity of DAMBO-Et-T (0.5% DMSO as a cosolvent) in sodium phosphate buffer (0.1 M). The fluorescence intensities were determined at 535 nm with excitation at 520 nm.

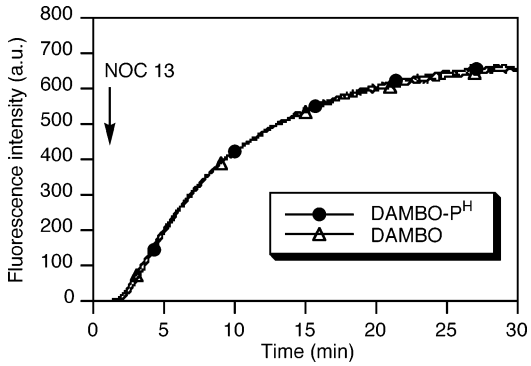


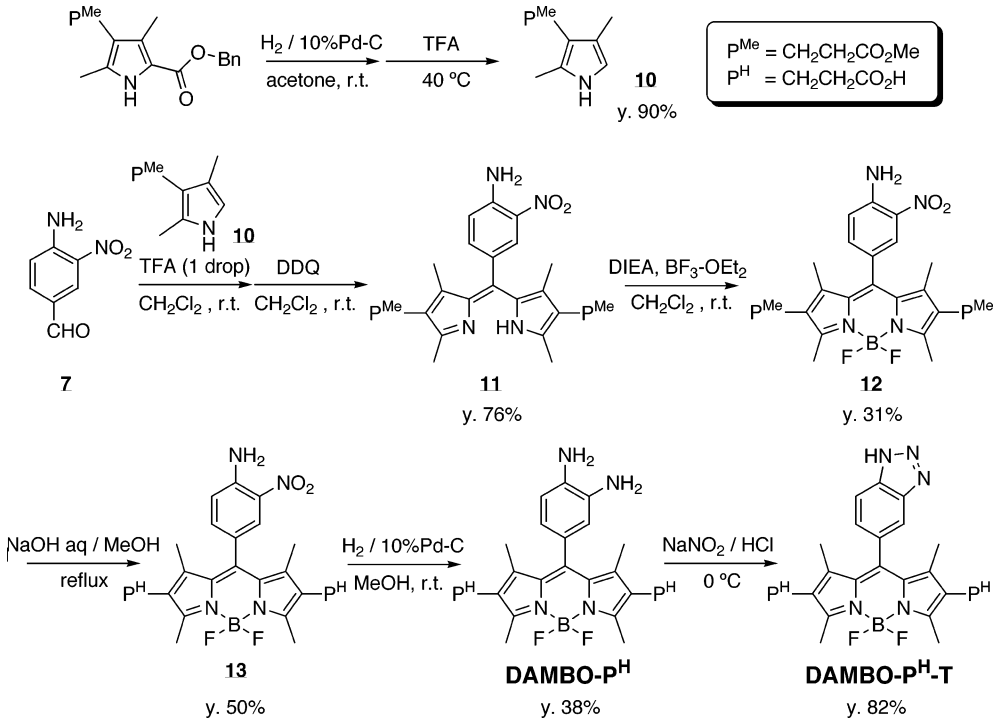
Figure 10. Fluorescence increase with DAMBOs (5 μM, 0.1% DMSO) upon reaction with NOC 13 (20 μM) in 0.1 M sodium phosphate buffer (pH 7.4). The fluorescence intensities of DAMBO-P^H and DAMBO were determined at 535 and 512 nm with excitation at 520 and 495 nm, respectively. NOC 13 was added at the point indicated by the arrow.

DAMBO-P^H and DAMBO were almost the same (Figure 10). We thus succeeded in the development of a highly sensitive and pH-independent NO probe, DAMBO-P^H.

Very recently, Zhang et al. reported a compound whose proposed structure was the same as that of one of our DAMBO derivatives.[21] However, their compounds were not well characterized, and the fluorescence properties were in marked

(21) Zhang, X.; Wang, H.; Li, J.-S.; Zhang, H.-S. *Anal. Chem. Acta* **2003**, *481*, 101−108.

Scheme 6. Synthetic Scheme of DAMBO-P^{H}s



contrast to those of our compounds. We characterized our compounds in detail by means of ^{1}H NMR, ^{13}C NMR, high-resolution mass spectroscopy, and elemental analysis, and their fluorescence properties could be well interpreted in terms of PeT theory. Moreover, other diamino BODIPYs reported in this paper also showed fluorescence properties similar to those of DAMBO, so we consider that DAMBO and its derivatives are novel fluorescence probes for NO.

The probe, DAMBO-P^H, can be used in buffer solution, so it should be potentially useful for detection of NO in biological applications.

Conclusion

BODIPY is a well-known chromophore that is highly fluorescent in almost all solvents but has not been utilized much in biological functional probes.

We found that the fluorescence properties of BODIPYs can be controlled through a PeT-dependent fluorescence off/on switching mechanism, and we succeeded in developing DAMBO-P^H as a sensitive probe for NO, based on the following strategy: (1) raising the electron density of the reaction site in order to acquire higher sensitivity for NO, (2) optimizing the PeT process by modulating the spectroscopic and electrochemical properties of the BODIPY chromophore to avoid the pH dependency of fluorescence intensity in the pH region of biological interest, and (3) introducing carboxyl functional groups to increase hydrophilicity, thereby preventing the quenching of fluorescence by stacking. Further studies on biological applications of DAMBO-P^H are in progress.

A similar rational design strategy may also be applicable to other fluorophores, such as cyanine dye, in addition to BODIPY, for the development of various biofunctional and practical fluorescence probes.

Experimental Section

Materials. General chemicals were of the best grade available, supplied by Tokyo Chemical Industries, Wako Pure Chemical, or Aldrich Chemical Co., and used without further purification. Special chemicals were dimethyl sulfoxide (DMSO, fluorometric grade, Dojindo) and tetrabutylammonium perchlorate (TBAP, electrochemical grade, dried over P_2O_5 before use, Fluka). All solvents were used after appropriate distillation or purification.

Instruments. NMR spectra were recorded on a JEOL JNM-LA300 instrument at 300 MHz for ^{1}H NMR and at 75 MHz for ^{13}C NMR. Mass spectra (MS) were measured with a JEOL SX-102A for EI and a JEOL JMS-T100LC AccuTOF for ESI. UV−visible spectra were obtained on a Shimadzu UV-1600. Fluorescence spectroscopic studies were performed on a Hitachi F4500. Determinations of pH profile of fluorescence and time course of fluorescence increase upon reaction with NO were performed on a Perkin-Elmer LS-50B.

Synthesis of 4-Acetamido-3-nitrobenzaldehyde (1). Fuming nitric acid (1.0 mL) was carefully added dropwise to concentrated sulfuric acid (6 mL) at 0 °C. 4-Acetamidobenzaldehyde (1.91 g, 11.7 mmol) was added to the solution at 0 °C. At the end of addition, the reaction mixture was poured onto ice. The precipitated yellow powder was collected by filtration, washed with cold water, dried, purified by silica gel chromatography (CH_2Cl_2), and recrystallized from water to afford light yellow needles (1.57 g, yield 64%). ^{1}H NMR ($CDCl_3$): δ 2.36 (s, 3H); 8.16 (dd, 1H, J = 1.7, 8.8 Hz); 8.74 (d, 1H, J = 1.7 Hz); 9.04 (d, 1H, J = 8.8 Hz); 9.99 (s, 1H); 10.63 (s, 1H). MS (EI): 208 (M^+). Mp: 156 °C.

Synthesis of 6-(4-Acetamido-3-nitrophenyl)-3,3′,5,5′-tetramethylpyrromethene (2). 1 (953 mg, 4.58 mmol) and 2,4-dimethylpyrrole (0.94 mL, 9.16 mmol) were dissolved in 250 mL of absolute CH_2Cl_2 under an Ar atmosphere. One drop of trifluoroacetic acid (TFA) was added, and the solution was stirred at room temperature overnight. When TLC monitoring (silica; CH_2Cl_2) showed complete consumption of the aldehyde, a solution of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (1.07 g, 4.58 mmol) in CH_2Cl_2 was added, and stirring was continued for 15 min. The reaction mixture was washed with water, dried over $MgSO_4$, filtered, and evaporated. The crude compound was purified by column chromatography on aluminum oxide (CH_2Cl_2) to afford a brown powder (442 mg, yield 26%). ^{1}H NMR ($CDCl_3$): δ 1.35 (s, 6H); 2.33 (s, 3H); 2.35 (s, 6H); 5.91 (s, 2H); 7.60 (dd, 1H, J = 2.0, 8.6 Hz); 8.20 (d, 1H, J = 2.0 Hz); 8.93 (d, 1H, J = 8.6 Hz); 10.46 (s, 1H). HRMS (EI^+): calcd for M^+, 378.1692; found, 378.1696.

Synthesis of 6-(4-Amino-3-nitrophenyl)-3,3′,5,5′-tetramethylpyrromethene (3). 2 (285 mg, 0.75 mmol) was dissolved in 20 mL of MeOH, then 20 mL of 1 N HCl was added, and the mixture was refluxed at 100 °C for 1 h. The reaction mixture was neutralized with 2 N NaOH. The aqueous solution was extracted with CH_2Cl_2. The combined organic extracts were dried over Na_2SO_4, filtered, and evaporated to afford a brown powder (250 mg, yield 99%). ^{1}H NMR ($CDCl_3$): δ 1.46 (s, 6H); 2.34 (s, 6H); 5.91 (s, 2H); 6.17 (s, 2H); 6.90 (d, 1H, J = 8.4 Hz); 7.29 (dd, 1H, J = 1.8, 8.4 Hz); 8.09 (d, 1H, J = 1.8 Hz). HRMS (EI$^+$): calcd for M$^+$, 336.1586; found, 336.1614.

Synthesis of 6-(3,4-Diaminophenyl)-3,3′,5,5′-tetramethylpyrromethene (4). 3 (250 mg, 0.74 mmol) was dissolved in 50 mL of CH_2Cl_2 saturated with concentrated HCl. The solution was stirred at 0 °C for 20 min, then $SnCl_2$–$2H_2O$ (3.35 g, 14.9 mmol) was added, and stirring was continued at room temperature overnight. When TLC monitoring (alumina; CH_2Cl_2/MeOH, 9:1) showed complete consumption of **3**, the reaction mixture was washed with 2 N NaOH. The aqueous solution was extracted with CH_2Cl_2. The combined organic extracts were dried over Na_2SO_4, filtered, and evaporated to afford a light brown powder (215 mg, yield 95%). ^{1}H NMR ($CDCl_3$): δ 1.46 (s, 6H); 2.33 (s, 6H); 3.39 (s, 2H); 3.49 (s, 2H); 5.89 (s, 2H); 6.62 (dd, 1H, J = 1.8, 8.3 Hz); 6.63 (d, 1H, J = 1.8 Hz); 6.75 (d, 1H, J = 8.3 Hz). HRMS (EI$^+$): calcd for M$^+$, 306.1844; found, 306.1843.

Synthesis of 8-(3,4-Diaminophenyl)-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene (DAMBO). 4 (129 mg, 0.42 mmol) and *N*,*N*-diisopropylethylamine (DIEA) (1.0 mL, 5.7 mmol) were dissolved in 20 mL of absolute CH_2Cl_2 under an Ar atmosphere, and the solution was stirred at room temperature for 10 min. BF_3-OEt_2 (1.0 mL, 7.9 mmol) was added, and stirring was continued for 40 min. The reaction mixture was washed with water and 2 N NaOH. The aqueous solution was extracted with CH_2Cl_2. The combined organic extracts were dried over Na_2SO_4, filtered, and evaporated. The crude compound was purified by column chromatography over aluminum oxide (CH_2Cl_2/MeOH, 20:1) and recrystallized from $CHCl_3$/*n*-hexane to afford orange crystals (105 mg, yield 71%). ^{1}H NMR ($CDCl_3$): δ 1.53 (s, 6H); 2.54 (s, 6H); 3.44 (s, 2H); 3.52 (s, 2H); 5.96 (s, 2H); 6.58 (d, 1H, J = 8.4 Hz); 6.59 (s, 1H); 6.79 (d, 1H, J = 8.4 Hz). ^{13}C NMR ($CDCl_3$): δ 14.6, 14.6, 116.0, 117.0, 119.8, 120.8, 126.5, 131.9, 135.4, 135.4, 142.7, 143.3, 154.9. HRMS (EI$^+$): calcd for M$^+$, 354.1827; found, 354.1838. Mp: 251−254 °C (dec). Anal. Calcd for $C_{19}H_{21}BF_2N_4$: C, 64.43; H, 5.98; N, 15.82. Found: C, 64.40; H, 5.92; N, 15.61.

Synthesis of 8-(5-Benzotriazolyl)-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene (DAMBO-T). An aqueous solution of $NaNO_2$ (10 mg, 0.15 mmol) was added to a suspension of **DAMBO** (50 mg, 0.14 mmol) in 25 mL of 2 N HCl at 0 °C. The mixture was stirred at room temperature for 15 min. The resulting mixture was extracted with CH_2Cl_2. The combined organic extracts were dried over Na_2SO_4, filtered, and evaporated. The crude compound was purified by preparative reversed-phase HPLC (ODS, eluent: CH_3CN/H_2O/TFA, 50:50:0.1) and recrystallized from $CHCl_3$ to afford orange crystals (15 mg, yield 29%). ^{1}H NMR ($CDCl_3$): δ 1.26 (s, 6H); 2.57 (s, 6H); 5.99 (s, 2H); 7.41 (d, 1H, J = 8.4 Hz); 7.87 (s, 1H); 8.07 (d, 1H, J = 8.4 Hz). HRMS (EI$^+$): calcd for M$^+$, 365.1623; found, 365.1630. Mp: 282−286 °C (dec).

Synthesis of 4,4′-Diethyl-3,3′,5,5′-tetramethyl-6-phenylpyrromethene (5a), 4,4′-Diethoxycarbonyl-3,3′,5,5′-tetramethyl-6-phenylpyrromethene (5b), and 3,3′,5,5′-Tetramethyl-6-phenylpyrromethene (5c). Benzaldehyde (0.10 mL, 1.0 mmol) and 2,4-dimethyl-3-ethylpyrrole (0.27 mL, 2.0 mmol) were dissolved in 50 mL of absolute CH_2Cl_2 under an Ar atmosphere. One drop of TFA was added, and the solution was stirred at room temperature overnight. When TLC monitoring (silica; CH_2Cl_2) showed complete consumption of the aldehyde, a solution of DDQ (234 mg, 1.0 mmol) in CH_2Cl_2 was added, and stirring was continued for 15 min. The reaction mixture was washed with water, dried over $MgSO_4$, filtered, and evaporated. The crude compound was purified by column chromatography over aluminum oxide (CH_2Cl_2) to afford a brown powder (138 mg, yield 42%). ^{1}H NMR ($CDCl_3$): δ 0.97 (t, 6H, J = 7.5 Hz); 1.19 (s, 6H); 2.27 (q, 4H, J = 7.5 Hz); 2.32 (s, 6H); 7.29−7.33 (m, 2H); 7.38−7.42 (m, 3H); 13.10 (s, 1H). HRMS (EI$^+$): calcd for M$^+$, 332.2252; found, 332.2263.

5b and **5c** were similarly prepared from ethyl 2,4-dimethylpyrrole-3-carboxylate[22] and 2,4-dimethylpyrrole in 63% and 39% yield, respectively.

5b. ^{1}H NMR ($CDCl_3$): δ 1.32 (t, 6H, J = 7.1 Hz); 1.59 (s, 6H); 2.59 (s, 6H); 4.25 (q, 4H, J = 7.1 Hz); 7.28−7.32 (m, 2H); 7.46−7.48 (m, 3H). HRMS (EI$^+$): calcd for M$^+$, 420.2049; found, 420.2079.

5c. ^{1}H NMR ($CDCl_3$): δ 1.29 (s, 6H); 2.34 (s, 6H); 5.88 (s, 2H); 7.28−7.31 (m, 2H); 7.40−7.42 (m, 3H). HRMS (EI$^+$): calcd for M$^+$, 276.1626; found, 276.1642.

Synthesis of 2,6-Diethyl-4,4-difluoro-1,3,5,7-tetramethyl-8-phenyl-4-bora-3a,4a-diaza-*s*-indacene (6a), 2,6-Diethoxycarbonyl-4,4-difluoro-1,3,5,7-tetramethyl-8-phenyl-4-bora-3a,4a-diaza-*s*-indacene (6b), and 4,4-Difluoro-1,3,5,7-tetramethyl-8-phenyl-4-bora-3a,4a-diaza-*s*-indacene (6c). 5a (109 mg, 0.33 mmol) and DIEA (2.0 mL, 11.5 mmol) were dissolved in 100 mL of absolute CH_2Cl_2 under an Ar atmosphere and stirred at room temperature for 10 min. BF_3-OEt_2 (2.0 mL, 15.8 mmol) was added, and stirring was continued for 1 h. The reaction mixture was washed with water and 2 N NaOH. The aqueous solution was extracted with CH_2Cl_2. The combined organic extracts were dried over Na_2SO_4, filtered, and evaporated. The crude compound was purified by silica gel chromatography (CH_2Cl_2/*n*-hexane, 2:1) and recrystallized from *n*-hexane to afford orange needles (118 mg, yield 95%). ^{1}H NMR ($CDCl_3$): δ 0.98 (t, 6H, J = 7.7 Hz); 1.27 (s, 6H); 2.30 (q, 4H, J = 7.7 Hz); 2.53 (s, 6H); 7.26−7.30 (m, 2H); 7.46−7.48 (m, 3H). ^{13}C NMR ($CDCl_3$): δ 11.6, 12.5, 14.6, 17.0, 128.2, 128.7, 129.0, 130.8, 132.7, 135.8, 138.4, 140.2, 153.6. HRMS (EI$^+$): calcd for M$^+$, 380.2235; found, 380.2245. Mp: 185−186 °C. Anal. Calcd for $C_{23}H_{27}BF_2N_2$: C, 72.64; H, 7.16; N, 7.37. Found: C, 72.66; H, 7.16; N, 7.26.

6b and **6c** were similarly prepared from **5b** and **5c** in 10% and 78% yield, respectively.

6b. Recrystallized from $CHCl_3$/*n*-hexane to afford orange needles. ^{1}H NMR ($CDCl_3$): δ 1.32 (t, 6H, J = 7.1 Hz); 1.66 (s, 6H); 2.84 (s, 6H); 4.28 (q, 4H, J = 7.1 Hz); 7.26−7.29 (m, 2H); 7.53−7.55 (m, 3H). ^{13}C NMR ($CDCl_3$): δ 13.6, 14.3, 15.0, 60.2, 122.6, 127.7, 129.6, 129.7, 131.4, 134.4, 145.8, 147.7, 159.5, 164.3. HRMS (EI$^+$): calcd for M$^+$, 468.2032; found, 468.2035. Mp: 204−205 °C. Anal. Calcd for $C_{25}H_{27}BF_2N_2O_4$: C, 64.12; H, 5.81; N, 5.98. Found: C, 63.99; H, 5.81; N, 5.97.

6c. Recrystallized from $CHCl_3$/*n*-hexane to afford green crystals. ^{1}H NMR ($CDCl_3$): δ 1.37 (s, 6H); 2.56 (s, 6H); 5.98 (s, 2H); 7.27−7.30 (m, 2H); 7.47−7.49 (m, 3H). ^{13}C NMR ($CDCl_3$): δ 14.3, 14.6, 121.2, 127.9, 128.9, 129.1, 131.4, 135.0, 141.7, 143.1, 155.4. HRMS (EI$^+$): calcd for M$^+$, 324.1609; found, 324.1596. Mp: 176−177 °C. Anal. Calcd for $C_{19}H_{19}BF_2N_2$: C, 70.39; H, 5.91; N, 8.64. Found: C, 70.67; H, 6.00; N, 8.82.

Synthesis of 4-Amino-3-nitrobenzaldehyde (7). 1 (2.08 g, 10 mmol) was dissolved in 200 mL of MeOH, then 50 mL of 2 N HCl was added, and the mixture was refluxed at 80 °C for 8 h under an Ar atmosphere. The reaction mixture was evaporated and washed with 2 N NaOH. The aqueous solution was extracted with CH_2Cl_2. The combined organic extracts were dried over Na_2SO_4, filtered, and evaporated to afford yellow needles (1.62 g, yield 98%). ^{1}H NMR ($CDCl_3$): δ 6.91 (1H, d, J = 8.6 Hz); 7.92 (dd, 1H, J = 2.0, 8.6 Hz); 8.63 (d, 1H, J = 2.0 Hz); 9.82 (s, 1H). MS (EI): 166 (M$^+$).

Synthesis of 6-(4-Amino-3-nitrophenyl)-4,4′-diethyl-3,3′,5,5′-tetramethylpyrromethene (8a) and 6-(4-Amino-3-nitrophenyl)-4,4′-diethoxycarbonyl-3,3′,5,5′-tetramethylpyrromethene (8b). 7 (424 mg,

(22) Alberola, A.; Ortega, A. G.; Sádaba, M. L.; Sañudo, C. *Tetrahedron* **1999**, *55*, 6555−6566.

2.5 mmol) and 2,4-dimethyl-3-ethylpyrrole (616 mg, 5.0 mmol) were dissolved in 100 mL of absolute CH_2Cl_2 under an Ar atmosphere. One drop of TFA was added, and the solution was stirred at room temperature overnight. A solution of DDQ (585 mg, 2.5 mmol) in CH_2Cl_2 was added, and stirring was continued for 15 min. The reaction mixture was washed with water, dried over $MgSO_4$, filtered, and evaporated. The crude compound was purified by column chromatography over aluminum oxide (CH_2Cl_2) to afford a brown powder (136 mg, yield 14%). ^{1}H NMR ($CDCl_3$): δ 0.98 (t, 6H, J = 7.5 Hz); 1.35 (s, 6H); 2.29 (q, 4H, J = 7.5 Hz); 2.31 (s, 6H); 6.17 (s, 2H); 6.89 (d, 1H, J = 8.6 Hz); 7.30 (dd, 1H, J = 2.0, 8.6 Hz); 8.09 (d, 1H, J = 2.0 Hz). HRMS (EI$^+$): calcd for M$^+$, 392.2212; found, 392.2200.

8b was similarly prepared from ethyl 2,4-dimethylpyrrole-3-carboxylate in 23% yield. ^{1}H NMR ($CDCl_3$): δ 1.33 (t, 6H, J = 7.1 Hz); 1.77 (s, 6H); 2.58 (s, 6H); 4.26 (q, 4H, J = 7.1 Hz); 6.28 (s, 2H); 6.95 (d, 1H, J = 8.6 Hz); 7.27 (d, 1H, J = 8.6 Hz); 8.08 (s, 1H). HRMS (EI$^+$): calcd for M$^+$, 480.2009; found, 480.1983.

Synthesis of 6-(3,4-Diaminophenyl)-4,4′-diethyl-3,3′,5,5′-tetramethylpyrromethene (9a) and 6-(3,4-Diaminophenyl)-4,4′-diethoxycarbonyl-3,3′,5,5′-tetramethylpyrromethene (9b). 9a and **9b** were prepared from **8a** and **8b** by the same method as that used to obtain **4**, in 93% and 98% yield, respectively.

9a. ^{1}H NMR ($CDCl_3$): δ 0.98 (t, 6H, J = 7.5 Hz); 1.35 (s, 6H); 2.29 (q, 4H, J = 7.5 Hz); 2.30 (s, 6H); 3.39 (s, 2H); 3.50 (s, 2H); 6.63 (dd, 1H, J = 1.5, 8.3 Hz); 6.64 (d, 1H, J = 1.5 Hz); 6.74 (d, 1H, J = 8.3 Hz). HRMS (EI$^+$): calcd for M$^+$, 362.2470; found, 362.2472.

9b. ^{1}H NMR ($CDCl_3$): δ 1.32 (t, 6H, J = 7.1 Hz); 1.76 (s, 6H); 2.57 (s, 6H); 3.42 (s, 2H); 3.61 (s, 2H); 4.25 (q, 4H, J = 7.1 Hz); 6.60 (s, 1H); 6.61 (d, 1H, J = 8.1 Hz); 6.76 (d, 1H, J = 8.1 Hz). HRMS (EI$^+$): calcd for M$^+$, 450.2267; found, 450.2266.

Synthesis of 8-(3,4-Diaminophenyl)-2,6-diethyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene (DAMBO-Et) and 8-(3,4-Diaminophenyl)-2,6-diethoxycarbonyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene (DAMBO-CO_2Et). DAMBO-Et and DAMBO-CO_2Et were prepared from **9a** and **9b** by the same method as that used to obtain **DAMBO**, in 43% and 44% yield, respectively.

DAMBO-Et. Recrystallized from $CHCl_3$/*n*-hexane to afford green crystals. ^{1}H NMR ($CDCl_3$): δ 0.98 (t, 6H, J = 7.5 Hz); 1.43 (s, 6H); 2.30 (q, 4H, J = 7.5 Hz); 2.51 (s, 6H); 3.46 (s, 2H); 3.52 (s, 2H); 6.57 (d, 1H, J = 8.4 Hz); 6.59 (s, 1H); 6.78 (d, 1H, J = 8.4 Hz). ^{13}C NMR ($CDCl_3$): δ 11.8, 12.5, 14.6, 17.1, 116.4, 117.0, 120.1, 127.3, 131.2, 132.4, 135.1, 135.3, 138.6, 141.1, 153.1. HRMS (EI$^+$): calcd for M$^+$, 410.2453; found, 410.2446. Mp: 280−283 °C (dec). Anal. Calcd for $C_{23}H_{29}BF_2N_4$·0.1$CHCl_3$: C, 65.71; H, 6.95; N, 13.27. Found: C, 66.09; H, 6.65; N, 13.28.

DAMBO-CO_2Et. Recrystallized from $CHCl_3$/*n*-hexane to afford green crystals. ^{1}H NMR ($CDCl_3$): δ 1.33 (t, 6H, J = 7.1 Hz); 1.82 (s, 6H); 2.82 (s, 6H); 3.50 (s, 2H); 3.62 (s, 2H); 4.28 (q, 4H, J = 7.1 Hz); 6.55 (dd, 1H, J = 1.8, 8.3 Hz); 6.56 (d, 1H, J = 1.8 Hz); 6.82 (d, 1H, J = 8.3 Hz). ^{13}C NMR ($CDCl_3$): δ 14.0, 14.3, 14.9, 60.2, 115.7, 117.2, 119.6, 122.2, 125.6, 131.9, 135.8, 136.2, 147.0, 147.9, 158.9, 164.5. HRMS (EI$^+$): calcd for M$^+$, 498.2250; found, 498.2222. Mp: 241−242 °C. Anal. Calcd for $C_{25}H_{29}BF_2N_4O_4$·0.5H_2O: C, 59.18; H, 5.96; N, 11.04. Found: C, 59.41; H, 5.75; N, 11.11.

Synthesis of 8-(5-Benzotriazolyl)-2,6-diethyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene (DAMBO-Et-T) and 8-(5-Benzotriazolyl)-2,6-diethoxycarbonyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene (DAMBO-CO_2Et-T). DAMBO-Et-T and DAMBO-CO_2Et-T were prepared from DAMBO-Et and DAMBO-CO_2Et by a method similar to that used for DAMBO-T, in 20% and 85% yield, respectively.

DAMBO-Et-T. Recrystallized from $CHCl_3$/*n*-hexane to afford brown needles. ^{1}H NMR ($CDCl_3$): δ 0.96 (t, 6H, J = 7.5 Hz); 1.17 (s, 6H); 2.28 (q, 4H, J = 7.5 Hz); 2.55 (s, 6H); 7.41 (d, 1H, J = 8.6 Hz); 7.86 (br, 1H); 8.07 (br, 1H); 12.93 (br, 1H). MS (EI): 421 (M$^+$). HRMS (EI$^+$): calcd for M$^+$, 421.2249; found, 421.2258. Mp: 206−207 °C. Anal. Calcd for $C_{23}H_{26}BF_2N_5$: C, 65.57; H, 6.22; N, 16.62. Found: C, 65.87; H, 6.41; N, 16.36.

DAMBO-CO_2Et-T. Recrystallized from $CHCl_3$/*n*-hexane to afford bright yellow needles. ^{1}H NMR ($CDCl_3$): δ 1.32 (t, 6H, J = 7.1 Hz); 1.55 (s, 6H); 2.86 (s, 6H); 4.28 (q, 4H, J = 7.1 Hz); 7.38 (dd, 1H, J = 1.1, 8.6 Hz); 7.88 (br, 1H); 8.10 (br, 1H). HRMS (EI$^+$): calcd for M$^+$, 509.2046; found, 509.2019. Mp: 229−230 °C. Anal. Calcd for $C_{25}H_{26}BF_2N_5O_4$·H_2O: C, 56.94; H, 5.35; N, 13.28. Found: C, 57.04; H, 5.19; N, 12.98.

Synthesis of 3-(2-Methoxycarbonylethyl)-2,4-dimethylpyrrole (10). Methyl 5-(benzyloxycarbonyl)-2,4-dimethyl-3-pyrrolepropionate (3.1 g, 9.8 mmol) was dissolved in 100 mL of acetone. After the addition of 10% Pd−C (50 mg), the mixture was stirred vigorously under a H_2 atmosphere overnight. The Pd−C was filtered off and washed with acetone. The residue after evaporation of the filtrate was dissolved in 10 mL of TFA and stirred at 40 °C for 10 min under an Ar atmosphere. $CHCl_3$ was added to the reaction mixture, and this was washed with water. The aqueous solution was back-extracted with $CHCl_3$, and the combined organic extracts were washed with aqueous sodium carbonate and water, dried over $MgSO_4$, filtered, and evaporated. The crude compound was purified by column chromatography over silica gel (CH_2Cl_2) to afford a yellow oil (1.6 g, yield 90%). ^{1}H NMR ($CDCl_3$): δ 2.03 (d, 3H, J = 0.9 Hz); 2.18 (s, 3H); 2.45 (m, 2H); 2.72 (m, 2H); 3.67 (s, 3H); 6.38 (d, 1H, J = 0.9 Hz); 7.53 (s, 1H). MS (EI): 181 (M$^+$).

Synthesis of 6-(4-Amino-3-nitrophenyl)-4,4′-bis-(2-methoxycarbonylethyl)-3,3′,5,5′-tetramethylpyrromethene (11). 11 was prepared from **7** and **10** by the same method as that used to obtain **8a**, in 76% yield. ^{1}H NMR ($CDCl_3$): δ 1.37 (s, 6H); 2.33 (s, 6H); 2.36 (t, 4H, J = 7.1, 8.4 Hz); 2.63 (t, 4H, J = J = 7.1, 8.4 Hz); 3.65 (s, 6H); 6.32 (s, 2H); 6.92 (d, 1H, J = 8.6 Hz); 7.26 (dd, 1H, J = 2.0, 8.6 Hz); 8.06 (d, 1H, J = 2.0 Hz). HRMS (EI$^+$): calcd for M$^+$, 508.2323; found, 508.2327.

Synthesis of 8-(4-Amino-3-nitrophenyl)-4,4-difluoro-2,6-bis-(2-methoxycarbonylethyl)-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene (12). 12 was prepared from **11** by the same method as that used to obtain **DAMBO**, in 31% yield. ^{1}H NMR ($CDCl_3$): δ 1.46 (s, 6H); 2.37 (t, 4H, J = 7.3, 8.3 Hz); 2.54 (s, 6H); 2.65 (t, 4H, J = 7.3, 8.3 Hz); 3.66 (s, 6H); 6.32 (s, 2H); 6.99 (d, 1H, J = 8.4 Hz); 7.24 (dd, 1H, J = 1.8, 8.4 Hz); 8.06 (d, 1H, J = 1.8 Hz). HRMS (EI$^+$): calcd for M$^+$, 556.2305; found, 556.2294.

Synthesis of 8-(4-Amino-3-nitrophenyl)-2,6-bis-(2-carboxyethyl)-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene (13). 12 (183 mg, 0.33 mmol) was dissolved in 100 mL of MeOH, then 10 mL of 0.2 N NaOH was added, and the mixture was refluxed at 80 °C for 1 h. The reaction mixture was evaporated and acidified with 2 N HCl. The aqueous solution was extracted with ethyl acetate. The combined organic extracts were dried over Na_2SO_4, filtered, and evaporated. The crude compound was purified by column chromatography over silica gel (CH_2Cl_2/ MeOH, 10:1) to afford an orange powder (87 mg, yield 50%). ^{1}H NMR (CD_3OD): δ 1.44 (s, 6H); 2.26 (t, 4H, J = 7.3, 7.9 Hz); 2.40 (s, 6H); 2.57 (t, 4H, J = 7.3, 7.9 Hz); 7.07 (d, 1H, J = 8.6 Hz); 7.17 (dd, 1H, J = 2.0, 8.6 Hz); 7.88 (d, 1H, J = 2.0 Hz). HRMS (ESI$^+$): calcd for [M+Na]$^+$, 551.1889; found, 551.1922.

Synthesis of 8-(3,4-Diaminophenyl)-2,6-bis(2-carboxyethyl)-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene (DAMBO-P^H). 13 (87 mg, 0.16 mmol) was dissolved in 100 mL of MeOH. After the addition of 10% Pd−C (50 mg), the mixture was stirred vigorously under a H_2 atmosphere. When TLC monitoring (silica; CH_2Cl_2/ MeOH, 5:1) showed complete consumption of **13**, the Pd−C was filtered off and washed with MeOH. The residue after evaporation of the filtrate was purified by column chromatography over silica gel (CH_3CN/H_2O, 10:1) and recrystallized from EtOH to afford orange needles (32 mg, yield 38%). ^{1}H NMR (CD_3OD): δ 1.51 (s, 6H); 2.28 (t, 4H, J = 7.1, 8.4 Hz); 2.47 (s, 6H); 2.64 (t, 4H, J = 7.1, 8.4 Hz); 6.46 (dd, 1H, J =

2.0, 7.9 Hz); 6.59 (d, 1H, $J = 2.0$ Hz); 6.83 (d, 1H, $J = 7.9$ Hz). ^{13}C NMR (CD_3OD): δ 12.3, 12.6, 20.7, 36.2, 116.9, 117.6, 119.9, 127.0, 130.5, 132.6, 137.1, 137.1, 140.9, 144.2, 154.3, 177.7. HRMS (ESI^+): calcd for $[M+Na]^+$, 521.2148; found, 521.2127. Mp: >300 °C. Anal. Calcd for $C_{25}H_{29}BF_2N_4O_4$: C, 60.25; H, 5.87; N, 11.24. Found: C, 60.31; H, 5.86; N, 11.42.

Synthesis of 8-(5-Benzotriazolyl)-2,6-bis(2-carboxyethyl)-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene (DAMBO-P^H-T). DAMBO-P^H-T was prepared from DAMBO-P^H by a method similar to that used for DAMBO-T. The resulting mixture was extracted with ethyl acetate. The combined organic extracts were dried over Na_2SO_4, filtered, and evaporated. The crude compound was purified by column chromatography over silica gel (CH_3CN/H_2O, 10:1) and recrystallized from EtOH to afford brown crystals (yield 82%). ^{1}H NMR (CD_3OD): δ 1.24 (s, 6H); 2.30 (t, 4H, $J = 7.1$, 8.3 Hz); 2.51 (s, 6H); 2.63 (t, 4H, $J = 7.1$, 8.3 Hz); 7.43 (d, 1H, $J = 8.4$ Hz); 7.89 (s, 1H); 8.08 (d, 1H, $J = 8.4$ Hz). HRMS (ESI^+): calcd for $[M+Na]^+$, 532.1944; found, 532.1927. Mp: 235−240 °C dec

Fluorometric Analysis. The slit width was 2.5 nm for both excitation and emission. The photon multiplier voltage was 700 V. Relative quantum efficiencies of fluorescence of BODIPY derivatives were obtained by comparing the area under the corrected emission spectrum of the test sample excited at 492 nm in 0.1 N NaOH with that of a solution of fluorescein, which has a quantum efficiency of 0.85 according to the literature.[23]

In the experiment to measure the pH profile of fluorescence and the time course of fluorescence increase, the slit width was 5.0 nm for excitation and 2.5 nm for emission and the photon multiplier voltage was 750 V. Compounds were dissolved in DMSO to make a 5 mM stock solution, which was diluted to the required concentration for measurement.

Computational Methods. All structures were computed using hybrid density functional theory (B3LYP)[24] with the 6-31G* basis set as implemented in Gaussian 98W.[25] Several starting geometries were used for the geometry optimization to ensure that the optimized structure corresponds to a global minimum.

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HPLC Analysis. HPLC analyses were performed on an Inertsil ODS-3 (4.6 × 250 mm) column using an HPLC system composed of a pump (PU-980, Jasco) and a detector (UV-970 or FP-920, Jasco).

Cyclic Voltammetry. Cyclic voltammetry was performed on a 600A electrochemical analyzer (ALS). A three-electrode arrangement in a single cell was used for the measurements: a Pt wire as the auxiliary electrode, a Pt electrode (i.d. = 1.6 mm) as the working electrode, and a Ag/Ag^+ electrode as the reference electrode. The sample solutions contained 1.0×10^{-3} M sample and 0.1 M tetrabutylammonium perchlorate (TBAP) as a supporting electrolyte in acetonitrile, and argon was bubbled for 10 min before each measurement. The scan rate was 0.1 V s^{-1}. Obtained potentials (vs Ag/Ag^+) were converted to those vs a saturated calomel electrode (SCE) by adding 0.25 V.

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