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A Photoinduced Electron-Transfer Reagent for Peroxyacetic Acid, 4-Ethylthioacetylamino-7-phenylsulfonyl-2,1,3-benzoxadiazole, Based on the Method for Predicting the Fluorescence Quantum Yields

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To develop new photoinduced electron-transfer (PET) reagents, we established a method for predicting the fluorescence quantum yields (Φ) of the benzofurazan compounds bearing an aliphatic substituent group having an n-electron. The PET process occurred sufficiently to reduce the Φ values in the benzofurazan compounds bearing an aliphatic moiety, which had a high quenching ability. The quenching ability was estimated by the molecular orbital calculation and Stern−Volmer plotting. The Φ values of the benzofurazan compounds could be controlled by changing the quenching ability of a substituent group. We succeeded in designing a PET reagent for peroxyacetic acid (PAA), 4-ethylthioacetylamino-7-phenylsulfonyl-2,1,3-benzoxadiazole (EPB), using the established method for predicting the Φ values. EPB and its oxidized derivative were separated by reversed-phase HPLC and fluorometrically detected at 479 nm with excitation at 362 nm. The attained detection limit for PAA was 105 fmol (S/N = 3) and the cross-reactivity toward hydrogen peroxide was very low, indicating EPB is a highly sensitive and selective reagent for PAA.

Fluorometric detection has been widely used in many fields of science such as analytical chemistry and biochemistry, because of its sensitivity and selectivity. Since most analytes do not fluoresce, their derivatization with fluorescent reagents is necessary. Many fluorescent reagents have been developed, and they are classified into two groups by their fluorescence properties. One is a "fluorescent labeling reagent", which is strongly fluorescent itself. The other is a "fluorescent 'on−off' reagent", which is nonfluorescent or weakly fluorescent itself and reacts with the analytes to form the fluorescent derivatives. The fluorescent on−off reagents are generally superior because they can avoid interference from the fluorescence of the reagents themselves.

Recently, reagents utilizing photoinduced electron transfer (PET)[1−3] have been developed as a new type of fluorescent on−off reagent, and they are called "PET reagents".[4] In general, the PET reagents consist of a fluorophore and a reacting group as an electron donor and an electron acceptor in one molecule. A donor and an acceptor are connected directly or through a spacer. These reagents are nonfluorescent or weakly fluorescent themselves, since the electron transfer from a donor to an acceptor in an excited state accelerates the radiationless relaxation. After the reaction with analytes, the electron transfer is suppressed because the electron-donating ability of a donor decreases, restoring the fluorescence of the fluorophore. A fluorophore and a reaction group of most previously reported PET reagents[1−3] are an acceptor and a donor, respectively. The donor is divided into a π-donor and an n-donor, to give a π-electron and n-electron, respectively, to an acceptor.[5−8] Diaminofluorescein (DAF-2) for nitric oxide[9] and *N*-(9-anthrylmethyl)diethanolamine for boronic and boric acids[10] are the representative reagents with a π-donor and an n-donor, respectively.

To develop new PET reagents more efficiently, we intended to establish a method for predicting the fluorescence quantum yields (Φ) of benzofurazan compounds bearing an aromatic substituent group as a π-donor.[11] Since some fluorescent coumarin dyes were quenched in the presence of other aromatic compounds[12,13] (intermolecular quenching) and a fluorescein derivative

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having an aminobenzene moiety had a markedly small Φ value[14] (intramolecular quenching), we estimated that these quenchings were derived from the electron transfer between the aromatic moiety (π-donor) and the fluorophore. Assuming that the mechanism of quenching by the intermolecular electron transfer was identical with that of the fluorescence "off" switching by the intramolecular electron transfer, we first investigated the intermolecular electron transfer between some benzofurazan compounds and other aromatic compounds using Stern−Volmer plots. As a result, fluorescence quenching of the benzofurazan compounds was observed by the addition of some aromatic compounds such as aniline and indole. We then synthesized the benzofurazan compounds bearing an aromatic substituent group to investigate the intramolecular electron transfer between the benzofurazan skeleton and an aromatic moiety. Moreover, molecular orbital theory was adopted as an explanation of intramolecular quenching by electron transfer, and we reached the following conclusion: the Φ values of the benzofurazan compounds bearing an aromatic moiety could be estimated by investigation of the electron-donating ability of the aromatic moiety. This ability was indicated by a HOMO energy (the semiempirical PM3/COSMO calculation) or a *K* value (the Stern−Volmer plotting). And finally, we succeeded in developing the first PET reagent for peroxycarboxylic acids, 4-(*p*-*N*,*N*-dimethylamino)-benzylamino-7-nitro-2,1,3-benzoxadiazole (NBD-Bz-*p*-NMe_2), by predicting the Φ values using the *K* values and the HOMO energies. Recently, it was also reported that the Φ values of the fluorescein derivatives bearing a benzoic acid moiety could be estimated by calculating the HOMO energies and then a PET reagent for singlet oxygen, 9-[2-(3-carboxy-9,10-dimethyl)anthryl]-6-hydroxy-3*H*-xanthen-3-one (DMAX), was developed.[15]

As mentioned above, the method for predicting the Φ values of the compounds bearing a π-donor was established and new PET reagents have been developed. We considered that the establishment of a method for predicting the Φ values of the compounds bearing an n-donor, which have not been yet reported, was also valuable for developing new PET reagents. The fluorescence quenching rate constants for some anthracene compounds by the addition of the aliphatic compounds having n-electrons increased with the decrease in the ionization potential of the aliphatic compounds[5−8] (intermolecular quenching). Furthermore, the PET process was observed on some compounds having an aliphatic amino moiety and a fluorophore such as anthracene,[16] 1,8-naphthalimide,[17,18] and benzofurazan[19,20] (intramolecular quenching). Taking these reports into consideration, the method for predicting the Φ values of the compounds bearing a π-donor[11,15] can be applied to the prediction of the Φ values of the compounds bearing an n-donor. In this study, we first synthesized the benzofurazan compounds bearing an aliphatic substituent group having an n-electron to investigate the relationship between the intramolecular electron transfer and their Φ values. We then investigated the intermolecular electron transfer by obtaining the HOMO energies of the aliphatic moiety (the semiempirical PM3/COSMO calculation) and the *K* values (the Stern−Volmer plotting) and tried to establish the prediction method.

The second purpose of this study is to develop a new PET reagent for peroxyacetic acid (PAA) based on the prediction. NBD-Bz-*p*-NMe_2, the above-mentioned PET reagent for peroxycarboxylic acids, reacted only with *m*-chloroperoxybenzoic acid (MCPBA) and did not react with any other peroxycarboxylic acids.[11] Among the peroxycarboxylic acids, PAA is one of the most practical peroxycarboxylic acids, since it has excellent antimicrobial, bactericidal, fungicidal, virucidal, and bleaching properties[21] and is used in industrial processes, including disinfection in the food and beverage industries and the bleaching of paper and textiles.[22] Various methods, such as titration,[23,24] photometry,[25] electrochemical sensing,[26] and chromatographic methods,[22,27,28] have been used to determine the concentration of PAA. Among these methods, the chromatographic methods offer both high selectivity and low detection limits. Di Furia et al. developed a gas chromatographic method for the determination of PAA using methyl-*p*-tolyl sulfide (MTS) as a precolumn derivatizing reagent.[27] MTS is oxidized to the corresponding sulfoxide, methyl-*p*-tolyl sulfoxide (MTSO). This method gave accurate results, but it was time-consuming due to the need for an extraction step with chloroform to obtain a solution suitable for gas chromatography. This problem could be avoided by employing HPLC.[28] The detection of MTSO was performed at 230 nm using a UV−visible detector. However, the colored matrix components interfered with the detection of MTSO at the low detection wavelength, so the azo dye functionalized sulfide reagent, 2-[(3-{2-[4-amino-2-(methylsulfanyl)phenyl]-1-diazenyl}phenyl)sulfonyl]-1-ethanol (ADS), was developed as a new derivatization reagent.[22] The HPLC method for the determination of PAA using ADS at 410 nm provided lower limits of detection and higher selectivity toward colored matrix components. However, to detect PAA more accurately and sensitively, it is necessary to adopt fluorescence detection. Therefore, we finally tried to develop a new PET reagent for PAA based on the established method for predicting Φ values and apply it to HPLC analysis.

EXPERIMENTAL SECTION

Materials. Water was purified using a Milli-Q reagent system (Millipore). Acetonitrile and methanol were of HPLC grade (Kanto Chemicals). Acecide (Saraya) was used as the disinfectant sample. All other reagents were of guaranteed reagent grade and used without further purification.

Apparatus. Mass spectra were measured using a Hitachi M-1200H mass spectrometer (atmospheric pressure chemical

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ionization (APCI) system). Melting points were measured on a Yanagimoto Micro Point apparatus and were uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained using a JEOL LA-500 spectrometer. The *J* values are given in hertz. UV−visible absorption spectra (30 μM) were measured using a Jasco Ubest-50 spectrometer. Fluorescence spectra (50 nM−30 μM) were measured using a Hitachi F-4010 fluorescence spectrometer.

Determination of Fluorescence Quantum Yield (Φ). The Φ values were determined using quinine sulfate in 0.1 M sulfuric acid ($\Phi_Q = 0.55$ with excitation of 366 nm) as a standard[29] using the following equation[30]

$$\Phi_S = \Phi_Q(F_S/F_Q)(A_Q/A_S)(n_S/n_Q)^2 \quad (1)$$

where *F* is the area under the fluorescence spectra, *A* is the absorbance, *n* is the refractive index of the solvent, and the subscripts Q and S represent quinine sulfate and sample, respectively.

Synthesis. 4-Methylamino-7-nitro-2,1,3-benzoxadiazole (NBD-NHMe),[31] 4-methylthio-7-aminosulfonyl-2,1,3-benzoxadiazole (ABD-SMe),[31] 4-acetylamino-7-phenylsulfonyl-2,1,3-benzoxadiazole (PSBD-NHAc),[31] 4-phenylthio-7-amino-2,1,3-benzoxadiazole (PTBD-NH_2),[31] and 4-(*N*,*N*-dimethylethylenediamino)-7-nitro-2,1,3-benzoxadiazole (NBD-NH$(CH_2)_2$$NMe_2$ (**1**))[19] were synthesized as previously described.

4-(*N*,*N*-Diethylethylenediamino)-7-nitro-2,1,3-benzoxadiazole (NBD-NH$(CH_2)_2$$NEt_2$ (2)). 4-Chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl) (100 mg, 0.50 mmol) was dissolved in acetonitrile (20 mL). After the addition of *N*,*N*-diethylethylenediamine (70 μL) in acetonitrile (20 mL), the solution was stirred at room temperature for 30 min. The reaction mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with dichloromethane−methanol (19:1) to afford **2** (54 mg, yield 39%) as brown crystals. APCI-MS: *m*/*z* 280 ((M + H)$^+$). mp: 96 °C. ^{1}H NMR (CD_3OD): δ 0.99 (t, 6H), 2.58 (q, 4H), 2.76 (t, 2H), 3.55 (br, 2H), 6.25 (d, 1H, $J = 8.9$), 8.40 (d, 1H, $J = 8.9$). Found: C, 51.52; H, 6.12; N, 24.79. Calcd for $C_{12}H_{17}N_5O_3$: C, 51.60; H, 6.14; N, 25.08.

4-(2-Methylaminoethylamino)-7-nitro-2,1,3-benzoxadiazole (NBD-NH$(CH_2)_2$NHMe (3)). *N*-Methylethylenediamine (400 μL) was dissolved in acetonitrile (10 mL). After the addition of NBD-Cl (200 mg, 1.0 mmol) in acetonitrile (20 mL), the solution was stirred at room temperature for 30 min. The reaction mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with dichloromethane−methanol (3:1) to afford **3** (94 mg, yield 39%) as brown crystals. APCI-MS: *m*/*z* 238 ((M + H)$^+$). mp: 121−122 °C. ^{1}H NMR ($CDCl_3$): δ 2.48 (s, 3H), 3.01 (t, 2H), 3.50 (br, 2H), 6.13 (d, 1H, $J = 8.6$), 8.48 (d, 1H, $J = 8.6$).

4-Ethylenediamino-7-nitro-2,1,3-benzoxadiazole (NBD-NH$(CH_2)_2$$NH_2$ (4)). A procedure similar to that for **3** yielded 64% of a product as brown crystals. APCI-MS: *m*/*z* 224 ((M + H)$^+$). mp: 145−146 °C. ^{1}H NMR (CD_3OD): δ 3.11 (t, 2H), 3.69 (br, 2H), 6.35 (d, 1H, $J = 8.9$), 8.44 (d, 1H, $J = 8.9$).

4-(2-Acetylaminoethylamino)-7-nitro-2,1,3-benzoxadiazole (NBD-NH$(CH_2)_2$NHAc (5)). NBD-NH$(CH_2)_2$$NH_2$ (37 mg, 0.17 mmol) was dissolved in acetonitrile (10 mL). After the addition of acetic anhydride (0.5 mL), the solution was stirred at room temperature for 1 h. The reaction mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with ethyl acetate−methanol (19:1) to afford **5** (24 mg, yield 55%) as yellow crystals. APCI-MS: *m*/*z* 266 ((M + H)$^+$). mp: 232−234 °C. ^{1}H NMR (CD_3OD): δ 1.84 (s, 3H), 3.42 (t, 2H), 3.55 (br, 2H), 6.33 (d, 1H, $J = 8.9$), 8.44 (d, 1H, $J = 8.9$). Found: C, 45.29; H, 4.19; N, 26.39. Calcd for $C_{10}H_{11}N_5O_4$: C, 45.28; H, 4.18; N, 26.41.

4-(2-Acetylmethylaminoethylamino)-7-nitro-2,1,3-benzoxadiazole (NBD-NH$(CH_2)_2$NMeAc (6)). A procedure similar to that for **5** yielded 25% of a product as orange crystals. APCI-MS: *m*/*z* 280 ((M + H)$^+$). mp: 238−240 °C. ^{1}H NMR ($CDCl_3$): δ 2.15 (s, 3H), 3.12 (s, 3H), 3.63 (br, 2H), 3.81 (t, 2H), 6.11 (d, 1H, $J = 8.9$), 8.46 (d, 1H, $J = 8.9$).

4-(2-Hydroxyethylamino)-7-nitro-2,1,3-benzoxadiazole (NBD-NH$(CH_2)_2$OH (7)). A procedure similar to that for **2** yielded 60% of a product as orange crystals. APCI-MS: *m*/*z* 225 ((M + H)$^+$). mp: 153 °C. ^{1}H NMR (CD_3OD): δ 3.57 (br, 2H), 3.76 (t, 2H), 6.32 (d, 1H, $J = 8.9$), 8.42(d, 1H, $J = 8.9$). Found: C, 42.90; H, 3.71; N, 24.73. Calcd for $C_8H_8N_4O_4$: C, 42.86; H, 3.60; N, 24.99.

4-(2-Methoxyethylamino)-7-nitro-2,1,3-benzoxadiazole (NBD-NH$(CH_2)_2$OMe (8)). A procedure similar to that for **2** yielded 37% of a product as orange crystals. APCI-MS: *m*/*z* 239 ((M + H)$^+$). mp: 170−172 °C. ^{1}H NMR ($CDCl_3$): δ 3.43 (s, 3H), 3.65 (t, 2H), 3.72 (t, 2H), 6.18 (d, 1H, $J = 8.6$), 6.49 (br, 1H), 8.48 (d, 1H, $J = 8.6$). Found: C, 45.27; H, 4.28; N, 23.28. Calcd for $C_9H_{10}N_4O_4$: C, 45.38; H, 4.23; N, 23.52.

4-(2-Acetoxyethylamino)-7-nitro-2,1,3-benzoxadiazole(NBD-NH$(CH_2)_2$OAc (9)). A procedure similar to that for **5** yielded 85% of a product as orange crystals. APCI-MS: *m*/*z* 267 ((M + H)$^+$). mp: 134−136 °C. ^{1}H NMR ($CDCl_3$): δ 2.06 (s, 3H), 3.72 (q, 2H), 4.40 (t, 2H), 6.18 (d, 1H, $J = 8.6$), 6.53 (br, 1H), 8.43 (d, 1H, $J = 8.6$). Found: C, 45.20; H, 4.02; N, 20.80. Calcd for $C_{10}H_{10}N_4O_5$: C, 45.12; H, 3.79; N, 21.05.

4-(2-Acetylaminoethylthio)-7-aminosulfonyl-2,1,3-benzoxadiazole (ABD-S$(CH_2)_2$NHAc (10)). ABD-F (50 mg, 0.23 mmol) was dissolved in a mixture of acetonitrile (2 mL) and saturated sodium hydrogen carbonate solution (2 mL). After the addition of 2-aminoethanethiol (19 mg) in acetonitrile (2 mL) and saturated sodium hydrogen carbonate solution (2 mL), the solution was stirred at room temperature for 1 h. Hydrochloric acid solution was then added to the reaction mixture until it became pH 2−3, the solution was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with dichloromethane−methanol (3:1) to afford ABD-S$(CH_2)_2$$NH_2$. APCI-MS: *m*/*z* 275 ((M + H)$^+$). ^{1}H NMR (CD_3OD): δ 3.01 (t, 2H), 3.36 (t, 2H), 7.37 (d, 1H, $J = 7.3$), 7.87 (d, 1H, $J = 7.3$). ABD-S$(CH_2)_2$$NH_2$ (50 mg, 0.18 mmol) was dissolved in acetonitrile (20 mL). After the addition of acetic anhydride (1 mL), the solution was stirred at room temperature for 6 h. Acetonitrile was then evaporated under reduced pressure, and the hydrochloric acid solution was

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added to the residue until it became pH 2−3. The solution was extracted with 3 × 50 mL of dichloromethane, the dichloromethane was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with ethyl acetate−methanol (20:1) to afford **10** (24 mg, yield 41%) as yellow crystals. APCI-MS: m/z 317 ($(M + H)^+$). mp: 184 °C. ^{1}H NMR (CD_3OD): δ 1.84 (s, 3H), 3.31 (t, 2H), 3.44 (t, 2H), 7.43 (d, 1H, $J = 7.3$), 7.86 (d, 1H, $J = 7.3$). Found: C, 38.23; H, 4.06; N, 17.57. Calcd for $C_{10}H_{12}N_4O_4S_2$: C, 37.97; H, 3.82; N, 17.71.

4-(2-Hydroxyethylthio)-7-aminosulfonyl-2,1,3-benzoxadiazole (ABD-S(CH$_2$)$_2$OH (11)). A procedure similar to that for ABD-S$(CH_2)_2NH_2$ yielded 93% of a product as yellow crystals. APCI-MS: m/z 276 ($(M + H)^+$). mp: 137−138 °C. ^{1}H NMR (CD_3OD): δ 3.33 (t, 2H), 3.79 (t, 2H), 7.30 (d, 1H, $J = 7.3$), 7.84 (d, 1H, $J = 7.3$). Found: C, 34.91; H, 3.45; N, 15.22. Calcd for $C_8H_9N_3O_4S_2$: C, 34.90; H, 3.30; N, 15.26.

4-(2-*N*-Oxydiethylaminoethylamino)-7-nitro-2,1,3-benzoxadiazole (NBD-NH(CH$_2$)$_2$NEt$_2$O (12)). NBD-NH$(CH_2)_2$-NEt_2 (80 mg, 0.29 mmol) was dissolved in chloroform (5 mL). After the addition of 70% MCPBA (75 mg) in chloroform (5 mL), the solution was stirred at room temperature for 15 min. The reaction mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with dichloromethane−methanol (3:1) to afford **12** (68 mg, yield 80%) as brown crystals. APCI-MS: m/z 280 ($(M - O + H)^+$). mp: 86−87 °C. ^{1}H NMR (CD_3OD): δ 1.26 (t, 6H), 3.27−3.40 (m, 4H), 3.55 (t, 2H), 4.80 (br, 2H), 6.21 (d, 1H, $J = 8.9$), 8.27 (d, 1H, $J = 8.9$).

4-Ethylthioacetylamino-7-phenylsulfonyl-2,1,3-benzoxadiazole (PSBD-NHCOCH$_2$SEt, EPB (13)). PTBD-NH_2 (100 mg, 0.41 mmol) was dissolved in dichloromethane (10 mL). After the addition of bromoacetyl chloride (100 μL) in dichloromethane (5 mL) and triethylamine (50 μL) in dichloromethane (5 mL), the solution was stirred at room temperature for 45 min. The reaction mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with dichloromethane−*n*-hexane (2:1) to afford PTBD-$NHCOCH_2$Br (126 mg, yield 84%). PTBD-$NHCOCH_2$Br (64 mg, 0.18 mmol) was dissolved in chloroform (2 mL). After the addition of 70% MCPBA (100 mg) in chloroform (5 mL), the solution was stirred at room temperature for 2 h. The chloroform was then evaporated to dryness under reduced pressure, and a saturated sodium hydrogen carbonate solution (50 mL) was added to the residue. The solution was extracted with 3 × 50 mL of dichloromethane, the dichloromethane was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with ethyl acetate−*n*-hexane (2:1) to afford PSBD-$NHCOCH_2$Br (50 mg, yield 71%) as white crystals. APCI-MS: m/z 395 ($(M - H)^-$). ^{1}H NMR ($CDCl_3$): δ 4.09 (s, 2H), 7.52 (m, 2H), 7.59 (m, 1H), 8.17 (m, 2H), 8.27 (d, 1H, $J = 7.9$), 8.37 (d, 1H, $J = 7.9$), 9.13 (br, 1H). Found: C, 42.65; H, 2.73; N, 10.50. Calcd for $C_{14}H_{10}BrN_3O_4S$: C, 42.44; H, 2.54; N, 10.61. PSBD-$NHCOCH_2$Br (150 mg, 0.38 mmol) was dissolved in *N*,*N*-dimethylformamide (DMF) (1 mL). After the addition of ethanethiol (50 μL) and potassium carbonate (100 mg), the solution was stirred at room temperature for 3 h. Hydrochloric acid solution was then added to the reaction mixture until it became pH 2−3. The solution was extracted with 3 × 50 mL of dichloromethane, the dichloromethane was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with ethyl acetate−*n*-hexane (1:2) to afford **13** (20 mg, yield 14%) as white crystals. APCI-MS: m/z 378 ($(M + H)^+$). mp: 136−138 °C. ^{1}H NMR ($CDCl_3$): δ 1.28 (t, 3H), 2.62 (q, 2H), 3.46 (s, 2H), 7.51 (m, 2H), 7.58 (m, 1H), 8.16 (d, 2H), 8.26 (d, 1H, $J = 7.9$), 8.38 (d, 1H, $J = 7.9$), 9.82 (br, 1H). Found: C, 50.86; H, 4.11; N, 11.14. Calcd for $C_{16}H_{15}N_3O_4S_2$: C, 50.91; H, 4.01; N, 11.13.

4-Ethylsulfinylacetylamino-7-phenylsulfonyl-2,1,3-benzoxadiazole (PSBD-NHCOCH$_2$SOEt, EPBO (14)). A procedure similar to that for **12** yielded 10% of a product as white crystals. APCI-MS: m/z 394 ($(M + H)^+$). mp: 222−223 °C. ^{1}H NMR ($CDCl_3$): δ 1.36 (t, 3H), 2.92 (q, 2H), 3.93 (s, 2H), 7.51 (m, 2H), 7.58 (m, 1H), 8.17 (d, 2H), 8.25 (d, 1H, $J = 7.9$), 8.35 (d, 1H, $J = 7.9$).

Computational Methods. We adopted the semiempirical PM3/COSMO method as described in our previous report.[11] The keywords, PM3, EF, PRECISE, and EPS = 37.5, were used for all calculations. The PM3/COSMO calculations were carried out using the program MOPAC 2000 in the WinMOPAC ver. 3.0 package (Fujitsu) with a DynaBook DB65C/4RC computer (Toshiba).

Stern−Volmer Plots. The method described in our previous report[11] was adopted and some modifications were made. To the acetonitrile solution (2.4 mL) of the benzofurazan compounds (NBD-NHMe (100 nM), ABD-SMe (1 μM) or PSBD-NHAc (200 nM)), a series of acetonitrile (100 μL) or acetonitrile solutions of aliphatic compounds (100 μL) were added 12 times and the fluorescence intensity of this mixture was defined as I_0 (control) or I (test), respectively. The excitation wavelengths were 459 (for NBD-NHMe), 384 (for ABD-SMe), and 368 nm (for PSBD-NHAc). The emission wavelengths were 522 (for NBD-NHMe), 510 (for ABD-SMe), and 471 nm (for PSBD-NHAc). According to the Stern−Volmer equation (eq 2), the K value was calculated from

$$I_0/I = 1 + K[Q] \qquad (2)$$

[Q] (the concentration of aliphatic compounds) and the obtained I_0/I by least-squares analysis. All the least-squares analyses were carried out using Microsoft EXCEL 2000.

High-Performance Liquid Chromatography. The high-performance liquid chromatograph consisted of a Hitachi L-6300 pump, a Hitachi L-1080 fluorescence detector, and a Hitachi D-2500 integrator. The separation for the derivative was studied on an analytical column, TSKgel ODS-80Ts (150 × 4.6 mm i.d., 5 μm) (TOSOH). The eluent for the derivative was acetonitrile−water (2:3). The eluate was monitored with fluorescence detection (excitation at 362 nm, emission at 479 nm).

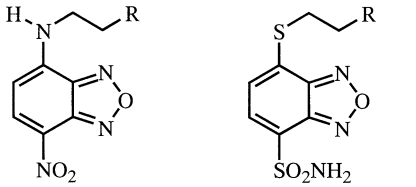
Time Course of the Reaction of 13 with PAA. 13 (100 μM) was reacted with PAA (10 μM) in acetonitrile at room temperature or 50 °C for 10, 30, or 60 min. An aliquot (5 μL) of each reaction mixture was subjected to HPLC. The reaction yields were determined by comparison with the peak area of authentic **14**.

Calibration Curve for the Derivative of 13 with PAA. 13 (100 μM) was reacted with PAA (0.020, 0.078, 0.31, 1.25, 5, and 20 μM) in acetonitrile at 50 °C for 30 min. An aliquot (5 μL) of each reaction mixture was subjected to HPLC.

Reaction Yields of the Derivative of 13 with Other Peroxycarboxylic Acids, Hydrogen Peroxide, and Hydroperoxides. With each MCPBA, peroxybenzoic acid, hydrogen peroxide,

Table 1. Fluorescence Characteristics of the Synthesized Benzofurazan Compounds

		in benzene				in acetonitrile				in methanol			
no.	R	λ_{ab} (nm)	ϵ (10^4 M^{-1} cm^{-1})	λ_{em} (nm)	Φ	λ_{ab} (nm)	ϵ (10^4 M^{-1} cm^{-1})	λ_{em} (nm)	Φ	λ_{ab} (nm)	ϵ (10^4 M^{-1} cm^{-1})	λ_{em} (nm)	Φ
						NBD-NH$(CH_2)_2$R							
1	NMe_2	451	1.69	511	0.0091	462	2.06	525	0.00038	461	2.00	522	0.0051
2	NEt_2	453	1.76	509	0.0084	464	2.09	526	0.00017	464	2.04	520	0.0089
3	NHMe	451	1.56	512	0.087	462	1.85	524	0.0018	458	1.69	522	0.046
4	NH_2	453	1.75	512	0.23	461	2.06	536	0.014	458	2.02	524	0.078
5	NHAc	453	1.50	511	0.26	461	1.85	525	0.30	461	1.93	528	0.17
6	NMeAc	450	1.51	510	0.43	461	2.13	526	0.32	460	1.78	528	0.16
7	OH	452	1.59	510	0.33	460	1.92	528	0.29	464	2.17	531	0.14
8	OMe	449	1.55	510	0.37	459	1.95	525	0.30	463	1.97	531	0.14
9	OAc	446	1.57	505	0.31	457	1.93	523	0.32	456	1.93	527	0.17
cf. NBD-NHMe		448	1.55	508	0.41	459	1.85	522	0.39	461	1.80	529	0.16
						ABD-S$(CH_2)_2$R							
10	NHAc	386	0.75	484	0.058	385	0.89	501	0.016	384	0.79	505	0.017
11	OH	387	0.79	499	0.21	386	0.87	508	0.083	384	0.86	508	0.028
cf. ABD-SMe		387	0.84	493	0.33	384	0.88	510	0.097	384	0.86	509	0.017



NBD-NH$(CH_2)_2$R
R=NH_2,NHMe,NMe_2, NEt_2,NHAc,NMeAc, OH,OMe,OAc

ABD-S$(CH_2)_2$R
R=NHAc,OH

Figure 1. Structures of the benzofurazan compounds used in this study.

tert-butyl hydroperoxide, or cumene hydroperoxide (20 μM), **13** (100 μM) was reacted in acetonitrile at 50 °C for 30 min. An aliquot (5 μL) of each reaction mixture was subjected to HPLC. The reaction yields were determined by comparison with the peak area of authentic **14**.

Analysis of PAA in Disinfectant Sample. The concentration of PAA in disinfectant sample was determined. The disinfectant samples were diluted with acetonitrile to an appropriate concentration for analysis. The diluted disinfectant sample was reacted with **13** (100 μM) at 50 °C for 30 min, and an aliquot (5 μL) of reaction mixture was subjected to HPLC. The result was compared with that of two-step titration method.[24] For the accuracy and precision study, the diluted disinfectant sample was added to acetonitrile containing 1 and 10 μM PAA and reacted with **13** (100 μM) at 50 °C for 30 min. An aliquot (5 μL) of each reaction mixture was subjected to HPLC.

RESULTS AND DISCUSSION

Fluorescence Characteristics of the Benzofurazan Compounds Bearing an Aliphatic Substituent Group Having an n-Electron. Nine NBD compounds (NBD-NH$(CH_2)_2$R, **1**−**9**) and two ABD compounds (ABD-S$(CH_2)_2$R, **10**, **11**) were synthesized for this study (Figure 1). The benzofurazan compounds having an NBD-NH− moiety and ABD-S− moiety were selected as the fluorophore, since they had relatively large Φ values.[32] Compounds **1**−**11** have an n-electron in the aliphatic moiety (R). In these compounds, the benzofurazan skeleton and an aliphatic moiety having an n-electron (R) are connected through the short methylene spacer. A previous paper reported that the PET process occurred more efficiently as the spacer length became shorter.[20] NBD-NHMe and ABD-SMe were also synthesized as the compounds without an aliphatic moiety having an n-electron (R). The fluorescence characteristics of the synthesized compounds in benzene, acetonitrile, and methanol are shown in Table 1. The absorption characteristics and the maximum emission wavelengths of NBD-NH$(CH_2)_2$R and ABD-S$(CH_2)_2$R are similar to those of NBD-NHMe and ABD-SMe, respectively. However, the Φ values of the compounds bearing a tertiary or secondary amino moiety in an aliphatic subsituent group (**1**−**3**) are very small compared with those of NBD-NHMe in all the solvents. These results indicated that the intramolecular electron transfer (the PET process) occurred between the excited benzofurazan skeleton and the aliphatic moiety (R) in compounds having small Φ values, and the tertiary and secondary amino moiety would work as an excellent n-donor. NBD-NH$(CH_2)_2NH_2$ (**4**) is weakly fluorescent in acetonitrile and methanol, whereas it fluoresces in benzene. These results could be explained by a previous report[33] that the PET process occurred more efficiently in polar solvents than in nonpolar solvents.

Method for Predicting the Φ Values of the Benzofurazan Compounds Bearing an Aliphatic Substituent Group Having an n-Electron. First, the molecular orbital calculation was carried out to obtain the HOMO energies of the aliphatic compounds having an n-electron (RCH_3 or RC_2H_5) in acetonitrile. The compounds, RCH_3 or RC_2H_5, corresponded to the aliphatic moiety (R) of the synthesized NBD compounds (**1**−**9**) (Table 2). For example, triethylamine (Et_3N) was chosen as the aliphatic compound corresponding to the aliphatic moiety of NBD-NH-$(CH_2)_2NEt_2$ (**2**), and similarly, *N*-methylacetamide (CH_3NHAc) was chosen corresponding to that of NBD-NH$(CH_2)_2$NHAc (**5**). The calculated HOMO energy of RCH_3 or RC_2H_5 denotes the

(32) Uchiyama, S.; Santa, T.; Imai, K. *J. Chem. Soc., Perkin Trans. 2* **1999**, 2525−2532.
(33) Bissell, R. A.; de Silva, A. P.; Fernando, W. T. M. L.; Patuwathavithana, S. T.; Samarasinghe, T. K. S. D. *Tetrahedron Lett.* **1991**, *32*, 425−428.

Table 2. HOMO Energy of the Aliphatic Compound (RCH_3 or RC_2H_5) Corresponding to the Aliphatic Moiety (R) of the NBD Compound

HOMO energy of the corresponding aliphatic compd		Φ value of the NBD-NH$(CH_2)_2$R		
corresponding aliphatic compd	HOMO (eV)	no.	R	Φ
triethylamine (Et_3N)	−9.657	**2**	NEt_2	0.00017
trimethylamine (Me_3N)	−9.753	**1**	NMe_2	0.00038
dimethylamine (Me_2NH)	−9.827	**3**	NHMe	0.0018
methylamine (CH_3NH_2)	−9.977	**4**	NH_2	0.014
dimethyl ether (Me_2O)	−11.066	**8**	OMe	0.30
methanol (CH_3OH)	−11.380	**7**	OH	0.29
N-methylacetamide (CH_3NHAc)	−11.429	**5**	NHAc	0.30
N,*N*-dimethylacetamide (Me_2NAc)	−11.509	**6**	NMeAc	0.32
acetic acid methyl ester (CH_3OAc)	−11.857	**9**	OAc	0.32

Table 3. *K* Value of the Aliphatic Compound (RCH_3 or RC_2H_5) Corresponding to the Aliphatic Moiety (R) of the Benzofurazan Compound

K value of the corresponding aliphatic compd					Φ value of the compd[c]		
fluorophore	added aliphatic compd	*c* (mM)[a]	*r*[b]	*K* (M^{-1})	no.	R	Φ
NBD-NHMe	triethylamine (Et_3N)	20	0.999	98.0	**2**	NEt_2	0.00017
NBD-NHMe	dimethylamine (Me_2NH)	20	0.998	74.4	**3**	NHMe	0.0018
NBD-NHMe	trimethylamine (Me_3N)	20	0.996	35.9	**1**	NMe_2	0.00038
NBD-NHMe	methylamine (CH_3NH_2)	50	0.992	23.8	**4**	NH_2	0.014
NBD-NHMe	*N*-methylacetamide (CH_3NHAc)	1000	0.989	0.2	**5**	NHAc	0.30
NBD-NHMe	*N*,*N*-dimethylacetamide (Me_2NAc)	1000	0.979	0.1	**6**	NMeAc	0.32
NBD-NHMe	methanol (CH_3OH)	1000	*d*	*d*	**7**	OH	0.29
NBD-NHMe	diethyl ether (Et_2O)	1000	*d*	*d*	**8**	OMe	0.30
NBD-NHMe	ethyl acetate (EtOAc)	1000	*d*	*d*	**9**	OAc	0.32
ABD-SMe	*N*-methylacetamide (CH_3NHAc)	1000	0.999	1.9	**10**	NHAc	0.013
ABD-SMe	methanol (CH_3OH)	1000	0.995	0.3	**11**	OH	0.083

[a] Concentration of the added aliphatic compound. [b] Correlation coefficient. [c] NBD-NH$(CH_2)_2$R for compounds **1**−**9**; ABD-S$(CH_2)_2$R for **10** and **11**. [d] Quenching was not observed.

electron-donating ability of the aliphatic moiety (R). As shown in Table 2, the Φ values of the NBD compounds (**1**−**9**) bearing an aliphatic moiety (R), which has a high electron-donating ability (i.e., the calculated HOMO energy of RCH_3 or RC_2H_5 is large), are greatly reduced, similar to those of the NBD compounds bearing an aromatic moiety.[11] Therefore, the Φ values of the benzofurazan compounds bearing an aliphatic moiety having an n-electron (R) are predictable by calculating the HOMO energies of the corresponding aliphatic compounds (RCH_3 or RC_2H_5).

Next, the Stern−Volmer plotting was carried out to obtain the *K* values of the aliphatic compounds having an n-electron (RCH_3 or RC_2H_5) in acetonitrile corresponding to the aliphatic moiety (R) of the synthesized compounds (**1**−**11**) (Table 3). The compound with the larger *K* value has a higher quenching ability. As shown in Table 3, the Φ values of compounds **1**−**11** bearing an aliphatic moiety (R), which has a high quenching ability (i.e., the *K* value of RCH_3 or RC_2H_5 is large), are reduced. These results indicated that the mechanism of the intramolecular electron transfer between the benzofurazan skeleton and an aliphatic moiety (R) was identical with that of the intermolecular electron transfer between the corresponding benzofurazan compound and aliphatic compound (RCH_3 or RC_2H_5), and the Φ values of the benzofurazan compounds bearing an aliphatic moiety having an n-electron (R) are also predictable by obtaining the *K* values of the corresponding aliphatic compounds (RCH_3 or RC_2H_5).

Design of New PET Reagents for PAA and Application to HPLC Analysis. The above results enable us to design the PET reagents by obtaining the HOMO energies using the molecular orbital calculations and the *K* values with Stern−Volmer plotting. First, an aliphatic moiety (R), which has a high quenching ability (i.e., RCH_3 or RC_2H_5 has a large HOMO energy or a large *K* value), must be selected as a reacting moiety of the PET reagent. We should then confirm that an aliphatic moiety corresponding to the reacted moiety has a low quenching ability (i.e., a small HOMO energy or a small *K* value). For example, methylamine (RCH_3, R = NH_2) has a large HOMO energy (−9.977 eV; see Table 2) and a large *K* value for NBD-NHMe (*K* = 23.8; see Table 3) and the acetylated derivative of methylamine, *N*-methylacetamide, has a small HOMO energy (−11.429 eV, Table 2) and a small *K* value for NBD-NHMe (*K* = 0.2, Table 3), suggesting that NBD-NH-$(CH_2)_2NH_2$ can be a PET reagent for carboxylic acids such as acetic acid. Actually, **4** (Φ = 0.014) was weakly fluorescent and its derivative with acetic acid, **5** (Φ = 0.30), was strongly fluorescent in acetonitrile as indicated in Table 1.

In the same way, we designed a new PET reagent for PAA. We first tried to improve the previously reported NBD-Bz-*p*-NMe_2[11] with respect to its reactivity to peroxycarboxylic acids. NBD-Bz-*p*-NMe_2 has a *N*,*N*-dialkylaniline moiety as a reacting moiety, but it reacted only with MCPBA and did not react with the less reactive peroxycarboxylic acids. Since a trialkylamine moiety seemed to be more reactive than an *N*,*N*-dialkylaniline moiety, NBD-NH$(CH_2)_2NEt_2$ (**2**) was chosen, expecting to be oxidized by PAA to the corresponding amine *N*-oxide, NBD-NH$(CH_2)_2$-NEt_2O (**12**). The calculated HOMO energy of triethylamine (as

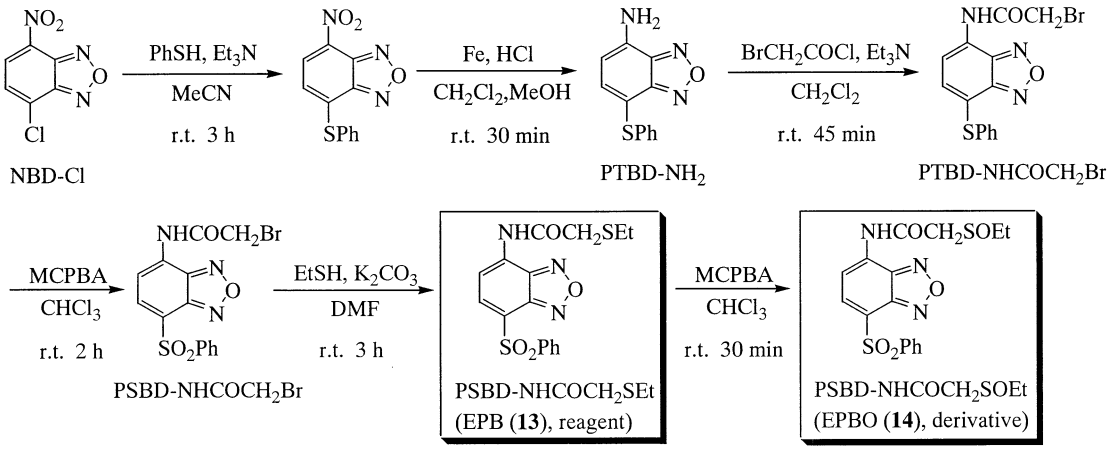


Figure 2. Synthesis of EPB (**13**) and its derivative, EPBO (**14**).

a reacting moiety, −9.657 eV, Table 2) was large, whereas that of triethylamine *N*-oxide (as a reacted moiety, −11.356 eV) was small. Therefore, it was estimated that NBD-NH$(CH_2)_2NEt_2$ (**2**) would be a PET reagent for PAA. As indicated in Table 1, **2** did not fluoresce ($\Phi = 0.00017$) in acetonitrile. **12** was then synthesized to obtain the fluorescence property. As expected, **12** fluoresced ($\Phi = 0.051$, $\lambda_{ab} = 474$ nm, $\lambda_{em} = 534$ nm) in acetonitrile and the Φ value of **12** was 294 times greater than that of **2**. The fluorescence properties of **2** and **12** in water were also obtained to apply the reagent, **2**, to the reversed-phase HPLC analysis, resulting in the fact that the Φ value of the derivative, **12** ($\Phi = 0.021$, $\lambda_{ab} = 469$ nm, $\lambda_{em} = 531$ nm), was smaller than that of the reagent, **2** ($\Phi = 0.026$, $\lambda_{ab} = 463$ nm, $\lambda_{em} = 528$ nm). Considering the pK_a value of triethylamine (p$K_a = 10.7$), the electron transfer from the n-electron of the $-NEt_2$ moiety to the benzofurazan skeleton would be suppressed because the $-NEt_2$ moiety would be protonated in water. These results indicated that this reagent was not suitable for reversed-phase HPLC analysis.

Next, the sulfide moiety was chosen as a reacting moiety with PAA, since the reacting moiety of the conventional derivatization reagents for PAA consisted of a sulfide moiety.[22,27,28] First, the NBD compounds bearing a sulfide moiety (R = SEt), NBD-NH-$(CH_2)_2$SEt, was chosen as a reagent, expecting to be oxidized by PAA to the corresponding sulfoxide, NBD-NH$(CH_2)_2$SOEt. As the HOMO energy of methyl ethyl sulfoxide (as a reacted moiety, −9.826 eV) was smaller than that of methyl ethyl sulfide (RCH_3, R = SEt) (as a reacting moiety, −9.751 eV), it was estimated that the Φ value of NBD-NH$(CH_2)_2$SOEt would be larger than that of NBD-NH$(CH_2)_2$SEt. However, considering the *K* value of methyl ethyl sulfide for NBD-NHMe ($K = 1.8$), NBD-NH$(CH_2)_2$SEt would be fluorescent, so NBD-NHMe was not suitable for the fluorophore of the PET reagent having a sulfide moiety. The PSBD compound bearing a sulfide moiety (R = SEt), **13** (see Figure 2), was then chosen, since PSBD-NHAc also had a large Φ value ($\Phi = 0.45$ in acetonitrile, measured in this study). From the *K* value of methyl ethyl sulfide (RCH_3, R = SEt) for PSBD-NHAc ($K = 60.1$) and that of dimethyl sulfoxide (RCH_3, R = SOMe, easily obtained, in place of R = SOEt) for PSBD-NHAc ($K = 8.5$), it was expected that the reagent, **13**, would not fluoresce and its derivative, **14**, would fluoresce. Finally, **13** and **14** were synthesized (Figure 2), and their fluorescence characteristics in acetonitrile are shown in Table 4. As expected, the Φ value of **14** was

Table 4. Fluorescence Characteristics of 13 (reagent) and 14 (derivative)

solvent	compd		λ_{ab} (nm)	λ_{em} (nm)	Φ	ratio[a]
acetonitrile	**13**	EPB	367	468	0.0014	254
	14	EPBO	365	466	0.34	
acetonitrile−water (2:3)	**13**	EPB	367	483	0.00049	53
	14	EPBO	362	479	0.026	

[a] The ratio of the Φ value of EPBO (**14**) to the Φ value of EPB (**13**).

254 times greater than that of **13** in acetonitrile. The fluorescence characteristics in water could not be obtained due to the poor solubility of **13** and **14**, but considering the pK_a value of the dialkyl sulfide (p$K_a = -5.3$), the PET process occurring in **13** would not be suppressed even in water. Therefore, **13** seemed to have the appropriate fluorescence property of a PET reagent for PAA. This is the first report showing that the PET process occurs between a fluorophore and an aliphatic sulfide moiety.

The reactivity of **13** as a derivatization reagent for PAA was then tested using reversed-phase HPLC. The peak for **14** was fluorometrically detected at ∼6 min using acetonitrile−water (2:3) as the eluent, as shown in Figure 3. The fluorescence characteristics of **13** and **14** in this eluent are also shown in Table 4. In the HPLC eluent, the Φ value of **14** is 53 times greater than that of **13**. The time course study of the derivatization of PAA (10 μM) with **13** (100 μM) was performed at room temperature or at 50 °C. The reaction was completed at 50 °C within 30 min in acetonitrile to give a fluorescent derivative of **14** (yield, 99.8%), whereas the reaction was not completed at room temperature for 60 min (yield: 84.3%). Thus, this condition (50 °C, 30 min) was adopted for the derivatization of **13** with PAA. The chromatogram of the reaction mixture of **13** (100 μM) and PAA (20 μM) is shown in Figure 3. The peak of the reagent, **13**, (b) is much smaller than that of the derivative, **14**, (a), though excess reagent was included in the reaction mixture. The calibration curve for PAA (0.020−20 μM) with **13** (100 μM) was linear over the range from 390 fmol (78 nM) to 100 pmol (20 μM) ($r > 0.999$). The detection limit (S/N = 3) of the derivative of the developed **13** was 105 fmol (21 nM), which was lower than that of the conventional reagent.[22,28] The reaction yields of the derivative of **13** (100 μM)

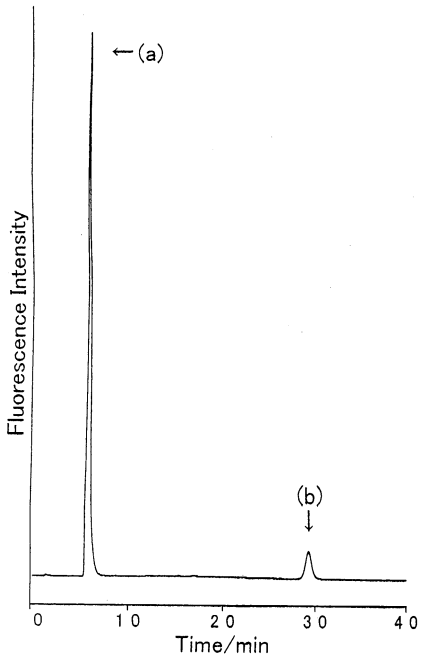


Figure 3. Chromatogram of PAA derivatized with excess **13**: (a) **14**, 100 pmol, (b) excess **13**, 400 pmol; column, TSKgel ODS-80Ts (150 × 4.6 mm, i.d. 5 μm); eluent, acetonitrile−water (2:3); flow rate, 1.0 mL min^{-1}; detection, excitation at 362 nm, emission at 479 nm.

with MCPBA, peroxybenzoic acid, hydrogen peroxide, *tert*-butyl hydroperoxide, and cumene hydroperoxide (20 μM) under the same conditions were 100.1%, 0.057%, 0.096%, 0.019%, and 0.005%, respectively, so the cross-reactivity of **13** toward hydrogen peroxide was negligible. The cross-reactivity of the reagent toward hydrogen peroxide must be remarkably low,[22] since PAA solutions always contain significant amounts of hydrogen peroxide.

The proposed method was applied to the determination of PAA in disinfectant sample. The determined concentration of PAA in disinfectant sample by this method was 0.94 ± 0.036 M (mean ± SD, $n = 5$). This result was comparable to the result obtained by the popular two-step titration method[24] (0.96 ± 0.055 M; mean ± SD, $n = 5$). The accuracy and precision data were shown in Table 5. Both intraday and interday accuracy and precision were satisfactory for the analysis of PAA in real samples. From these results, **13** was indicated to be an excellent reagent for PAA with high sensitivity and selectivity.

Table 5. Precision and Accuracy in the Determination of PAA in Disinfectant Sample

	added amounts (μM)		
	0	1	10
	Intraday ($n = 5$)		
found (μM)	2.20	3.29	12.23
RSD (%)	5.4	5.0	1.5
accuracy (%)		109.6	100.3
	Interday ($n = 5$)		
found (μM)	2.21	3.13	11.98
RSD (%)	5.8	4.3	2.5
accuracy (%)		92.1	97.7

CONCLUSIONS

The Φ values of the benzofurazan compounds bearing an aliphatic substituent group having an n-electron became predictable based on the molecular orbital calculations and the Stern−Volmer plotting. It is expected that various new PET reagents can be developed using this method for prediction. The fluorescent reagents have been used not only for HPLC analysis but also for bioimaging, so this method can be widely applicable to the development of the fluorescent reagents, such as derivatization reagents, sensors, probes, and so on. We succeeded in developing a highly sensitive and selective PET reagent for PAA, **13**, based on this method. In the future, the simultaneous determination of peroxycarboxylic acids by postcolumn derivatization seems to be possible because of the fluorescent on−off property of this reagent.

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