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Study on the fluorescent 'on–off' properties of benzofurazan compounds bearing an aromatic substituent group and design of fluorescent 'on–off' derivatization reagents

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In order to elucidate the fluorescence 'on–off' switching mechanism of benzofurazan compounds bearing an aromatic substituent group, we first studied the intermolecular electron transfer between fluorescent benzofurazan compounds and other aromatic compounds. The fluorescence was quenched by the added aromatic compounds without changing the absorption and excitation wavelengths, indicating that quenching was caused by intermolecular electron transfer. The quenching abilities of the added aromatic compounds reflected the *K* values of the Stern–Volmer plot. The *K* values were also co-related with the molecular orbital energies of the benzofurazan and the added aromatic compounds calculated with the semi-empirical PM3/COSMO method. Next, 11 benzofurazan compounds bearing an aromatic substituent group were synthesized and the effects of the intramolecular electron transfer between the benzofurazan skeleton and the aromatic substituent group were studied. A benzofurazan compound bearing an aromatic substituent group which has a high quenching ability (large *K* value) in the study of intermolecular electron transfer did not fluoresce (fluorescence 'off') because of the intramolecular electron transfer, whereas a compound bearing an aromatic substituent group which has a low quenching ability (small *K* value) fluoresced (fluorescence 'on'). From the results, the fluorescence 'on–off' properties of benzofurazan compounds bearing an aromatic substituent group were predictable from the estimation of the quenching ability of the aromatic substituent group using the Stern–Volmer plot. Finally, the prediction of the fluorescence 'on–off' properties of benzofurazan compounds was applied to the development of a new fluorescent 'on–off' reagent for oxidation. The results are useful in the context of the development of new fluorescent 'on–off' reagents for use in analytical chemistry.

Introduction

Since most analytes do not fluoresce, their derivatization with various fluorescent reagents[1,2] is necessary for their sensitive and selective detection. Among many fluorescent reagents reported previously, those which are non-fluorescent themselves (fluorescence 'off') and react with analytes to fluoresce (fluorescence 'on'), seemed to be the most favorable for the detection of small amounts of analytes, since such fluorescence 'on-off' reagents can avoid interference from the fluorescence of the reagents themselves.

Previously, the following fluorescence 'on–off' derivatization reagents having a benzofurazan (2,1,3-benzoxadiazole) skeleton were developed: 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F)[3] for amines, 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadazole (DBD-F)[4] for amines and thiols, 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F)[5] for thiols, 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F)[6] for thiols, 4-hydrazino-7-nitro-2,1,3-benzoxadiazole (NBD-H)[7] for aldehydes and ketones and *N*-methyl-4-hydrazino-7-nitro-2,1,3-benzoxadiazole (MNBDH)[8] for ozone, nitrogen dioxide and nitrite. These reagents have been effectively applied to the detection of small amounts of various analytes in biological samples.[9] However, they were developed by trial and error because we did not have any general rules which predict the fluorescence 'on–off' properties based on the compounds involved. For the efficient development of new fluorescence 'on–off' reagents with the benzofurazan skeleton, knowledge of the fluorescence 'on–off' switching mechanism is necessary.

For this purpose, we first investigated the fluorescence 'on–off' properties (the state of fluorescence switching) of 4,7-disubstituted benzofurazan compounds. Since the electronic state of the benzofurazan skeleton seemed to be related to the fluorescence 'on–off' property, we estimated the electron density and the dipole moment of these benzofurazan compounds using the sum and difference of the Hammett substituent constants (σ_p)[10,11] at the 4- and 7-positions. We found that the sum and difference of σ_p were well related to the fluorescence 'on–off' properties of these compounds.[12] The total electron density and the dipole moment directed from the 4- to 7-positions of the benzofurazan skeleton, obtained by semi-empirical PM3 calculation, were also related to the fluorescence 'on–off' properties.[13] Based on these relationships, we were able to predict the fluorescence 'on–off' properties of the 4,7-disubstituted benzofurazan compounds and thus developed the new fluorescence 'on–off' derivatization reagents, 7-phenylsulfonyl-4-(2,1,3-benzoxadiazolyl) isocyanate (PSBD-NCO),[14] 7-acetylamino-4-mercapto-2,1,3-benzoxadiazole (AABD-SH)[15] and 7-methylthio-4-(2,1,3-benzoxadiazolyl) isothiocyanate (MTBD-NCS)[16] for alcohols, carboxylic acids and amino acid sequencing analysis, respectively. Next, we studied the fluorescence 'on–off' switching properties of benzofurazan compounds using the detailed semi-empirical PM3-CAS/CI calculation. This calculation indicated that in the benzofurazan compounds, the fluorescence 'on–off' property was determined by the probability of the non-radiative $S_1 \rightarrow T_2$ intersystem crossing in the benzofurazan skeleton.[17,18]

However, there are some benzofurazan compounds for which the fluorescence 'on–off' property cannot be explained by the electronic state of the benzofurazan skeleton. For example, NBD-Trp (the derivative of NBD-F with tryptophan) did not fluoresce,[19] although it should fluoresce like the other NBD-

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amine derivatives according to the prediction using σ_p or semi-empirical PM3 calculation. We considered that another fluorescence 'on–off' switching mechanism may exist in NBD-Trp. Because the fluorescence of NBD-Trp was restored by the degradation of its indole skeleton,[19,20] this fluorescence 'off' property seemed to be derived from the photoinduced electron transfer (PET)[21–25] between the benzofurazan ring and aromatic ring in the substituent group (intramolecular electron transfer).

Considering that some fluorescent coumarin dyes have been reported to be quenched in the presence of other aromatic compounds,[26,27] we expected that the fluorescent benzofurazan compounds would also be quenched by the addition of another aromatic compound (intermolecular electron transfer[26,27]). Assuming that the mechanism of the quenching by the intermolecular electron transfer was identical with that of the fluorescence 'off' switching by intramolecular electron transfer, the knowledge of the intermolecular electron transfer could be applied to predict the fluorescence 'on–off' properties of the benzofurazan compounds. In this work, therefore, we first investigated the intermolecular electron transfer between fluorescent benzofurazan compounds and other aromatic compounds. Next, the benzofurazan compounds bearing an aromatic substituent group were synthesized and the intramolecular electron transfer between the benzofurazan skeleton and the aromatic substituent was investigated. Finally, we propose a method for predicting the fluorescence 'on–off' properties of benzofurazan compounds based on the above data and for the development of a new type of fluorescence 'on–off' reagent based on the prediction.

Experimental

Materials

4-Methylamino-7-nitro-2,1,3-benzoxadiazole (NBD-NHMe),[12] 4-methylthio-7-aminosulfonyl-2,1,3-benzoxadiazole (ABD-SMe),[12] 4-acetylamino-7-phenylsulfonyl-2,1,3-benzoxadiazole (PSBD-NHAc),[12] and dimethylaniline *N*-oxide[28] were synthesized and purified as described previously. 2-, 3- and 4-aminobenzylamine, *p*-anisidine, anisole, benzimidazole, benzotrifluoride (α,α,α-trifluorotoluene), benzylamine, 4-chlorobenzylamine, 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl), cyanobenzene, 4-dimethylaminobenzylamine dihydrochloride, *p*-dinitrobenzene, indole, 4-methoxybenzylamine and *N*-methylaniline were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Acetic anhydride, acetonitrile, *p*-aminophenol, aniline, benzene, chlorobenzene, chloroform, ethyl acetate, hexane, methanol, 4-methylbenzylamine, nitrobenzene, pyridine and toluene were purchased from Kanto Chemicals (Tokyo, Japan). 4-Nitrobenzylamine hydrochloride and *tert*-butyl hydroperoxide were obtained from Sigma-Aldrich Japan (Tokyo, Japan). Acetanilide, *m*-chloroperbenzoic acid (MCPBA), *N*,*N*-dimethylaniline, *p*-*N*,*N*-dimethylphenylenediamine, hydrogen peroxide and phenol were purchased from Wako Pure Chemicals (Osaka, Japan). Imidazole and silica gel 60 were supplied by Merck (Darmstadt, Germany). All chemicals were of HPLC or guaranteed reagent grade and were used without further purification.

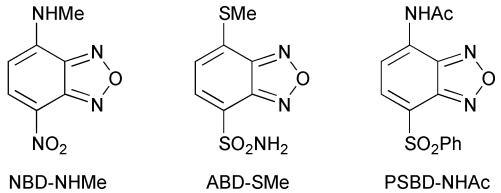

Fig. 1 Structures of three fluorescent benzofurazan compounds used in this study.

Apparatus

Melting-points were measured on a Yanagimoto (Tokyo, Japan) micro melting point apparatus and were uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a JEOL (Tokyo, Japan) LA-500 spectrometer with tetramethylsilane as an internal standard (abbreviations used: s = singlet, d = doublet, t = triplet, m = multiplet). *J* values are given in hertz. Mass spectra were measured on a Hitachi (Tokyo, Japan) M-1200 H mass spectrometer [atmospheric pressure chemical ionization (APCI) system] . UV-VIS absorption spectra were measured on a JASCO (Tokyo, Japan) Ubest-50 spectrometer. Fluorescence spectra were measured on a Hitachi F-4010 and a Hitachi F-4500 spectrofluorimeter.

Synthesis

4-(*o*-Aminobenzylamino)-7-nitro-2,1,3-benzoxadiazole [NBD-Bz-*o*-NH$_2$ (21)]. 2-Aminobenzylamine (300 mg) was dissolved in 5 ml of acetonitrile. After the addition of 4-chloro-7-nitro-2,1,3-benzoxaziazole (NBD-Cl, 100 mg, 0.50 mmol) solution (in 10 ml of acetonitrile), the mixture was stirred for 30 min at room temperature. The reaction mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with ethyl acetate–hexane (1 + 1) as the developing solvent to afford NBD-Bz-*o*-NH$_2$ (55 mg, 39%) as a brown powder, m.p. 227 °C. ^{1}H NMR δ_H ($CDCl_3$) 8.52 (1H, d, J 8.5), 6.81–7.05 (4H, m), 6.32 (2H, m), 4.56 (2H, d, *J* 4.9), 3.77 (2H, br). APCI-MS: *m/z* 286 ([M + H]$^+$).

4-(*m*-Aminobenzylamino)-7-nitro-2,1,3-benzoxadiazole [NBD-Bz-*m*-NH$_2$(22)]. A similar procedure to that for NBD-Bz-*o*-NH$_2$ yielded 51% of product as a brown powder, m.p. 175–176 °C. ^{1}H NMR δ_H ($CDCl_3$) 8.48 (1H, d, *J* 8.5), 7.19 (1H, t), 6.74 (1H, d, *J* 7.1), 6.68 (2H, m), 6.49 (1H, m), 6.22 (1H, d, *J* 8.5), 4.57 (2H, d, *J* 5.5), 3.76 (2H, br). APCI-MS: *m/z* 286 ([M + H]$^+$).

4-(*p*-Aminobenzylamino)-7-nitro-2,1,3-benzoxadiazole [NBD-Bz-*p*-NH$_2$ (23)]. A similar procedure to that for NBD-Bz-*o*-NH$_2$ yielded 10% of product as a brown powder, m.p. 205–206 °C. ^{1}H NMR: δ_H ($CDCl_3$) 8.49 (1H, d, *J* 8.5), 7.17 (2H, d, *J* 8.8), 6.71 (2H, d, *J* 8.8), 6.39 (1H, br), 6.24 (1H, d, *J* 8.5), 4.53 (2H, d, *J* 5.5), 3.80 (2H, br). Found: C, 55.01; H, 3.97; N, 24.17. Calc. for $C_{13}H_{11}N_5O_3$: C, 54.74; H, 3.89; N, 24.55%. APCI-MS: *m*/*z* 284 ([M − H]$^-$).

4-(*o*-Acetylaminobenzylamino)-7-nitro-2,1,3-benzoxadiazole [NBD-Bz-*o*-NHAc(24)]. NBD-Bz-*o*-NH$_2$ (25 mg, 0.09 mmol) was dissolved in the mixture of acetonitrile (15 ml) and acetic anhydride (200 μl). The mixture was stirred for 3 h at room temperature. The reaction mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with ethyl acetate as the developing solvent to afford NBD-Bz-*o*-NHAc (25 mg, 87%) as an orange powder, m.p. 218 °C. ^{1}H NMR: δ_H ($CDCl_3$) 8.47 (1H, d, *J* 8.5), 7.42 (2H, m), 7.32 (2H, m), 7.05 (1H, br), 6.27 (1H, d, *J* 8.5), 4.68 (2H, d, *J* 5.5), 2.05 (3H,s). APCI-MS: *m/z* 328 ([M + H]$^+$).

4-(*m*-Acetylaminobenzylamino)-7-nitro-2,1,3-benzoxadiazole [NBD-Bz-*m*-NHAc (25)]. A similar procedure to that for NBD-Bz-*o*-NHAc yielded 99% of product as an orange powder, m.p. 228–229 °C. ^{1}H NMR: δ_H ($CDCl_3$) 8.46 (1H, d, *J*

8.5), 7.98 (1H, s), 7.49–7.86 (4H, m), 6.58 (1H, br), 6.20 (1H, d, *J* 8.5), 4.67 (2H, d, *J* 5.5), 2.16 (3H, s). APCI-MS: *m/z* 328 ($[M + H]^+$).

4-(*p*-Acetylaminobenzylamino)-7-nitro-2,1,3-benzoxadiazole [NBD-Bz-*p*-NHAc (26)]. A similar procedure to that for NBD-Bz-*o*-NHAc yielded 73% of product as an orange powder, m.p. 265 °C (decomp.). ^{1}H NMR: δ_H ($CDCl_3$) 8.47 (1H, d, *J* 8.5), 7.93 (1H, s), 7.47–7.57 (4H, m), 6.45 (1H, br), 6.21 (1H, d, *J* 8.5), 4.64 (2H, d, *J* 5.5), 2.04 (3H, s). APCI-MS: *m/z* 326 ($[M - H]^-$).

4-(*p*-Methoxybenzylamino)-7-nitro-2,1,3-benzoxadiazole [NBD-Bz-*p*-OMe (27)]. 4-Chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl, 30 mg, 0.15 mmol) was dissolved in 2 ml of acetonitrile. After the addition of 4-methoxybenzylamine (50 μl), the mixture was stirred for 30 min at room temperature. The reaction mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with dichloromethane as the developing solvent to afford NBD-Bz-*p*-OMe (40 mg, 89%) as an orange powder, m.p. 178 °C. ^{1}H NMR: δ_H ($CDCl_3$) 8.48 (1H, d, *J* 8.5), 7.31 (2H, d, *J* 8.8), 6.94 (2H, d, *J* 8.8), 6.43 (1H, br), 6.24 (1H, d, J 8.5), 4.60 (2H, d, *J* 5.1), 3.83 (3H,s). Found: C, 55.75; H, 4.25; N, 18.57. Calc. for $C_{14}H_{12}N_4O_4$: C, 56.00; H, 4.03; N, 18.66%, APCI-MS: *m/z* 299 ($[M - H]^-$).

4-(*p*-Methylbenzylamino)-7-nitro-2,1,3-benzoxadiazole [NBD-Bz-*p*-Me (28)]. A similar procedure to that for NBD-Bz-*p*-OMe yielded 80% of product as an orange powder, m.p. 208–209 °C. ^{1}H NMR δ_H ($CDCl_3$) 8.47 (1H, d, *J* 8.5), 7.22–7.28 (4H, m), 6.49 (1H, br), 6.22 (1H, d, J 8.5), 4.63 (2H, d, *J* 5.5), 2.38 (3H, s). Found: C, 59.25; H, 4.35; N, 19.63. Calc. for $C_{14}H_{12}N_4O_3$: C, 59.15; H, 4.25; N, 19.71%. APCI-MS: *m/z* 285 ($[M + H]^+$).

4-Benzylamino-7-nitro-2,1,3-benzoxadiazole [NBD-Bz (29)]. A similar procedure to that for NBD-Bz-*p*-OMe yielded 51% of product as an orange powder, m.p. 210 °C. ^{1}H NMR: δ_H ($CDCl_3$) 8.48 (1H, d, *J* 8.5), 7.39–7.44 (5H, m), 6.48 (1H, br), 6.22 (1H, d, *J* 8.5), 4.68 (2H, d, *J* 5.5). Found: C, 57.72; H, 3.90; N, 20.61. Calc. for $C_{13}H_{10}N_4O_3$: C, 57.78; H, 3.73; N, 20.73%. APCI-MS: *m/z* 271 ($[M + H]^+$).

4-(*p*-Chlorobenzylamino)-7-nitro-2,1,3-benzoxadiazole [NBD-Bz-*p*-Cl (30)]. A similar procedure to that for NBD-Bz-*p*-OMe yielded 81% of product as an orange powder, m.p. 220–221 °C. ^{1}H NMR: δ_H ($CDCl_3$) 8.46 (1H, d, *J* 8.5), 7.40 (2H, d, *J* 8.8), 7.32 (2H, d, *J* 8.8), 6.48 (1H, br), 6.19 (1H, d, *J* 8.5), 4.66 (2H, d, *J* 5.5). Found: C, 51.15; H, 3.15; N, 18.41. Calc. for $C_{13}H_9ClN_4O_3$: C, 51.25; H, 2.98; N, 18.39%. APCI-MS: *m/z* 303 ($[M - H]^-$).

4-(*p*-Nitrobenzylamino)-7-nitro-2,1,3-benzoxadiazole [NBD-Bz-*p*-NO_2 (31)]. A similar procedure to that for NBD-Bz-*p*-OMe yielded 22% of product as an orange powder, m.p. 213 °C. ^{1}H NMR: δ_H ($CDCl_3$) 8.45 (1H, d, *J* 8.5), 8.28 (2H, d, *J* 8.5), 7.57 (2H, d, *J* 8.5), 6.59 (1H, br), 6.41 (1H, d, *J* 8.5), 4.85 (2H, d, *J* 5.5). APCI-MS: *m/z* 314 ($[M - H]^-$).

4-(*p*-*N*,*N*-Dimethylamino)benzylamino-7-nitro-2,1,3-benzoxadiazole [NBD-Bz-*p*-NMe_2(32)]. A similar procedure to that for NBD-Bz-*p*-OMe yielded 35% of product as black crystals, m.p. 214 °C. ^{1}H NMR: δ_H ($CDCl_3$) 8.49 (1H, d, *J* 8.5), 7.24 (2H, d, *J* 8.8), 6.74 (2H, d, *J* 8.8), 6.39 (1H, br), 6.25 (1H, d, *J* 8.5), 4.54 (2H, d, *J* 5.0), 2.98 (6H, s). Found: C, 57.46; H, 4.95; N, 22.06. Calc. for $C_{15}H_{15}N_5O_3$: C, 57.50; H, 4.83; N, 22.35%; APCI-MS: *m/z* 314 ($[M + H]^+$).

4-(*p*-*N*-Oxy-*N*,*N*-dimethylamino)benzylamino-7-nitro-2,1,3-benzoxadiazole [NBD-Bz-*p*-NMe_2O (33)]. NBD-Bz-*p*-NMe_2 (14 mg, 0.44 mmol) was dissolved in 5 ml of chloroform. After the addition of *m*-chloroperbenzoic acid (MCPBA, 11 mg), the mixture was stirred for 3 h at room temperature. The reaction mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on alumina with chloroform–methanol (1 + 1) as the developing solvent to afford NBD-Bz-*p*-NMe_2O (14 mg, 95%) as an orange powder, m.p. 135–137 °C. ^{1}H NMR: δ_H (CD_3CN) 8.35 (1H, d, *J* 7.0), 7.76 (2H, br), 7.52 (2H, br), 6.15 (1H, d, *J* 7.0), 4.73 (2H, br), 3.77 (6H, s). APCI-MS: *m/z* 330 ($[M + H]^+$).

Measurement of UV-VIS absorption spectra of benzofurazan compounds with aromatic compounds

The mixture of a benzofurazan compound (NBD-NHMe, ABD-SMe or PSBD-NHAc) (30 μmol l^{-1}) and aromatic compounds [*p*-*N*,*N*-dimethylphenylenediamine (**1**), indole (**2**), *N*,*N*-dimethylaniline (**5**), benzene (**15**), nitrobenzene (**19**) or *p*-dinitrobenzene (**20**)] (1 mol l^{-1}) were used for the measurement of the UV-VIS absorption spectra of benzofurazan compounds with aromatic compounds.

Measurement of fluorescence spectra of benzofurazan compounds with aromatic compounds

The mixture of a benzofurazan compound (NBD-NHMe, ABD-SMe or PSBD-NHAc) (1 μmol l^{-1}) and aromatic compounds [*p*-*N*,*N*-dimethylphenylenediamine (**1**), indole (**2**), *N*,*N*-dimethylaniline (**5**), benzene (**15**), nitrobenzene (**19**) or *p*-dinitrobenzene (**20**)] (1 mol l^{-1}) were used for the measurement of the fluorescence spectra of benzofurazan compounds with aromatic compounds.

Stern–Volmer plots

Solutions (acetonitrile, 2.5 ml) of benzofurazan compounds [NBD-NHMe (100 nmol l^{-1}), ABD-SMe (1 μmol l^{-1}) or PSBD-NHAc (200 nmol l^{-1})] were prepared. A series of aliquots of acetonitrile (100 μl) were added 12 times to the benzofurazan solution and the fluorescence intensity of this solution was defined as I_0 (control). Similarly, a series of acetonitrile solutions of aromatic compounds [100 μl, concentrations summarized in Table 1, dimethylaniline *N*-oxide (10 mmol l^{-1})] were added 12 times to the solution of benzofurazan compounds and the fluorescence intensity of this mixture was defined as *I* (test). The excitation wavelengths were at 458 nm (for NBD-NHMe), 385 nm (for ABD-SMe) and 348 nm (for PSBD-NHAc). The emission wavelengths were at 524 nm (for NBD-NHMe), 510 nm (for ABD-SMe) and 460 nm (for PSBD-NHAc). The quenching ratio (I_0/I) varied linearly with the concentration of quencher (aromatic compound in this study, [Ar]) according to the Stern-Volmer expression:

$$I_0/I = 1 + K\,[\mathrm{Ar}] \quad (1)$$

The *K* value was calculated from [Ar] and the observed I_0/I by least-squares analyses. All least-squares analyses were carried out with Microsoft EXCEL 98.

Computational methods

We employed the PM3 (MNDO-parametric method 3) method[29,30] for our calculations because this method gave good results in the calculation of molecular orbital energies of benzofurazan compounds compared with the AM1 (Austin Model 1) method in our previous report.[13] All PM3/COSMO

(conductor-like screening model)[31] semi-empirical molecular orbital calculations were carried out using the program MOPAC97 in the WinMOPAC ver. 2.0 package (Fujitsu, Tokyo, Japan) by means of a DELL Dimension XPS R450/512K computer. The geometry in acetonitrile (keyword EPS = 37.5) were optimized completely (keyword PRECISE) by the eigenvector following routine (keyword EF) to obtained the molecular orbital energies.

Measurement of UV-VIS absorption and fluorescence spectra of benzofurazan compounds bearing an aromatic substituent group

The UV-VIS absorption spectra of the benzofurazan compounds bearing an aromatic substituent group were measured in acetonitrile (30 μmol l^{-1}) at room temperature. The fluorescence spectra were measured in acetonitrile (1 μmol l^{-1}) at room temperature. Each emission spectrum was obtained by excitation at the wavelength of maximum absorption. The fluorescence quantum yields (Φ) were determined using quinine sulfate in 0.1 mol l^{-1} sulfuric acid (Φ = 0.55, excitation wavelength 355 nm, at room temperature) as a standard.

Calibration curve for the derivative of NBD-Bz-*p*-NMe_2 with MCPBA

NBD-Bz-*p*-NMe_2 (100 μmol l^{-1}) was reacted with MCPBA (0098, 0.39, 1.56, 6.25 and 25 μmol l^{-1}) in chloroform at room temperature for 20 min. An aliquot (2 μl) of each reaction mixture was subjected to HPLC.

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-80 Ts (150 × 4.6 mm id, 5 μm) (TOSOH, Tokyo Japan). The column temperature was ambient. The eluent for the derivative was methanol–water (2 + 5). The eluate was monitored with fluorescence detection (excitation at 474 nm, emission at 531 nm).

Table 1 Concentrations (10^{-3} mol l^{-1}) of aromatic compounds **1**–**20** in acetonitrile. These aromatic compounds were added to acetonitrile solutions of three fluorescent benzofurazan compounds for Stern–Volmer plot.

No.	Compound	NBD-NHMe	ABD-SMe	PSBD-NHAc
1	*p*-*N*,*N*-Dimethylphenylenediamine	10	10	10
2	Indole	50	20	20
3	*p*-Aminophenol	20	20	20
4	*p*-Anisidine	10	10	10
5	*N*,*N*-Dimethylaniline	20	20	20
6	*N*-Methylaniline	20	20	20
7	Aniline	20	20	20
8	Benzimidazole	20	40	40
9	Acetanilide	1000	100	100
10	Phenol	1000	100	100
11	Anisole	1000	100	100
12	Chlorobenzene	1000	1000	1000
13	Imidazole	1000	100	100
14	Toluene	1000	1000	1000
15	Benzene	4000	1000	1000
16	Cyanobenzene	1000	1000	1000
17	Pyridine	1000	100	100
18	Benzotrifluoride	1000	1000	1000
19	Nitrobenzene	1000	20	20
20	*p*-Dinitrobenzene	20	20	20

Results and discussion

Studies on the intermolecular electron transfer between the fluorescent benzofurazan compounds and other aromatic compounds

NBD-NHMe, ABD-SMe and PSBD-NHAc (Fig. 1) were selected as typical fluorescent benzofurazan compounds for the study. Their absorption spectra were measured in the presence of other aromatic compounds [*p*-*N*,*N*-dimethylphenylenediamine (**1**), indole (**2**), *N*,*N*-dimethylaniline (**5**), benezene (**15**), nitrobenzene (**19**) or *p*-dinitrobenzene (**20**); the compound numbers are defined as shown in Table 1, see Experimental section] in acetonitrile. The results showed that their absorption spectra were not influenced by the addition of other aromatic compounds, suggesting that the benzofurazan compounds in the ground state in acetonitrile were not influenced by the added aromatic compounds.

Concerning the effects on the fluorescence spectra, the fluorescent benzofurazan compounds were substantially quenched by the addition of **1**, **2**, **5**, **19** and **20**, but not **15**, whereas the emission wavelengths were not changed. The quenching of the fluorescence was observed in the presence of certain aromatic compounds such as indole. In order to examine the quenching ability of aromatic compounds (**1**–**20**) on the fluorescent benzofurazan compounds, Stern–Volmer plotting was carried out and the *K* values were obtained (see Experimental section) as summarized in Table 2. Previous reports indicated that quenching without a change of absorption and fluorescence wavelengths was caused by intermolecular electron transfer.[26,27] Therefore, the quenching of the fluorescent benzofurazan compounds observed seemed to be derived from the intermolecular electron transfer.

We tried to correlate the *K* values of the Stern–Volmer plots with the electronic properties of the added aromatic compounds. Previous reports indicated that aromatic compounds with a smaller ionization potential have a higher quenching ability (of larger *K* value).[32,33] Since the molecular orbital energies have sometimes been adopted for the explanation of quenching by intermolecular electron transfer[2,21,34] similarly to the ionization potential, we calculated the HOMO (highest occupied molecular orbital) and LUMO (lowest unoccupied molecular orbital) energies of 20 aromatic compounds and three fluorescent

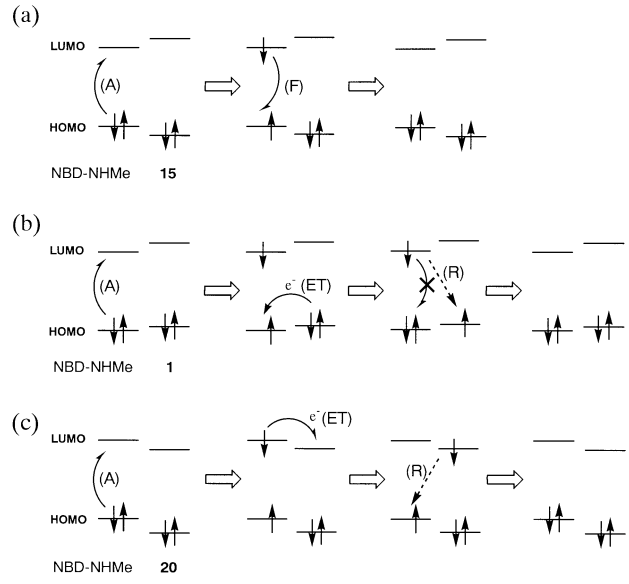

Fig. 2 Relationships between the molecular orbital energies and occurrence of the quenching of NBD-NHMe. A, absorption, F, fluorescence; ET electron transfer; R, non-radiative relaxation. The stabilization of the excited benzofurazan compound was ignored for simplification.

benzofurazan compounds with the semi-empirical PM3/COSMO method (Table 2).

Comparing the *K* values with the molecular orbital energies, the quenching mechanism of NBD-NHMe will be explained with three benzofurazan compounds as typical examples as shown in Fig. 2 (a) and (b). When an aromatic compound (**8**–**19**) such as benzene (**15**) with a sufficiently smaller HOMO energy and a larger LUMO energy than those of NBD-NHMe was added [Fig. 2(a)], intermolecular electron transfer did not occur and the original fluorescence of NBD-NHMe was preserved (fluorescene 'on'). When an aromatic compound (**1**–**7**) such as *p*-*N*,*N*-dimethylphenylenediamine (**1**) with a larger HOMO energy than NBD-NHMe was added [Fig. 2(b)], intermolecular electron transfer occurred from the HOMO of the added aromatic compound to the HOMO of the excited NBD-NHMe, and thus the fluorescence of NBD-NHMe was quenched (fluorescence 'off'). These HOMO → HOMO intermolecular electron transfers could well explain the results in Table 2, except for *p*-dinitrobenzene (**20**), which had a small HOMO energy. However, this exception was also explained by another intermolecular electron transfer. Since the LUMO energy of *p*-dinitrobenzene (**20**) was very small, the intermolecular electron transfer could occur from the LUMO of NBD-NHMe to the LUMO of the added *p*-dinitrobenzene (**20**) [Fig. 2(c)], and thus the fluorescence of NBD-NHMe was also quenched (fluorescence 'off'). Although the calculated molecular orbital energies of NBD-NHMe (the HOMO and LUMO energies obtained with the PM3/COSMO method were −8.878 and −1.892 eV, respectively), and aromatic compounds (**5**–**7**, **20**) did not completely satisfy the relationship suggested in Fig. 2(b) or (c), the fluorescence of NBD-NHMe was quenched by these compounds (**5**–**7**, **20**). This disagreement seemed to be caused by the inaccuracy of the semi-empirical PM3 molecular orbital calculation or the ignorance of the energy distribution of the molecular vibration. The use of a more accurate method such as an *ab initio* approach for obtaining molecular orbital energies and the energy distribution of molecular vibration would solve this issue.

In a similar way to NBD-NHMe, the fluorescence quenching of ABD-SMe and PSBD-NHAc with the addition of the aromatic compounds could be explained by the intermolecular electron transfer between the excited benzofurazan compound and the added aromatic compound. The fluorescence of ABD-SMe or PSBD-NHAc was quenched even with the addition of benzimidazole (**8**), whereas that of NBD-NHMe was not (Table 2). These results suggest that the HOMO energies of ABD-SMe and PSBD-NHAc were lower than that of NBD-NHMe. In fact, the HOMO energies of ABD-SMe (−9.361 eV) and PSBD-NHAc (−9.268 eV) obtained with the PM3/COSMO calculation were lower than that of NBD-NHMe (−8.878 eV).

Studies on the intramolecular electron transfer between the benzofurazan skeleton and an aromatic substituent group

From the above results, it was assumed that the benzofurazan compounds bearing an aromatic substituent group which quenched the fluorescence more strongly in the study of intermolecular electron transfer would fluoresce more weakly. Then, 11 NBD-NHCH$_2$PhR [NBD-Bz (benzylamine)] derivatives (**21**–**31**) bearing various aromatic substituent moieties, which correspond to the aromatic compounds used above, were synthesized and their absorption and fluorescence spectra and fluorescence quantum yields (Φ) in acetonitrile were obtained. The results are summarized in Table 3.

As can be seen, the Φ values of some NBD-Bz derivatives (**21**–**23**) were very small (Φ < 0.002) compared with that of NBD-NHMe (Φ = 0.37), while the absorption characteristics and the maximum fluorescence wavelengths of the derivatives (**21**–**31**) were similar to those of NBD-NHMe. The results indicated that intramolecular electron transfer [photoinduced electron transfer (PET)[21–25]] occurred between the excited benzofurazan skeleton and the aromatic substituent group in the derivatives (**21**–**23**). As expected, an aromatic substituent group such as aniline (**7**) which quenched the fluorescence of NBD-NHMe in the study of intermolecular electron transfer (*K* = 119.4, Table 2) reduced the Φ values of the derivatives (**21**–**23**), whereas the aromatic substituent groups (**9**, **11**, **12**, **14**, **15**, **19**) with small *K* values (*K* < 3.0, Table 2) preserved the fluorescence of the derivatives (**24**–**31**).

It has been proved that NBD-Trp did not fluoresce[19,20] because of quenching (fluorescence 'off') by intramolecular electron transfer (PET) from the indole (*K* = 93.1) skeleton to

Table 2 Parameters for the Stern–Volmer plots for three benzofurazan compounds and 20 aromatic compounds and HOMO/LUMO energies of the added aromatic compounds obtained with the semi-empirical PM3/COSMO calculation.

		NBD-NHMe[a]		ABD-SMe[b]		PSBD-NHAc[c]			
No.	Compound	*K*/l mol^{-1}	r^d	*K*/l mol^{-1}	r^d	*K*/l mol^{-1}	r^d	HOMO/eV	LUMO/eV
1	*p*-*N*,*N*-Dimethylphenylenediamine	210.5	1.000	99.0	1.000	—[e]	—[e]	−8.516	0.003
2	Indole	93.1	1.000	54.8	0.999	126.6	1.000	−8.617	−0.188
3	*p*-Aminophenol	192.7	1.000	155.2	0.999	249.6	1.000	−8.743	0.005
4	*p*-Anisidine	212.3	0.995	72.6	1.000	171.7	0.999	−8.759	0.002
5	*N*,*N*-Dimethylaniline	91.4	0.998	76.5	0.999	199.9	1.000	−8.920	0.040
6	*N*-Methylaniline	136.2	1.000	74.2	1.000	180.5	1.000	−8.983	0.052
7	Aniline	119.4	1.000	66.1	1.000	149.8	1.000	−9.015	0.080
8	Benzimidazole	—	—	16.8	1.000	59.8	1.000	−9.166	−0.342
9	Acetanilide	2.3	0.999	29.8	1.000	55.0	0.999	−9.232	−0.164
10	Phenol	2.0	0.996	28.3	1.000	48.6	0.998	−9.461	0.046
11	Anisole	0.2	0.987	15.8	1.000	52.6	1.000	−9.471	0.040
12	Chlorobenzene	—	—	—	—	0.1	0.898	−9.652	−0.139
13	Imidazole	3.1	0.998	64.1	1.000	38.3	0.997	−9.694	0.705
14	Toluene	—	—	—	—	—	—	−9.715	0.080
15	Benzene	—	—	—	—	—	—	−10.018	0.125
16	Cyanobenzene	—	—	—	—	—	—	−10.116	−0.646
17	Pyridine	—	—	12.0	1.000	22.6	1.000	−10.207	−0.263
18	Benzotrifluoride	—	—	—	—	—	—	−10.237	−0.459
19	Nitrobenzene	2.5	1.000	40.2	1.000	137.4	0.998	−10.270	−1.058
20	*p*-Dinitrobenzene	164.3	0.999	439.4	0.992	1661.0	0.971	−10.469	−1.713

[a] HOMO and LUMO energies of NBD-NHMe were −8.878 and −1.892 eV, respectively. [b] HOMO and LUMO energies of ABD-SMe were −9.361 and −2.163 eV, respectively. [c] HOMO and LUMO energies of PSBD-NHAc were −9.268 and −2.125 eV, respectively. [d] Correlation coefficient. [e] Parameters could not be obtained because the fluorescence spectra of PSBD-NHAc and **1** overlapped.

Table 3 Absorption and fluorescence characteristics of eleven NBD-Bz derivatives in acetonitrile

No.	Compound	Ar[a]	λ_{ab}/nm	$\varepsilon/10^4$ l mol^{-1} cm^{-1}	λ_{em}/nm	Φ	RFI[b]
21	NBD-Bz-*o*-NH_2	**7**	458	1.89	533	—	0.0022
22	NBD-Bz-*m*-NH_2	**7**	460	2.32	518	0.0015	0.0029
23	NBD-Bz-*p*-NH_2	**7**	464	2.11	522	—	0.0022
24	NBD-Bz-*o*-NHAc	**9**	459	1.74	525	0.26	0.56
25	NBD-Bz-*m*-NHAc	**9**	457	2.21	522	0.18	0.48
26	NBD-Bz-*p*-NHAc	**9**	461	2.11	523	0.14	0.37
27	NBD-Bz-*p*-OMe	**11**	459	2.32	524	0.15	0.44
28	NBD-Bz-*p*-Me	**14**	458	2.36	522	0.27	0.80
29	NBD-Bz	**15**	457	2.16	524	0.37	0.98
30	NBD-Bz-*p*-Cl	**12**	455	2.34	521	0.29	0.77
31	NBD-Bz-*p*-NO_2	**19**	452	1.99	520	0.31	0.75
	(*cf.*, NBD-NHMe)		458	2.30	524	0.38	1.00

[a] Aromatic structure in the substituent group. The number corresponds to the aromatic compound (Table 2). [b] RFI = relative fluorescence intensity. The fluorescence intensity of NBD-NHMe was arbitrarily taken as 1.00.

the excited benzofurazan skeleton. In fact, the fluorescence was restored (fluorescence 'on') by suppression of the intramolecular electron transfer (PET) by the degradation of the indole skeleton by photochemical[19] or electrochemical[20] reaction.

Design of the fluorescent 'on–off' reagent by the estimation of quenching ability of an aromatic substituent group

Considering the above data, we propose a method for designing a fluorescent benzofurazan 'on–off' reagent bearing a reactive aromatic substituent group. First, a certain fluorophore skeleton is selected as a reagent and taken into solution. Then the two respective aromatic compounds for the supposed substituent groups in the reagent and the derivative are added to obtain the *K* value for the estimation of the quenching ability of substituent groups. The substituent in the 'on–off' reagent should have a high quenching ability (large *K* value) whereas that in the derivatives should have a low quenching ability (small *K* value). For example, aniline (**7**, *K* = 119.4 in Table 2) has a high quenching ability for NBD-NHMe where the acetylated derivative of aniline (acetanilide) (**9**, *K* = 2.3) does not. Accordingly, NBD-Bz-*p*-NH_2 (**23**, Φ not detected in Table 3) can be a fluorescent 'on–off' reagent for acetylation. Actually, the derivative with acetic acid, NBD-Bz-*p*-NHAc (**26**, Φ = 0.14) fluoresced.

In the same way, a fluorescent 'on–off' reagent for oxidation, 4-(*p*-*N*,*N*-dimethylamino)benzylamino-7-nitro-2,1,3-benzoxadiazole (NBD-Bz-*p*-NMe_2, for **32**) was designed with NBD-NHMe as the fluorophore. Considering the Stern–Volmer plot for NBD-NHMe with *N*,*N*-dimethylaniline (**5**) (*K* = 91.4, Table 2), and that of NBD-NHMe with *N*,*N*-dimethylaniline *N*-oxide (the product of the oxidation of *N*,*N*-dimethylaniline) (*K* not given, quenching was not observed), NBD-Bz-*p*-NMe_2 (**32**) would not fluoresce and 4-(*p*-*N*-oxy-*N*,*N*-dimethylamino)benzylamino7-nitro-2,1,3-benzoxadiazole (NBD-Bz-*p*-NMe_2O, **33**) would fluoresce.

The prediction was also supported by molecular orbital calculations with the PM3/COSMO method for 4-methyl-*N*,*N*-dimethylaniline (as a reacting moiety and a spacer) and 4-methyl-*N*,*N*-dimethylaniline *N*-oxide (as a reacted moiety and a spacer), since the HOMO energy of 4-methyl-*N*,*N*-dimethylaniline (−8.825 eV) is higher than that of NBD-NHMe (−8.878 eV), whereas the HOMO energy of 4-methyl-*N*,*N*-dimethylaniline *N*-oxide (−10.028 eV) is smaller than that of NBD-NHMe.

Finally, NBD-Bz-*p*-NMe_2 (**32**) and NBD-Bz-*p*-NMe_2O (**33**) were synthesized and the fluorescence spectra of these compounds were measured to confirm the hypothesis. As expected,

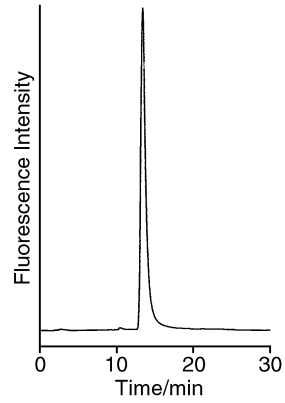

Fig. 3 Chromatogram of MCPBA derivatized with NBD-Bz-*p*-NMe_2 (**32**): 13.2 min. NBD-Bz-*p*-NMe_2O, 25 pmol; column, TSK gel ODS-80Ts (150 × 4.6 mm, id 5 μm); eluent, methanol-water (2 + 5): flow rate, 1.0 ml min^{-1}; detection, excitation at 474 nm, emission at 531 nm.

NBD-Bz-*p*-NMe_2O (**33**) fluoresced (Φ = 0.27, excitation at 454 nm, emission at 521 nm in acetonitrile), whereas NBD-Bz-*p*-NMe_2 (**32**) did not fluoresce.

Then, NBD-Bz-*p*-NMe_2 (**32**) was tested as a fluorogenic reagent for *m*-chloroperbenzoic acid (MCPBA) since *N*,*N*-dimethylaniline reacted well with MCPBA. As a result, the reaction of NBD-Bz-*p*-NMe_2 (**32**) (100 μmol l^{-1}) with MCPBA (25 μmol l^{-1}) occurred and was completed at room temperature within 20 min in chloroform to give a fluorescent NBD-Bz-*p*-NMe_2O (**33**) derivative. The chromatogram of the reaction mixture thus obtained is shown in Fig. 3. There was only a single peak and no other interfering peaks were detected. The calibration curve for MCPBA with NBD-Bz-*p*-NMe_2 (**32**) (100 μmol l^{-1}) was linear (r = 0.999) over the range from 195 fmol (0.098 μmol l^{-1}) to 50 pmol (25 μmol l^{-1}) per injection into the HPLC column. The detection limit (signal-to-noise ratio = 3) for the derivative was 59 fmol. NBD-Bz-*p*-NMe_2 (**32**) did not react with other peracids such as peracetic acid under the mild conditions used. A more highly reactive fluorogenic benzofurazan compound having a more reactive trialkylamine moiety than *N*,*N*-dimethylaniline should be the reagent for a variety of peracids, considering the report[34] that PET occurred even between an aliphatic substituent group such as trimethylamine moiety and a benzofurazan skeleton.

References

1 Y. Ohkura, M. Kai and H. Nohta, *J. Chromatogr. B*, 1994, **659**, 85.
2 A. P. Silva, H. Q. N. Gunaratne, T. Gunnlaugsson, A. J. M. Huxley, C. P. McCoy, J. T. Rademacher and T. E. Rice, *Chem. Rev.*, 1997, **97**, 1515.

3 K. Imai and Y. Watanabe, *Anal. Chim. Acta*, 1981, **130**, 377.
4 T. Toyo'oka, T. Suzuki, Y. Saito, S. Uzu and K. Imai, *Analyst*, 1989, **114**, 413.
5 T. Toyo'oka and K. Imai, *Anal. Chem.*, 1984, **56**, 2461.
6 K. Imai, T. Toyo'oka and Y. Watanabe, *Anal. Biochem.*, 1983, **128**, 471.
7 G. Gubitz, R. Wintersteiger and R. W. Frei, *J. Liq. Chromatogr.*, 1984, **7**, 839.
8 A. Buldt and U. Karst, *Anal. Chem.*, 1999, **71**, 1893.
9 K. Imai, S. Uzu and S. Kanda, *Anal. Chim. Acta*, 1994, **290**, 3.
10 L. P. Hammett, *J. Am. Chem. Soc.*, 1937, **59**, 96.
11 C. Hansch, A. Leo and R. W. Taft, *Chem. Rev.*, 1991, **91**, 165.
12 S. Uchiyama, T. Santa, T. Fukushima, H. Homma and K. Imai, *J. Chem. Soc., Perkin Trans. 2*, 1998, 2165.
13 S. Uchiyama, T. Santa and K. Imai, *J. Chem. Soc., Perkin Trans. 2*, 1999, 569.
14 S. Uchiyama, T. Santa, S. Suzuki, H. Yokosu and K. Imai, *Anal. Chem.*, 1999, **71**, 5367.
15 T. Santa, T. Okamoto, S. Uchiyama, K. Mitsuhashi and K. Imai, *Analyst*, 1999, **124**, 1689.
16 A. Toriba, K. Adzuma, T. Santa and K. Imai, *Anal. Chem.*, 2000, **72**, 732.
17 S. Uchiyama, T. Santa and K. Imai, *J. Chem. Soc., Perkin Trans. 2*, 1999, 2525.
18 S. Uchiyama, T. Santa, N. Okiyama, K. Aduma and K. Imai, *J. Chem. Soc., Perkin Trans. 2*, 2000, 1199.
19 K. Imai, E. Ueda and T. Toyo'oka, *Anal. Chim. Acta*, 1988, **205**, 7.
20 N. Watanabe, T. Toyo'oka and K. Imai, *Biomed. Chromatogr.*, 1987, **2**, 99.
21 R. A. Bissell, A. P. Silva, H. Q. N. Gunaratre, P. L. M. Lynch, G. E. M. Maguire and K. R. A. S. Sandanayake, *Chem. Soc. Rev.*, 1992, 187.
22 L. Fabbrizzi and A. Poggi, *Chem. Soc. Rev.*, 1995, 197.
23 A. P. Silva, T. Gunnlaugsson and T. E. Rice, *Analyst*, 1996, **121**, 1759.
24 G. L. Closs and J. R. Miller, *Science*, 1988, **240**, 440.
25 M. N. Paddon-Row, *Acc. Chem. Res.*, 1994, **27**, 18.
26 G. Jones II, S. F. Griffin, C. Choi and W. R. Bergmark, *J. Org. Chem.*, 1984, **49**, 2705.
27 C. A. M. Seidel, A. Schulz and M. H. M. Sauer, *J. Phys. Chem.*, 1996, **100**, 5541.
28 J. C. Craig and K. K. Purushothaman, *J. Org. Chem.*, 1970, **35**, 1721.
29 J. J. P. Stewart, *J. Comput. Chem.*, 1989, **10**, 209.
30 J. J. P. Stewart, *J. Comput. Chem.*, 1989, **10**, 221.
31 A. Klamt and G. Schuurmann, *J. Chem. Soc., Perkin Trans. 2*, 1993, 799.
32 P. Jacques, E. Haselbach, A. Henseler, D. Pilloud and P. Suppan, *J. Chem. Soc., Faraday Trans.*, 1991, **87**, 3811.
33 P. Jacques, *J. Photochem. Photobiol. A: Chem.*, 1991, **56**, 159.
34 B. Ramachandram and A. Samanta, *J. Phys. Chem. A*, 1998, **102**, 10579.