Effects of the substituent groups at the 4- and 7-positions on the fluorescence characteristics of benzofurazan compounds

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To develop new fluorogenic reagents having the benzofurazan structure, we investigated the effects of the substituent groups at the 4- and 7-positions of the benzofurazan skeleton on the fluorescence characteristics (fluorescence intensity, maximum excitation wavelength and maximum emission wavelength). Seventy benzofurazan compounds substituted at the 4- and 7-positions were obtained for this purpose. The Hammett substituent constant (σ_p) was adopted as a parameter for electronic effects by substituent groups. The study using the sum and the difference of the Hammett substituent constants (σ_p) at the 4- and 7-positions revealed that the highly fluorescent benzofurazan compounds were classified into two groups and that singlet excitation energies, calculated by the maximum excitation and emission wavelengths, of the benzofurazan compounds were different between these two groups. The fluorescence characteristics of benzofurazan compounds substituted at the 4- and 7-positions were empirically predictable by these relationships and σ_p values. A new fluorogenic reagent, 4-phenylaminosulfonyl-7-fluoro-2,1,3-benzoxadiazole, for amines was developed based on this method and applied to the amino acids analysis.

Introduction

Fluorometric detection has been widely used in many fields of science. Above all, it has often been adopted for the detection method of high-performance liquid chromatography (HPLC). However, most compounds do not fluoresce and derivatization or chemical transformation of the analytes with various fluorescent derivatization reagents has to be carried out to make the method useful for a much wider range of analytes.

Until now, numerous fluorescent derivatization reagents have been reported[1–8] and these can be classified in two categories. One is a "fluorescent tagging or labeling reagent" composed of a highly fluorescent moiety and a tagging moiety which reacts with the functional group of analytes to form fluorescent-tagging derivatives. The other is a "fluorogenic reagent" which is non-fluorescent itself and reacts with the analytes to form the fluorescent derivatives. The fluorogenic reagents are generally superior as they avoid interference from fluorescence of the reagent itself.

In the course of our studies on the development of fluorogenic reagents having a 2,1,3-benzoxadiazole (benzofurazan) skeleton, the following were synthesized: 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F)[9] for amines, 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F)[10] for amines and thiols, 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F),[11] 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F)[12] for thiols, 4-hydrazino-7-nitro-2,1,3-benzoxadiazole (NBD-H),[13] 4-(*N*,*N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBD-H), 4-aminosulfonyl-7-hydrazino-2,1,3-benzoxadiazole (ABD-H)[14] for aldehydes and ketones. However, we did not have a general rule which could predict the fluorescence characteristics (fluorescence intensity, maximum excitation and emission wavelengths) of each molecule accurately from its chemical structure, although there have been many reports on the effects of the chemical structure[15,16] or substituent groups[17–20] on the fluorescence characteristics. Therefore, the understanding of the effects of the substituent groups on the fluorescence characteristics of the benzofurazan compounds is necessary for the further development of new fluorogenic reagents.

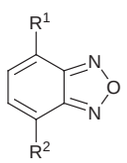

Fig. 1 Chemical structure of benzofurazan compounds (R^1, R^2; substituent groups). The substituent groups used in this study are described in Table 1.

In this article, we try to elucidate the relationship between the substituent groups and fluorescence characteristics of 4,7-disubstituted benzofurazan compounds. A new fluorogenic reagent obtained based on the relationship is also described.

Results and discussion

The fluorescence characteristics of the synthesized benzofurazan compounds

The seventy benzofurazan compounds having various kinds of substituent groups, R^1 and R^2, denoted R^1/R^2 in the text, at the 4- and 7-positions of the benzofurazan structure were obtained (Fig. 1). The substituent groups, the relative fluorescence intensities, the maximum excitation wavelength and the maximum emission wavelength of these compounds are summarized in Table 1.

The relationship between the relative fluorescence intensity and the Hammett substituent constant of the substituent groups at the 4- and 7-positions

In order to know the contribution of the substituent groups at the 4- and 7-positions of the benzofurazan structure to the fluorescence characteristics, a suitable, easily available, parameter was searched for. Since the fluorescence characteristics of benzofurazan compounds were assumed to be determined by the electronic effects (resonance plus field) of substituent groups at the 4- and 7-positions, the Hammett substituent constant (σ_p)[21–23] values seemed to be suitable to represent the total electronic effects, although these constants are commonly used

Table 1 Fluorescence characteristics of the compounds having the benzofuran skeleton

No.	R^1	R^2	R.F.I.[a]	λ(ex)/nm	λ(em)/nm
1	SO_2Cl	F	0.7	352	459
2	SO_2F	OPh	0.0		
3	SO_2F	NMe_2	1.6	440	533
4	NO_2	Cl	0.0		
5	NO_2	SPh	0.0		
6	NO_2	F	0.0		
7	NO_2	SEt	0.3	428	495
8	NO_2	NHAc	0.4	416	503
9	NO_2	SMe	0.6	427	507
10	NO_2	OPh	0.0		
11	NO_2	OMe	0.0		
12	NO_2	$NHNH_2$	0.0		
13	NO_2	NHPh	0.0		
14	NO_2	NH_2	54.5	460	532
15	NO_2	NHMe	62.7	467	528
16	NO_2	OH	3.7	468	545
17	NO_2	NMe_2	7.2	481	533
18	NO_2	NPr^n_2	5.2	489	538
19	SO_2Ph	NHAc	84.4	366	486
20	SO_2Ph	NHCOPh	172.6	368	492
21	SO_2NMe_2	NCS	0.0		
22	SO_2NMe_2	F	0.0		
23	SO_2NMe_2	SEt	2.3	393	508
24	SO_2NMe_2	NHAc	2.4	369	492
25	SO_2NMe_2	SMe	2.8	390	507
26	SO_2NMe_2	OEt	0.2	353	469
27	SO_2NMe_2	OMe	0.0		
28	SO_2NMe_2	NHPh	0.0		
29	SO_2NMe_2	NH_2	1.0	428	559
30	SO_2NMe_2	NHMe	5.9	430	552
31	SO_2NMe_2	NMe_2	6.7	448	563
32	SO_2NMe_2	NPr^n_2	8.8	459	563
33	SO_2NH_2	NCS	0.3	356	475
34	SO_2NH_2	F	0.0		
35	SO_2NH_2	SEt	3.4	387	511
36	SO_2NH_2	SMe	4.2	385	511
37	SO_2NH_2	OEt	7.9	353	471
38	SO_2NH_2	OMe	6.0	352	468
39	SO_2NH_2	$NHNH_2$	0.0		
40	SO_2NH_2	NH_2	0.4	424	568
41	SO_2NH_2	NHMe	2.0	430	563
42	SO_2NH_2	NMe_2	2.9	448	570
43	SO_2NH_2	NPr^n_2	4.9	459	573
44	SOPh	NHAc	1.6	369	506
45	SOPh	NHCOPh	1.9	368	506
46	NCS	Cl	0.1	416	519
47	NCS	OMe	1.4	384	523
48	NCS	NMe_2	0.0		
49	SO_3^-	F	0.0		
50	SO_3^-	SEt	41.5	380	508
51	SO_3^-	NHAc	14.0	358	492
52	SO_3^-	SMe	33.3	379	508
53	SO_3^-	NHCOPh	10.8	358	495
54	SO_3^-	OEt	5.4	352	472
55	SO_3^-	OMe	6.2	348	470
56	SO_3^-	NH_2	0.1	414	572
57	SO_3^-	NHMe	0.3	428	569
58	Cl	NHAc	2.1	368	521
59	Cl	NHCOPh	3.1	366	520
60	Cl	NH_2	0.0		
61	SPh	NHAc	0.5	392	554
62	SPh	NHCOPh	0.8	400	559
63	SPh	NH_2	0.0		
64	F	NHAc	0.6	363	520
65	F	NH_2	0.0		
66	NHAc	OMe	0.1	416	540
67	NHAc	NMe_2	0.0		
68	OMe	NH_2	0.0		
69	NH_2	NH_2	0.0		
70	NH_2	NMe_2	0.0		

[a] R.F.I. = Fluorescence intensity of SO_2NMe_2/NH_2 (No. 29) was arbitrarily taken as 1.0.

for estimation of reactivity and there is no theoretical connection between σ_p constants and fluorescence.

At first, the relationship between the relative fluorescence intensity and the substituent groups of 4,7-disubstituted benzofurazan compounds was investigated for the compounds having OMe and NMe_2 substituents. Fig. 2 shows the relationship between the relative fluorescence intensity and the Hammett substituent constant for the substituent group at the position *para* to the OMe group (a) and the NMe_2 group (b). There were some relations between the fluorescence intensity of these compounds and the σ_p value for the substituent group at the position *para* to both the OMe group and the NMe_2 group. The Hammett substituent constants seemed to be related to the electronic parameters of the substituent groups which affect the fluorescence intensity of benzofurazan compounds. Therefore, a further study was performed on all the benzofurazan compounds synthesized.

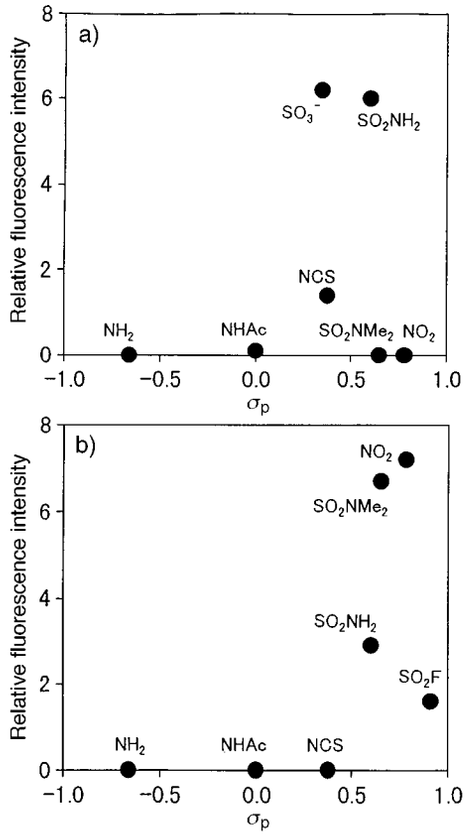

Fig. 2 The relationship between the relative fluorescence intensity and the Hammett substituent constant of the substituent group *para* to the OMe group (a) and the NMe_2 group (b) of 4,7-substituted benzofurazan compounds.

In order to simplify the relations, the sum of the Hammett substituent constants at the 4- and 7-positions was taken at the abscissa as a parameter for the electron density of the benzofurazan skeleton, and the difference of the Hammett substituent constants was taken at the vertical axis as a parameter for the magnitude of the dipole moment directed from the 4- to the 7-position. Then, the seventy benzofurazan compounds were first classified into three groups according to their relative fluorescence intensity (R.F.I.) (R.F.I = 0–1, having no or weak fluorescence; R.F.I. = 1–5, having moderate fluorescence; and R.F.I. >5, having strong fluorescence) and plotted on this graph (Fig. 3).

As shown in Fig. 3, the fluorescent compounds, represented as closed squares and closed triangles, were concentrated in two areas (areas A and B, named for convenience), in contrast the non-fluorescent compounds scattered out of these two areas. These results suggest that the fluorescence intensities of these compounds are strongly influenced by a certain range of both the electronic density and the dipole moment of the benzofurazan skeleton and thus the fluorescence intensity may be predicted using this graph. In fact, the relationship between the substituent groups and the fluorescence intensity revealed

in this study explains well the data in previous reports.[24–28] For example, it was reported that NO_2/F reacted with alkylamines such as methylamine and/or dimethylamine to form a strong fluorescent compound,[24–28] whereas it reacted with arylamine such as aniline to form a non-fluorescent compound.[8] As shown in Fig. 3, NO_2/NHMe (the abscissa and the ordinate are 0.05 and 1.51, respectively), the reaction product of NO_2/F and methylamine, and NO_2/NMe_2 (−0.05, 1.61), the reaction product of NO_2/F and dimethylamine, belong to the fluorescent area A, whereas NO_2/NHPh (0.22, 1.34) belongs to the non-fluorescent area. Therefore, only alkylamine derivatives of NO_2/F are expected to fluoresce. Further, NO_2/OH can be assumed to exist as NO_2/O^- (−0.03, 1.59) in the neutral medium and exists as NO_2/OH (0.41, 1.15) in the acidic medium. Therefore NO_2/OH can be expected to fluoresce in the neutral medium but not in the acidic medium, because only NO_2/O^- appears in the fluorescent area A in Fig. 3. Certainly, it was reported that NO_2/OH fluoresces in the neutral medium, however, it did not in the acidic medium.[24] These results demonstrate that the fluorescence intensity of benzofurazan compounds can be predicted using Hammett substituent constants of the substituent groups at the 4- and 7-positions.

The relationship between the maximum excitation and emission wavelength and Hammett substituent constants of the substituent groups at the 4- and 7-positions

Next, we tried to use further Hammett substituent constants to elucidate the relationship between the substituent groups and the maximum excitation and emission wavelength of the benzofurazan compounds. Since the wavelengths are related to the singlet excitation energy, *i.e.* the difference between the ground state and the excited singlet state energy level, we adopted it as a parameter. The singlet excitation energies were only calculated for the compounds having stronger fluorescence intensity than SO_2NMe_2/NH_2 (R.F.I = 1.0) according to eqn. (1), and were summarized in Table 2.

$$\text{Singlet excitation energy/nm}^{-1} = [1/\lambda(\text{ex}) + 1/\lambda(\text{em})]/2 \quad (1)$$

As shown in Table 2, all the compounds having a larger singlet excitation energy appear in area B, whereas the compounds having a smaller singlet excitation energy appear in the area A. That is to say, the maximum excitation and emission wavelengths of the compounds in area A are longer than those in area B. It was reported that the absorption bands around 360 nm were associated with $\pi\rightarrow\pi^*$ transitions,[29] whereas the absorption bands over 420 nm were associated with intramolecular charge transfer transition[29–32] in 4,7-disubstituted benzofurazan compounds. Therefore, the compounds in area A were presumed to fluoresce *via* the excitation associated with intramolecular charge transfer transitions, whereas the compounds in area B *via* $\pi\rightarrow\pi^*$ transitions. And the large difference in the Hammett substituent constants for the 4- and 7-positions of the compounds in area A agrees with the fact that the compounds exhibiting intramolecular charge transfer absorption have large dipole moments.[32] As a result, the trend of maximum excitation and emission wavelengths of fluorescent benzofurazan compounds is also predictable using Hammett substituent constants.

The relationship thus obtained between the substituent group and the maximum excitation and emission wavelengths agreed with previous reports.[10,33] It was reported that SO_2NMe_2/F (DBD-F) reacted with an alkylamine such as dimethylamine to form a fluorescent compound having longer excitation and emission wavelengths,[33] whereas it reacted with alkylthiols such as methanethiol to form fluorescent compounds having shorter excitation and emission wavelengths.[10] The reaction product of SO_2NMe_2/F and dimethylamine, SO_2NMe_2/NMe_2 (−0.18, 1.48), appears in area A and the reaction product of SO_2NMe_2/F and methane thiol, SO_2NMe_2/SMe (0.65, 0.65) appears in area B. Therefore, the maximum excitation and emission wavelengths of SO_2NMe_2/NMe_2 were expected to be longer than those of SO_2NMe_2/SMe. These results suggest that this relationship is valid for the prediction of the fluorescence characteristics of 4,7-disubstituted benzofurazan compounds.

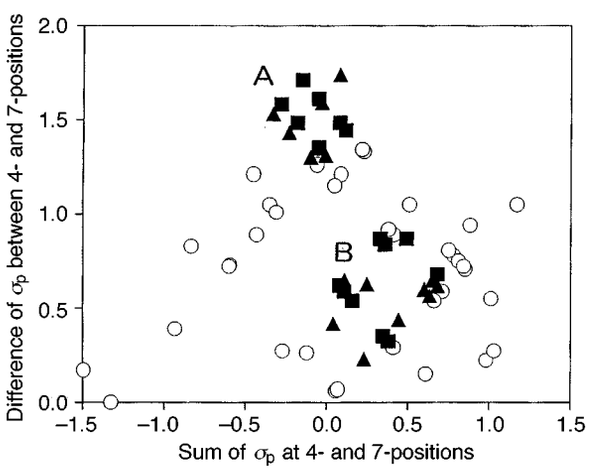

Fig. 3 The relationship between the relative fluorescence intensity of benzofurazan compounds and Hammett substituent constants of the substituent groups at the 4- and 7-positions; ■, R.F.I. ⩾5.0; ▲, 5.0 > R.F.I ⩾ 1.0; ○, 1.0 > R.F.I.

A new fluorogenic reagent synthesized according to the relationship

We tried to develop a new fluorogenic reagent for amines

Table 2 Singlet excitation energies of fluorescent benzofurazan compounds (R.F.I. ⩾ 1.0)

Compound	Energy/10^{-3} nm^{-1}	Area	Compound	Energy/10^{-3} nm^{-1}	Area
SO_3^-/OMe	2.50	B	SO_2NMe_2/SMe	2.27	B
SO_2NH_2/OMe	2.49	B	NCS/OMe	2.26	B
SO_3^-/OEt	2.48	B	SO_2NMe_2/SEt	2.26	B
SO_2NH_2/OEt	2.48	B	SO_2F/NMe_2	2.07	A
SO_3^-/NHAc	2.41	B	SO_2NMe_2/NHMe	2.07	A
SO_3^-/NHCOPh	2.41	B	SO_2NMe_2/NH_2	2.06	A
SO_2Ph/NHAc	2.39	B	SO_2NH_2/NHMe	2.05	A
SO_2Ph/NHCOPh	2.37	B	NO_2/NH_2	2.03	A
SO_2NMe_2/NHAc	2.37	B	NO_2/NHMe	2.02	A
SOPh/NHCOPh	2.35	B	SO_2NMe_2/NMe_2	2.00	A
SOPh/NHAc	2.34	B	SO_2NH_2/NMe_2	1.99	A
Cl/NHAc	2.32	B	NO_2/O^-	1.99	A
SO_3^-/SMe	2.30	B	NO_2/NMe_2	1.98	A
SO_3^-/SEt	2.30	B	SO_2NMe_2/NPr^n_2	1.98	A
SO_2NH_2/SMe	2.28	B	SO_2NH_2/NPr^n_2	1.96	A
SO_2NH_2/SEt	2.27	B	NO_2/NPr^n_2	1.95	A

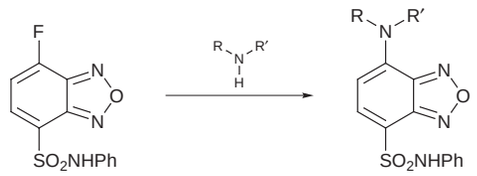

Fig. 4 Chemical structure of SO_2NHPh/F and its reaction with amines.

according to the obtained relationship. First, fluoride (F) group was selected as the reaction group at the 7-position for amines. Then the relationship suggests that a substituent group at the 4- position with a σ_p value from 0.6 to 0.8 is an appropriate fluorogenic reagent for amines (σ_p is about −0.8) without fluorescence from the reagent itself, resulting in the production of a fluorescent compound which falls in area A (longer excitation and emission wavelengths group). SO_2Ph (0.68), SO_2NHPh (0.65) and SO_2Me (0.72) groups should be suited to this criteria. We synthesized SO_2NHPh/F and SO_2NHPh/NMe_2 as a fluorogenic reagent and the reaction product with dimethylamine, respectively, to demonstrate the validity of the relationship (Fig. 4). The reagent SO_2NHPh/F was not fluorescent (R.F.I. = 0.0), but the derivative with dimethylamine, SO_2NHPh/NMe_2, was highly fluorescent (R.F.I. = 6.7) with excitation at 449 nm and emission at 562 nm. The derivative was stable for more than a week in methanol or dichloromethane at room temperature in the glass tube, suggesting the usefulness of SO_2NHPh/F as a fluorogenic reagent. These results further demonstrated the validity of the relationship.

The derivatization of amino acid standards was performed with the new fluorogenic reagent, SO_2NHPh/F, and the chromatogram of amino acid derivatives is shown in Fig. 5. No interfering peak was observed. The elution order of the SO_2NHPh/amino acid was as follows: alanine, proline, valine and leucine, and the detection limits (signal to noise ratio = 3) were 10, 1.0, 14 and 10 fmol, respectively. The emission wavelengths of SO_2NHPh/F derivatives (around 560 nm) are longer than those of NBD-F or Dns-Cl (5-dimethylaminonaphthalene-1-sulfonyl chloride)[34] derivatives (around 530 nm and 510 nm, respectively), providing superiority to the latter with regard to the avoidance of the fluorescent interferences derived from biomatrices. Moreover, SO_2NHPh/F reacts with secondary amines to form fluorescent compounds, whereas fluorescamine[35] and OPA (*o*-phthalaldehyde)[36] do not give fluorescent adducts.

In conclusion, using Hammett substituent constants (σ_p) as a parameter for the electronic effects of the substituent groups on the benzofurazan skeleton, the relationship between the substituent groups at the 4- and 7-positions of the benzofurazan skeleton and the fluorescence characteristics was established, enabling us to predict the fluorescence characteristics of 4,7-disubstituted benzofurazan compounds. In future, the fluorescence characteristics could be predicted more precisely if the electronic effects of substituent groups in the excited states were estimated by the study using the computer calculation.

Experimental

Materials

NBD-Cl, NBD-F, DBD-F, methylamine solution (40% in water), phenol and methyl mercaptan sodium salt solution (15% in water) were obtained from Tokyo Kasei (Tokyo, Japan). Aniline, ammonia solution (29% in water), dimethylamine solution (40% in water), di-*n*-propylamine, triethylamine, thiophenol, ethanethiol, dichloromethane, hydrochloric acid, iron powder, sodium hydroxide, sodium sulfate, pyridine, acetic anhydride, benzoic anhydride, sodium periodate and hexane were purchased from Kanto Chemicals (Tokyo, Japan). ABD-F and SBD-F were obtained from Wako Pure Chemicals (Osaka, Japan). Alanine, leucine, proline and valine were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Silica gel 60 was obtained from Merck (Darmstadt, Germany). Acetonitrile, methanol and ethanol were of HPLC grade (Kanto Chemicals, Tokyo, Japan). Water was purified using a Milli-Q reagent system (Millipore, Bedford, MA, USA). All other chemicals were of analytical or guaranteed reagent grade and were used without further purification.

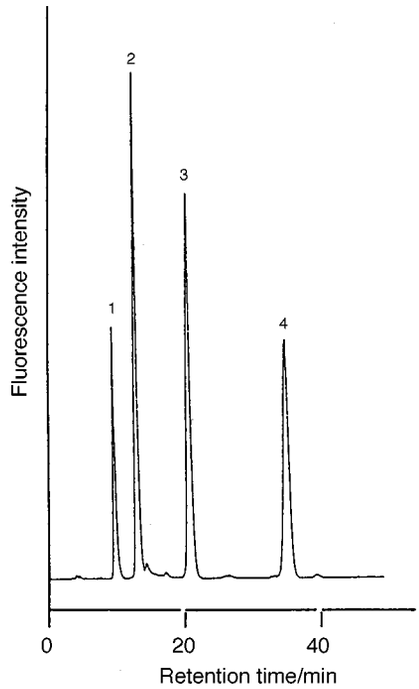

Fig. 5 Chromatogram of amino acids derivatized with SO_2NHPh/F: (1) alanine 4.0 pmol, (2) proline 0.8 pmol, (3) valine 4.0 pmol, (4) leucine 4.0 pmol; column, TSK gel ODS-80Ts (150 × 4.6 mm, i.d. 5 μm); eluent CH_3CN–water (7 : 12) containing TFA (0.01%); flow rate, 1.0 ml min^{-1}; detection, excitation 450 nm, emission 560 nm.

Apparatus

Melting points were measured on a Yanagimoto Micro Melting Point Apparatus (Tokyo, Japan) and uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were obtained on a JEOL GSX-400 spectrometer (Tokyo, Japan) with tetramethylsilane as an internal standard in $CDCl_3$ (abbreviations used: s = singlet, d = doublet, t = triplet, q = quartet and m = multiplet), *J* values are given in Hz. Mass spectra were measured on a Hitachi M-1200 H mass spectrometer [atmospheric pressure chemical ionization (APCI) system and electrospray ionization (ESI) system] (Tokyo, Japan). Fluorescence spectra were measured with a Hitachi F-4010 fluorescence spectrometer (Tokyo, Japan).

Synthesis

4-Amino-7-*N*,*N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole (DBD-NH_2: SO_2NMe_2/NH_2),[37] 4-amino-7-aminosulfonyl-2,1,3-benzoxadiazole (ABD-NH_2: SO_2NH_2/NH_2),[37] 7-*N*,*N*-dimethylaminosulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate (DBD-NCS: SO_2NMe_2/NCS),[37] 7-aminosulfonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanate (ABD-NCS: SO_2NH_2/NCS),[37] NBD-H ($NO_2/NHNH_2$),[13] ABD-H (SO_2NH_2/$NHNH_2$),[14] 4-fluorosulfonyl-7-phenoxy-2,1,3-benzoxadiazole (PBD-SO_2F: SO_2F/OPh),[33] 4-*N*,*N*-dimethylamino-7-fluorosulfonyl-2,1,3-benzoxadiazole (DBD-SO_2F: SO_2F/NMe_2),[10] 4-*N*,*N*-dimethylamino-7-*N*,*N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole (DDB: SO_2NMe_2/NMe_2),[10] 4-chlorosulfonyl-7-fluoro-2,1,3-benzoxadiazole (CBD-F: SO_2Cl/F),[11] 4-*N*,*N*-dimethylamino-7-nitro-2,1,3-benzoxadiazole (NO_2/NMe_2),[20] 4-*N*,*N*-dimethylamino-7-amino-2,1,3-benzoxadiazole (NH_2/

NMe_2),[20] 7-*N*,*N*-dimethylamino-4-(2,1,3-benzoxadiazolyl) isothiocyanate (NCS/NMe_2),[20] 4-methoxy-7-nitro-2,1,3-benzoxadiazole (NO_2/OMe),[20] 4-methoxy-7-amino-2,1,3-benzoxadiazole (OMe/NH_2),[20] 7-methoxy-4-(2,1,3-benzoxadiazolyl) isothiocyanate (NCS/OMe),[20] 4-amino-7-chloro-2,1,3-benzoxadiazole (Cl/NH_2)[20] and 7-chloro-4-(2,1,3-benzoxadiazolyl) isothiocyanate (NCS/Cl)[20] were synthesized and purified as described previously.

4-Phenylamino-7-nitro-2,1,3-benzoxadiazole (NO_2/NHPh). NBD-Cl (200 mg, 1.00 mmol) was dissolved in 20 ml of acetonitrile. After the addition of 1 ml of aniline, the mixture was stirred for 30 min. The reaction mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with CH_2Cl_2 to afford NO_2/NHPh (131 mg, 51%) as a red powder, mp: 152–153 °C. δ_H 8.46 (1H, d, *J* 8.0), 7.82 (1H, br), 7.38–7.53 (5H, m), 6.74 (1H, d, *J* 8.0). Found: C, 55.98; H, 2.82; N, 22.09. Calc. for $C_{12}H_8N_4O_3$: C, 56.25; H, 3.15; N, 21.87%; APCI-MS: *m*/*z* 257 ((M + H)$^+$).

4-Amino-7-nitro-2,1,3-benzoxadiazole (NO_2/NH_2). NBD-Cl (1 g, 5.01 mmol) was dissolved in 25 ml of acetonitrile. After the addition of 3 ml of ammonia solution, the mixture was stirred for 60 min. The reaction mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with AcOEt–hexane (3 : 2) to afford NO_2/NH_2 (380 mg, 42 %) as a brown powder, mp: 272–273 °C. δ_H 8.46 (1H, d, *J* 8.0), 6.41 (1H, d, *J* 8.0). Found: C, 40.23; H, 2.22; N, 30.84. Calc. for $C_6H_4N_4O_3$: C, 40.01; H, 2.24; N, 31.10%; APCI-MS: *m*/*z* 181 ((M + H)$^+$).

4-Methylamino-7-nitro-2,1,3-benzoxadizole (NO_2/NHMe). NBD-F (40 mg, 0.22 mmol) was dissolved in 10 ml of acetonitrile. After the addition of 0.5 ml of methylamine solution, the mixture was stirred for 60 min. The reaction mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with AcOEt–hexane (1 : 1) to afford NO_2/NHMe (31 mg, 73%) as a brown powder, mp: 274–275 °C. δ_H 8.53 (1H, d, *J* 8.0), 6.28 (1H, br), 6.18 (1H, d, *J* 8.0), 3.23 (3H, d, *J* 5.0). Found: C, 43.60; H, 2.96; N, 29.04. Calc. for $C_7H_6N_4O_3$: C, 43.31; H, 3.11; N, 28.86%; APCI-MS: *m*/*z* 195 ((M + H)$^+$).

4-*N*,*N*-Di-*n*-propylamino-7-nitro-2,1,3-benzoxadiazole (NO_2/NPr^n_2). NBD-Cl (120 mg, 0.60 mmol) was dissolved in 10 ml of acetonitrile. After the addition of 0.3 ml of di-*n*-propylamine, the mixture was stirred for 30 min. The reaction mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with CH_2Cl_2–hexane (1 : 1) to afford NO_2/NPr^n_2 (110 mg, 69%) as an orange powder, mp: 92–93 °C. δ_H 8.41 (1H, d, *J* 8.0), 6.10 (1H, d, *J* 8.0), 3.84 (4H, br), 1.80 (4H, q), 1.06 (6H, t). Found: C, 54.73; H, 5.88; N, 20.96. Calc. for $C_{12}H_{16}N_4O_3$: C, 54.54; H, 6.10; N, 21.20%; APCI-MS: *m*/*z* 265 ((M + H)$^+$).

4-Phenylamino-7-*N*,*N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole (SO_2NMe_2/NHPh). DBD-F (50 mg, 0.20 mmol) was dissolved in 5 ml of acetonitrile. After the addition of 0.25 ml of aniline, the mixture was stirred for 60 min. The reaction mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with CH_2Cl_2–MeOH (10 : 1) to afford SO_2NMe_2/NHPh (4 mg, 6%) as a yellow powder, mp: 134–135 °C. δ_H 7.88 (1H, d, *J* 8.0), 7.26–7.50 (5H, m), 6.77 (1H, d, *J* 8.0), 2.90 (6H, s). Found: C, 52.62; H, 4.16; N, 17.30. Calc. for $C_{14}H_{14}N_4O_3S$: C, 52.82; H, 4.43; N, 17.60%; APCI-MS: *m*/*z* 319 ((M + H)$^+$).

4-Methylamino-7-*N*,*N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole (SO_2NMe_2/NHMe). DBD-F (50 mg, 0.20 mmol) was dissolved in 2 ml of acetonitrile. After the addition of 5 ml of methylamine solution, the mixture was stirred for 60 min. The reaction mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with CH_2Cl_2–MeOH (10 : 1) to afford SO_2NMe_2/NHMe (26 mg, 51%) as a yellow powder, mp: 173–174 °C. δ_H 7.93 (1H, d, *J* 8.0), 6.13 (1H, d, *J* 8.0), 5.71 (1H, br), 3.13 (3H, d, *J* 5.0), 2.88 (6H, s). Found: C, 42.47; H, 4.55; N, 21.58. Calc. for $C_9H_{12}N_4O_3S$: C, 42.18; H, 4.72; N, 21.86%; APCI-MS: *m*/*z* 257 ((M + H)$^+$).

4-*N*,*N*-Dimethylamino-7-*N*,*N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole (SO_2NMe_2/NMe_2). DBD-F (100 mg, 0.41 mmol) was dissolved in 5 ml of acetonitrile. After the addition of 1 ml of dimethylamine solution, the mixture was stirred for 30 min. The reaction mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with CH_2Cl_2–MeOH (20 : 1) to afford SO_2NMe_2/NMe_2 (59 mg, 53%) as an orange powder, mp: 145–146 °C. δ_H 7.87 (1H, d, *J* 8.0), 6.04 (1H, d, *J* 8.0), 3.51 (6H, s), 2.87 (6H, s). Found: C, 44.20; H, 5.07; N, 20.73. Calc. for $C_{10}H_{14}N_4O_3S$: C, 44.43; H, 5.22; N, 20.73%; APCI-MS: *m*/*z* 271 ((M + H)$^+$).

4-*N*,*N*-Di-*n*-propylamino-7-*N*,*N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole (SO_2NMe_2/NPr^n_2). DBD-F (30 mg, 0.12 mmol) was dissolved in 5 ml of acetonitrile. After the addition of 0.2 ml of di-*n*-propylamine, the mixture was stirred for 30 min. The reaction mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with CH_2Cl_2 to afford SO_2NMe_2/NPr^n_2 (32 mg, 82%) as an orange powder, mp: 87–88 °C. δ_H 7.84 (1H, d, *J* 8.0), 6.02 (1H, d, *J* 8.0), 3.75 (4H, t), 2.87 (6H, s), 1.76 (4H, m), 1.02 (6H, m). Found: C, 51.68; H, 6.86; N, 17.08. Calc. for $C_{14}H_{22}N_4O_3S$: C, 51.52; H, 6.79; N, 17.16%; APCI-MS: *m*/*z* 327 ((M + H)$^+$).

4-Methylamino-7-aminosulfonyl-2,1,3-benzoxadiazole (SO_2NH_2/NHMe). ABD-F (50 mg, 0.23 mmol) was dissolved in 10 ml of acetonitrile. After the addition of 1 ml of methylamine solution, the mixture was stirred for 3 hours. The reaction mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with CH_2Cl_2–MeOH (20 : 1) to afford SO_2NH_2/NHMe (42 mg, 80%) as an orange powder, mp: 249–250 °C. δ_H 7.91 (1H, d, *J* 8.0), 6.99 (1H, br), 6.24 (2H, br), 6.02 (1H, d, *J* 8.0), 3.07 (3H, d, *J* 5.0). Found: C, 36.93; H, 3.50; N, 24.64. Calc. for $C_7H_8N_4O_3S$: C, 36.84; H, 3.53; N, 24.55%; APCI-MS: *m*/*z* 229 ((M + H)$^+$).

4-*N*,*N*-Dimethylamino-7-aminosulfonyl-2,1,3-benzoxadiazole (SO_2NH_2/NMe_2). ABD-F (40 mg, 0.18 mmol) was dissolved in 10 ml of acetonitrile. After the addition of 0.4 ml of dimethylamine solution, the mixture was stirred for 30 min. The reaction mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with CH_2Cl_2–MeOH (20 : 1) to afford SO_2NH_2/NMe_2 (34 mg, 76%) as an orange powder, mp: 236 °C. δ_H 7.86 (1H, d, *J* 8.0), 6.47 (2H, br), 6.03 (1H, d, *J* 8.0), 3.46 (6H, s). Found: C, 39.83; H, 4.00; N, 22.84. Calc. for $C_8H_{10}N_4O_3S$: C, 39.66; H, 4.16; N, 23.13%; APCI-MS: *m*/*z* 243 ((M + H)$^+$).

4-*N*,*N*-Di-*n*-propylamino-7-aminosulfonyl-2,1,3-benzoxadiazole (SO_2NH_2/NPr^n_2). ABD-F (40 mg, 0.18 mmol) was dissolved in 5 ml of acetonitrile. After the addition of 0.1 ml of di-*n*-propylamine, the mixture was stirred for 60 min. The reaction mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with CH_2Cl_2–MeOH (20 : 1) to afford SO_2NH_2/NPr^n_2 (33 mg, 61%) as an orange powder, mp: 120–121 °C. δ_H 7.87 (1H, d, *J* 8.0), 6.00 (1H, d, *J* 8.0), 5.03 (2H, br), 3.75 (4H, t), 1.74 (4H, m), 1.01 (6H, m). Found: C, 48.31; H, 6.18; N, 18.67. Calc. for $C_{12}H_{18}N_4O_3S$: C, 48.31; H, 6.08; N, 18.78%; APCI-MS: *m*/*z* 299 ((M + H)$^+$).

4-Amino-2,1,3-benzoxadiazole-7-sulfonate (SO_3^-/NH_2). SBD-F (200 mg, 0.85 mmol) was dissolved in 3 ml of ammonia solution. The mixture was stirred at 60 °C for 2 hours. The reaction mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with CH_2Cl_2–MeOH (4 : 1) to afford SO_3^-/NH_2 (180 mg, 91%) as an orange powder, mp: >280 °C. δ_H 7.47 (1H, d, *J* 8.0), 6.66 (2H, br), 6.18 (1H, d, *J* 8.0). Found: C, 31.13; H, 3.32; N, 24.08.

Calc. for $C_6H_8N_4O_4S$ (with NH_4^+): C, 31.13; H, 3.47; N, 24.13%; ESI-MS: *m*/*z* 214 (M^-).

4-Methylamino-2,1,3-benzoxadiazole-7-sulfonate (SO_3^-/NHMe). SBD-F (40 mg, 0.17 mmol) was dissolved in 3 ml of methylamine solution. The mixture was stirred at 60 °C for 2 hours. The reaction mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with CH_2Cl_2 to afford SO_3^-/NHMe (40 mg, 96%) as an orange powder, mp: 198 °C. δ_H 7.81 (1H, d, *J* 8.0), 6.57 (1H, br), 5.97 (1H, d, *J* 8.0), 3.01 (3H, s). ESI-MS: *m*/*z* 228 (M^-).

4-Ethoxy-7-*N*,*N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole (SO_2NMe_2/OEt). DBD-F (50 mg, 0.20 mmol) was dissolved in 20 ml of ethanol. After the addition of triethylamine (0.2 ml), the mixture was stirred for 6 hours at 60 °C. The mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with CH_2Cl_2 to afford SO_2NMe_2/OEt (38 mg, 70%) as a white powder, mp: 142–144 °C. δ_H 7.99 (1H, d, *J* 8.0), 6.60 (1H, d, *J* 8.0), 4.38 (2H, q), 2.92 (6H, s), 1.61 (3H, t). Found: C, 44.31; H, 4.54; N, 15.28. Calc. for $C_{10}H_{13}N_3O_4S$: C, 44.27; H, 4.83; N, 15.49%; APCI-MS: *m*/*z* 272 ((M + H)$^+$).

4-Methoxy-7-*N*,*N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole (SO_2NMe_2/OMe). DBD-F (50 mg, 0.20 mmol) was dissolved in 20 ml of methanol. After the addition of triethylamine (0.2 ml), the mixture was stirred for 6 hours at 60 °C. The mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with CH_2Cl_2 to afford SO_2NMe_2/OMe (40 mg, 78%) as a white powder, mp: 169 °C. δ_H 8.01 (1H, d, *J* 8.0), 6.63 (1H, d, *J* 8.0), 4.16 (3H, s), 2.93 (6H, s). Found: C, 42.18; H, 4.37; N, 16.16. Calc. for $C_9H_{11}N_3O_4S$: C, 42.02; H, 4.31; N, 16.33%; APCI-MS: *m*/*z* 258 ((M + H)$^+$).

4-Ethoxy-7-aminosulfonyl-2,1,3-benzoxadiazole (SO_2NH_2/OEt). ABD-F (50 mg, 0.23 mmol) was dissolved in 20 ml of ethanol. After the addition of triethylamine (0.2 ml), the mixture was stirred for 6 hours at 60 °C. The mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with CH_2Cl_2 to afford SO_2NH_2/OEt (36 mg, 64%) as a white powder, mp: 174–176 °C. δ_H 8.00 (1H, d, *J* 8.0), 6.58 (1H, d, *J* 8.0), 6.22 (2H, br), 4.38 (2H, q), 1.60 (3H, t). Found: C, 39.46; H, 3.53; N, 16.99. Calc. for $C_8H_9N_3O_4S$: C, 39.50; H, 3.73; N, 17.28%; APCI-MS: *m*/*z* 244 ((M + H)$^+$).

4-Methoxy-7-aminosulfonyl-2,1,3-benzoxadiazole (SO_2NH_2/OMe). ABD-F (50 mg, 0.23 mmol) was dissolved in 20 ml of methanol. After the addition of triethylamine (0.2 ml), the mixture was stirred for 6 hours at 60 °C. The mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with CH_2Cl_2 to afford SO_2NH_2/OMe (38 mg, 72%) as a white powder, mp: 202–203 °C. δ_H 8.04 (1H, d, *J* 8.0), 6.61 (1H, d, *J* 8.0), 5.21 (2H, br), 4.16 (3H, s). Found: C, 36.59; H, 3.09; N, 18.18. Calc. for $C_7H_7N_3O_4S$: C, 36.68; H, 3.08; N, 18.33%; APCI-MS: *m*/*z* 230 ((M + H)$^+$).

4-Ethoxy-2,1,3-benzoxadiazole-7-sulfonate (SO_3^-/OEt). SBD-F (50 mg, 0.21 mmol) was dissolved in 20 ml of ethanol. After the addition of triethylamine (0.2 ml), the mixture was stirred for 6 hours at 60 °C. The mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with CH_2Cl_2–MeOH (10 : 1) to afford SO_3^-/OEt (43 mg, 78%) as a white powder, mp: >280 °C. δ_H 7.88 (1H, d, *J* 8.0), 6.55 (1H, d, *J* 8.0), 4.32 (2H, q), 1.56 (3H, t). ESI-MS: *m*/*z* 243 (M^-).

4-Methoxy-2,1,3-benzoxadiazole-7-sulfonate (SO_3^-/OMe). SBD-F (50 mg, 0.21 mmol) was dissolved in 20 ml of methanol. After the addition of triethylamine (0.2 ml), the mixture was stirred for 6 hours at 60 °C. The mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with CH_2Cl_2–MeOH (10 : 1) to afford SO_3^-/OMe (47 mg, 91%) as a white powder, mp: >280 °C. δ_H 7.87 (1H, d, *J* 8.0), 6.58 (1H, d, *J* 8.0), 4.08 (3H, s). ESI-MS: *m*/*z* 229 (M^-).

4-Phenoxy-7-nitro-2,1,3-benzoxadiazole (NO_2/OPh). NBD-Cl (200 mg, 1.00 mmol) was dissolved in a mixture of acetonitrile (20 ml) and 0.1 M borate buffer (pH 9.5, 1 ml). After the addition of 100 mg of phenol, the mixture was stirred for 24 hours. The mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with CH_2Cl_2 –hexane (1 : 3) to afford NO_2/OPh (30 mg, 12%) as a yellow powder, mp: 121 °C. δ_H 8.43 (1H, d, *J* 8.0), 7.26–7.58 (5H, m), 6.54 (1H, d, *J* 8.0). Found: C, 55.97; H, 2.59; N, 16.29. Calc. for $C_{12}H_7N_3O_4$: C, 56.04; H, 2.74; N, 16.34%; APCI-MS: *m*/*z* 258 ((M + H)$^+$).

4-Phenylthio-7-nitro-2,1,3-benzoxadiazole (NO_2/SPh). NBD-Cl (1 g, 5.01 mmol) was dissolved in 20 ml of acetonitrile. After the addition of 1 ml of thiophenol and 0.5 ml of triethylamine, the mixture was stirred for 3 hours and NO_2/SPh (587 mg, 43%) was obtained as a yellow powder, mp: 157–158 °C. δ_H 8.24 (1H, d, *J* 8.0), 7.57–7.70 (5H, m), 6.64 (1H, d, *J* 8.0). Found: C, 52.57; H, 2.42; N, 15.09. Calc. for $C_{12}H_7N_3O_3S$: C, 52.74; H, 2.58; N, 15.38%; APCI-MS: *m*/*z* 274 ((M + H)$^+$).

4-Ethylthio-7-nitro-2,1,3-benzoxadiazole (NO_2/SEt). NBD-Cl (110 mg, 0.55 mmol) was dissolved in the mixture of acetonitrile (15 ml) and 0.1 M phosphate buffer (pH 8.0, 15 ml). After the addition of 0.1 ml of ethanethiol, the mixture was stirred for 4 hours. The mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with AcOEt–hexane (3 : 2) to afford NO_2/SEt (48 mg, 39%) as a yellow powder, mp: 100–101 °C. δ_H 8.43 (1H, d, *J* 8.0), 7.17 (1H, d, *J* 8.0), 3.32 (2H, q), 1.55 (3H, t). Found: C, 42.59; H, 3.01; N, 18.45. Calc. for $C_8H_7N_3O_3S$: C, 42.66; H, 3.13; N, 18.66%; APCI-MS: *m*/*z* 226 ((M + H)$^+$).

4-Methylthio-7-nitro-2,1,3-benzoxadiazole (NO_2/SMe). NBD-F (40 mg, 0.22 mmol) was dissolved in the mixture of acetonitrile (5 ml) and 0.1 M phosphate buffer (pH 8.0, 15 ml). After the addition of 0.1 ml of methyl mercaptan sodium salt solution, the mixture was stirred for 5 min. The mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with CH_2Cl_2–hexane (1 : 1) to afford NO_2/SMe (10 mg, 22%) as an orange powder, mp: 123–124 °C. δ_H 8.43 (1H, d, *J* 8.0), 7.10 (1H, d, *J* 8.0), 2.78 (3H, s). Found: C, 39.66; H, 2.30; N, 19.69. Calc. for $C_7H_5N_3O_3S$: C, 39.81; H, 2.39; N, 19.90%; APCI-MS: *m*/*z* 212 ((M + H)$^+$).

4-Ethylthio-7-*N*,*N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole (SO_2NMe_2/SEt). DBD-F (40 mg, 0.16 mmol) was dissolved in the mixture of acetonitrile (14 ml) and 0.1 M phosphate buffer (pH 8.0, 13 ml). After the addition of 0.1 ml of ethanethiol, the mixture was stirred for 1 hour. The mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with CH_2Cl_2 to afford SO_2NMe_2/SEt (31 mg, 67%) as a yellow powder, mp: 149–150 °C. δ_H 7.90 (1H, d, *J* 8.0), 7.12 (1H, d, *J* 8.0), 3.26 (2H, q), 2.94 (6H, s), 1.50 (3H, t). Found: C, 41.96; H, 4.35; N, 14.52. Calc. for $C_{10}H_{13}N_3O_3S_2$: C, 41.80; H, 4.56; N, 14.62%; APCI-MS: *m*/*z* 288 ((M + H)$^+$).

4-Methylthio-7-*N*,*N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole (SO_2NMe_2/SMe). DBD-F (50 mg, 0.20 mmol) was dissolved in the mixture of acetonitrile (10 ml) and 0.1 M phosphate buffer (pH 8.0, 15 ml). After the addition of 0.2 ml of methyl mercaptan sodium salt solution, the mixture was stirred for 3 hours. The mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with CH_2Cl_2–MeOH (10 : 1) to afford SO_2NMe_2/SMe (6 mg, 11%) as a yellow powder, mp: 147–158 °C. δ_H 7.92 (1H, d, *J* 8.0), 7.05 (1H, d, *J* 8.0), 2.93 (6H, s), 2.71 (3H, s). Found: C, 39.66; H, 3.89; N, 15.18. Calc. for $C_9H_{11}N_3O_3S_2$: C, 39.55; H, 4.06; N, 15.37%; APCI-MS: *m*/*z* 274 ((M + H)$^+$).

4-Ethylthio-7-aminosulfonyl-2,1,3-benzoxadiazole (SO_2NH_2/SEt). ABD-F (40 mg, 0.18 mmol) was dissolved in the mixture of acetonitrile (15 ml) and 0.1 M phosphate buffer (pH 8.0, 15

ml). After the addition of 0.2 ml of ethanethiol, the mixture was stirred at 50 °C for 30 min. The mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with AcOEt–hexane (1:1) to afford SO_2NH_2/SEt (18 mg, 39%) as a yellow powder, mp: 154–156 °C. δ_H 7.92 (1H, d, *J* 8.0), 7.14 (1H, d, *J* 8.0), 6.99 (2H, br), 3.27 (2H, q), 1.50 (3H, t). Found: C, 37.02; H, 3.26; N, 16.26. Calc. for $C_8H_9N_3O_3S_2$: C, 37.06; H, 3.50; N, 16.21%; APCI-MS: *m*/*z* 260 ($(M + H)^+$).

4-Methylthio-7-aminosulfonyl-2,1,3-benzoxadiazole (SO_2NH_2/SMe). ABD-F (50 mg, 0.23 mmol) was dissolved in the mixture of acetonitrile (15 ml) and 0.1 M phosphate buffer (pH 8.0, 15 ml). After the addition of 0.2 ml of methyl mercaptan sodium salt solution, the mixture was stirred for 60 min. The mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with CH_2Cl_2–MeOH (10:1) to afford SO_2NH_2/SMe (33 mg, 58%) as a yellow powder, mp: 185 °C (decomp.). δ_H 7.93 (1H, d, *J* 8.0), 7.06 (1H, d, *J* 8.0), 6.97 (2H, br), 2.72 (3H, s). Found: C, 34.25; H, 2.76; N, 17.20. Calc. for $C_7H_7N_3O_3S_2$: C, 34.28; H, 2.88; N, 17.13%; APCI-MS: *m*/*z* 246 ($(M + H)^+$).

4-Ethylthio-2,1,3-benzoxadiazole-7-sulfonate (SO_3^-/SEt). SBD-F (50 mg, 0.21 mmol) was dissolved in the mixture of acetonitrile (5 ml) and 0.1 M phosphate buffer (pH 8.0, 15 ml). After the addition of 0.1 ml of ethanethiol, the mixture was stirred for 30 min. The mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with CH_2Cl_2–MeOH (10:1) to afford SO_3^-/SEt (9 mg, 15%) as yellow powder, mp: >280 °C. δ_H 7.94 (1H, d, *J* 8.0), 7.15 (1H, d, *J* 8.0), 3.36 (2H, q), 1.44 (3H, t). ESI-MS: *m*/*z* 259 (M^-).

4-Methylthio-2,1,3-benzoxadiazole-7-sulfonate (SO_3^-/SMe). SBD-F (50 mg, 0.21 mmol) was dissolved in the mixture of acetonitrile (5 ml) and 0.1 M phosphate buffer (pH 8.0, 15 ml). After the addition of 0.1 ml of methyl mercaptan sodium salt solution, the mixture was stirred for 2 hours. The mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with CH_2Cl_2–MeOH (10:1) to afford SO_3^-/SMe (13 mg, 24%) as a yellow powder, mp: >280 °C. δ_H 7.83 (1H, d, *J* 8.0), 7.05 (1H, d, *J* 8.0), 2.67 (3H, s). ESI-MS: *m*/*z* 245 (M^-).

4-Phenylthio-7-amino-2,1,3-benzoxadiazole (SPh/NH_2). NO_2/SPh (300 mg, 1.10 mmol) was dissolved in a mixture of dichloromethane (10 ml), methanol (10 ml) and conc. hydrochloric acid (2 ml). After addition of 300 mg of iron powder, the mixture was vigorously stirred for 30 minutes. The reaction mixture was poured into a 1 M NaOH solution (100 ml) and extracted with methylene chloride (100 ml). The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel with CH_2Cl_2–hexane (2:1) to afford SPh/NH_2 (138 mg, 52%) as an orange powder, mp: 110–111 °C. δ_H 7.38 (1H, d, *J* 8.0), 7.16–7.31 (5H, m), 6.34 (1H, d, *J* 8.0), 4.76 (2H, br). Found: C, 58.99; H, 3.50; N, 17.15. Calc. for $C_{12}H_9N_3OS$: C, 59.24; H, 3.73; N, 17.27%; APCI-MS: *m*/*z* 244 ($(M + H)^+$).

4-Amino-7-fluoro-2,1,3-benzoxadiazole (F/NH_2). NO_2/F (50 mg, 0.27 mmol) was dissolved in a mixture of dichloromethane (10 ml), methanol (5 ml) and conc. hydrochloric acid (2 ml). After the addition of 70 mg of iron powder, the mixture was vigorously stirred for 30 minutes. The reaction mixture was poured into a 1 M NaOH solution (100 ml) and extracted with methylene chloride (100 ml). The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel with CH_2Cl_2 to afford F/NH_2 (11 mg, 27%) as orange crystals, mp: 93–94 °C. δ_H 6.87 (1H, q), 6.22 (1H, q), 4.46 (2H, br). Found: C, 47.88; H, 2.60; N, 26.08. Calc. for $C_6H_4FN_3O$: C, 47.07; H, 2.63; N, 27.44%; APCI-MS: *m*/*z* 152 ($(M - H)^-$).

4,7-Diamino-2,1,3-benzoxadiazole (NH_2/NH_2). NO_2/NH_2 (40 mg, 0.22 mmol) was dissolved in a mixture of dichloromethane (10 ml), methanol (5 ml) and conc. hydrochloric acid (2 ml). After addition of 70 mg of iron powder, the mixture was vigorously stirred for 30 minutes. The reaction mixture was poured into a 1 M NaOH solution (100 ml) and extracted with methylene chloride (100 ml). The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel with CH_2Cl_2 to afford NH_2/NH_2 (20 mg, 61%) as brown crystals, mp: 190 °C (decomp.). δ_H 6.27 (2H, s), 4.01 (4H, br). APCI-MS: *m*/*z* 151 ($(M + H)^+$).

4-Acetylamino-7-nitro-2,1,3-benzoxadiazole (NO_2/NHAc). NO_2/NH_2 (10 mg, 0.06 mmol) was dissolved in pyridine (2 ml). After the addition of acetic anhydride (0.5 ml), the mixture was stirred at 60 °C for 30 min. The mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with CH_2Cl_2 to afford NO_2/NHAc (6 mg, 49%) as red crystals, mp: 213–214 °C. δ_H 8.59 (1H, d, *J* 8.0), 8.53 (1H, d, *J* 8.0), 2.38 (3H, s). APCI-MS: *m*/*z* 223 ($(M + H)^+$).

4-Acetylamino-7-*N*,*N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole (SO_2NMe_2/NHAc). SO_2NMe_2/NH_2 (20 mg, 0.08 mmol) was dissolved in pyridine (4 ml). After the addition of acetic anhydride (0.5 ml), the mixture was stirred at 60 °C for 30 min. The mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with CH_2Cl_2–MeOH (10:1) to afford SO_2NMe_2/NHAc (4 mg, 17%) as purple crystals, mp: 170 °C. δ_H 8.39 (1H, d, *J* 8.0), 8.33 (1H, br), 8.03 (1H, d, J 8.0), 2.93 (6H, s), 2.37 (3H, s). APCI-MS: *m*/*z* 285 ($(M + H)^+$).

4-Acetylamino-2,1,3-benzoxadiazole-7-sulfonate (SO_3^-/NHAc). SO_3^-/NH_2 (20 mg, 0.09 mmol) was dissolved in pyridine (3 ml). After the addition of acetic anhydride (0.2 ml), the mixture was stirred at 70 °C for 1 hour. The mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel to afford SO_3^-/NHAc (9 mg, 38%) as a yellow powder, mp: 205 °C (decomp.). δ_H 8.20 (1H, d, *J* 8.0), 7.88 (1H, d, *J* 8.0), 2.27 (3H, s). EI-MS: *m*/*z* 256 (M^-).

4-Benzoylamino-2,1,3-benzoxadiazole-7-sulfonate (SO_3^-/NHCOPh). SO_3^-/NH_2 (30 mg, 0.13 mmol) was dissolved in pyridine (4 ml). After the addition of benzoic anhydride (0.6 ml), the mixture was stirred for 30 min. The mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with CH_2Cl_2–MeOH (10:1) to afford SO_3^-/NHCOPh (6 mg, 14%) as a yellow powder, mp: >280 °C. δ_H 9.73 (1H, s), 8.27 (1H, d, *J* 8.0), 7.98–8.07 (5H, m), 7.43 (1H, d, *J* 8.0). EI-MS: *m*/*z* 318 (M^-).

4-Acetylamino-7-chloro-2,1,3-benzoxadiazole (Cl/NHAc). Cl/NH_2 (15 mg, 0.09 mmol) was dissolved in pyridine (0.5 ml). After the addition of acetic anhydride (0.2 ml), the mixture was stirred at 60 °C for 30 min. The mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with CH_2Cl_2 to afford Cl/NHAc (8 mg, 43%) as a yellow powder, mp: 137–138 °C. δ_H 8.24 (1H, d, *J* 8.0), 8.08 (1H, br), 7.40 (1H, d, *J* 8.0), 2.37 (3H, s). Found: C, 45.50; H, 2.74; N, 19.79. Calc. for $C_8H_6ClN_3O_2$: C, 45.41; H, 2.86; N, 19.86%; APCI-MS: *m*/*z* 212 ($(M + H)^+$).

4-Benzoylamino-7-chloro-2,1,3-benzoxadiazole (Cl/NHCOPh). Cl/NH_2 (15 mg, 0.09 mmol) was dissolved in pyridine (0.5 ml). After the addition of benzoic anhydride (0.2 ml), the mixture was stirred at 60 °C for 30 min. The mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with CH_2Cl_2 to afford Cl/NHCOPh (5 mg, 21%) as a yellow powder, mp: 136 °C. Found: C, 57.08; H, 2.70; N, 15.57. Calc. for $C_{13}H_8ClN_3O_2$: C, 57.05; H, 2.95; N, 15.35%; APCI-MS: *m*/*z* 274 ($(M + H)^+$).

4-Phenylthio-7-acetylamino-2,1,3-benzoxadiazole (SPh/NHAc). SPh/NH_2 (100 mg, 0.41 mmol) was dissolved in pyridine (3 ml). After the addition of acetic anhydride (0.8 ml), the mixture was stirred at 90 °C for 30 min. The mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with CH_2Cl_2 to afford SPh/

NHAc (83 mg, 73%) as a yellow powder, mp: 115–116 °C. δ_H 8.15 (1H, d, *J* 8.0), 7.97 (1H, br), 7.36–7.52 (5H, m), 7.13 (1H, d, *J* 8.0), 2.30 (3H, s). Found: C, 58.67; H, 3.68; N, 14.78. Calc. for $C_{14}H_{11}N_3O_2S$: C, 58.94; H, 3.89; N, 14.73%; APCI-MS: *m*/*z* 286 ($(M + H)^+$).

4-Phenylthio-7-benzoylamino-2,1,3-benzoxadiazole (SPh/NHCOPh). SPh/NH_2 (100 mg, 0.41 mmol) was dissolved in pyridine (3 ml). After the addition of benzoic anhydride (2 ml), the mixture was stirred at 90 °C for 2 hours. The mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with AcOEt–hexane (1 : 3) to afford SPh/NHCOPh (36 mg, 25%) as a yellow powder, mp: 123 °C. δ_H 8.64 (1H, br), 8.34 (1H, d, *J* 8.0), 7.37–7.95 (10H, m), 7.20 (1H, d, *J* 8.0). Found: C, 65.95; H, 4.07; N, 11.91. Calc. for $C_{19}H_{13}N_3O_2S$: C, 65.69; H, 3.77; N, 12.10%. APCI-MS: *m*/*z* 348 ($(M + H)^+$).

4-Acetylamino-7-fluoro-2,1,3-benzoxadiazole (F/NHAc). F/NH_2 (4 mg, 0.03 mmol) was dissolved in pyridine (0.5 ml). After the addition of acetic anhydride (0.2 ml), the mixture was stirred at 60 °C for 30 min. The mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with CH_2Cl_2 to afford F/NHAc (2 mg, 39%) as pale yellow powder, mp: 105–106 °C. δ_H 8.21 (1H, d, *J* 8.0), 7.97 (1H, br), 7.06 (1H, d, *J* 8.0), 2.32 (3H, s). APCI-MS: *m*/*z* 194 ($(M - H)^-$).

4-Methoxy-7-acetylamino-2,1,3-benzoxadiazole (NHAc/OMe). OMe/NH_2 (14 mg, 0.08 mmol) was dissolved in pyridine (2 ml). After the addition of acetic anhydride (0.7 ml), the mixture was stirred for 3 hours. The mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with CH_2Cl_2 to afford NHAc/OMe (13 mg, 74%) as a yellow powder, mp: 165 °C. δ_H 8.15 (1H, d, *J* 8.0), 7.81 (1H, br), 6.52 (1H, d, *J* 8.0), 4.03 (3H, s), 2.28 (3H, s). APCI-MS: *m*/*z* 208 ($(M + H)^+$).

4-Acetylamino-7-*N*,*N*-dimethylamino-2,1,3-benzoxadiazole (NHAc/NMe_2). NH_2/NMe_2 (20 mg, 0.11 mmol) was dissolved in pyridine (1 ml). After the addition of acetic anhydride (0.4 ml), the mixture was stirred for 2 hours. The mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with CH_2Cl_2 to afford NHAc/NMe_2 (15 mg, 61%) as brown crystals, mp: 152–153 °C. δ_H 8.03 (1H, d, *J* 8.0), 7.63 (1H, br), 6.09 (1H, d, *J* 8.0), 3.25 (6H, s), 2.25 (3H, s). APCI-MS: *m*/*z* 221 ($(M + H)^+$).

4-Acetylamino-7-phenylsulfonyl-2,1,3-benzoxadiazole (SO_2Ph/NHAc). SPh/NHAc (20 mg, 0.07 mmol) was dissolved in methanol (20 ml). After the addition of 0.5 M sodium periodate solution (30 ml), the mixture was stirred at 70 °C for 2 hours. The mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with AcOEt–hexane (1 : 2) to afford SO_2Ph/NHAc (11 mg, 50%) as a yellow powder, mp: 233 °C. δ_H 8.41 (1H, d, *J* 8.0), 8.27 (1H, d, *J* 8.0), 8.24 (1H, br), 8.19 (2H, m), 7.56 (3H, m), 2.35 (3H, s). Found: C, 52.86; H, 3.44; N, 12.94. Calc. for $C_{14}H_{11}N_3O_4S$: C, 52.99; H, 3.49; N, 13.24%; APCI-MS: *m*/*z* 318 ($(M + H)^+$).

4-Benzoylamino-7-phenylsulfonyl-2,1,3-benzoxadiazole (SO_2Ph/NHCOPh). SPh/NHCOPh (28 mg, 0.08 mmol) was dissolved in methanol (20 ml). After the addition of 0.5 M sodium periodate solution (30 ml), the mixture was stirred at 70 °C for 2 hours. The mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with AcOEt–hexane (1 : 2) to afford SO_2Ph/NHCOPh (2 mg, 7%) as white crystals, mp: 207–208 °C. δ_H 8.89 (1H, br), 8.60 (1H, d, *J* 8.0), 8.34 (1H, d, *J* 8.0), 8.20 (2H, m), 7.95 (3H, m), 7.61 (6H, m). APCI-MS: *m*/*z* 380 ($(M + H)^+$).

4-Acetylamino-7-phenylsulfinyl-2,1,3-benzoxadiazole (SOPh/NHAc). SPh/NHAc (30 mg, 0.11 mmol) was dissolved in methanol (20 ml). After the addition of 0.5 M sodium periodate solution (30 ml), the mixture was stirred for 4 hours. The mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with AcOEt–hexane (1 : 1) to afford SOPh/NHAc (11 mg, 35%) as a yellow powder, mp: 207–208 °C. δ_H 8.43 (1H, d, *J* 8.0), 8.11 (1H, br), 8.08 (1H, d, *J* 8.0), 7.88 (2H, m), 7.47 (3H, m), 2.32 (3H, s). Found: C, 55.80; H, 3.56; N, 13.68. Calc. for $C_{14}H_{11}N_3O_3S$: C, 55.81; H, 3.68; N, 13.95%; APCI-MS: *m*/*z* 302 ($(M + H)^+$).

4-Benzoylamino-7-phenylsulfinyl-2,1,3-benzoxadiazole (SOPh/NHCOPh). SPh/NHCOPh (30 mg, 0.09 mmol) was dissolved in methanol (20 ml). After the addition of 0.5 M sodium periodate solution (30 ml), the mixture was stirred for 4 hours. The mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with AcOEt–hexane (1 : 1) to afford SOPh/NHCOPh (11 mg, 35%) as a yellow powder, mp: 208 °C. δ_H 8.77 (1H, br), 8.62 (1H, d, *J* 8.0), 8.15 (1H, d, *J* 8.0), 7.88–7.95 (4H, m), 7.47–7.64 (6H, m). Found: C, 62.61; H, 3.39; N, 11.30. Calc. for $C_{19}H_{13}N_3O_3S$: C, 62.80; H, 3.61; N, 11.56%; APCI-MS: *m*/*z* 364 ($(M + H)^+$).

Measurement of fluorescence spectra

Benzofurazan compounds in MeOH (5 μM) were used for the measurement of the fluorescence intensity, the maximum excitation wavelength and the maximum emission wavelength.

Hammett substituent constants

The values of the Hammett substituent constants (σ_p) used in this study were cited from the review by Hansch *et al.*[21] as follows; 1.11 for SO_2Cl, 0.91 for SO_2F, 0.78 for NO_2, 0.68 for SO_2Ph, 0.65 for SO_2NMe_2, 0.60 for SO_2NH_2, 0.44 for SOPh, 0.38 for NCS, 0.35 for SO_3^-, 0.23 for Cl, 0.07 for SPh, 0.06 for F, 0.03 for SEt, 0.00 for SMe and NHAc, −0.03 for OPh, −0.19 for NHCOPh, −0.24 for OEt, −0.27 for OMe, −0.55 for $NHNH_2$, −0.56 for NHPh, −0.66 for NH_2, −0.70 for NHMe, −0.81 for O^-, −0.83 for NMe_2 and −0.93 for NPr^n_2.

Synthesis of 4-phenylaminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (SO_2NHPh/F)

SO_2Cl/F (300 mg, 1.27 mmol) was dissolved in acetonitrile (4 ml). After the addition of aniline (160 μl), the mixture was stirred at room temperature for 1 hour. The reaction mixture was poured into 100 ml of a 1 M HCl solution and extracted with 100 ml of methylene chloride. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel with CH_2Cl_2 to afford SO_2NHPh/F (10 mg, 3%) as a white powder and SO_2F/NHPh (250 mg, by-product) as a red powder. SO_2NHPh/F; mp: 116–117 °C. δ_H 8.01 (1H, q), 7.05–7.26 (6H, m). Found: C, 49.21; H, 2.66; N, 14.55. Calc. for $C_{12}H_8FN_3O_3S$: C, 49.15; H, 2.75; N, 14.33%; APCI-MS: *m*/*z* 292 ($(M - H)^-$). SO_2F/NHPh; mp: 119 °C. δ_H 8.09 (1H, d, *J* 8.0), 7.66 (1H, br), 7.36–7.55 (5H, m), 6.75 (1H, d, *J* 8.0). APCI-MS: *m*/*z* 292 ($(M - H)^-$).

Synthesis of 4-*N*,*N*-dimethylamino-7-phenylaminosulfonyl-2,1,3-benzoxadiazole (SO_2NHPh/NMe_2)

SO_2NHPh/F (3 mg, 0.01 mmol) was dissolved in acetonitrile (5 ml). After the addition of dimethylamine solution (30 μl), the mixture was stirred at room temperature for 10 minutes. The mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with CH_2Cl_2–MeOH (10 : 1) to afford SO_2NHPh/NMe_2 (3 mg, 94%) as an orange powder, mp: 165–166. δ_H 7.84 (1H, d, *J* 8.0), 7.02–7.16 (6H, m), 5.92 (1H, d, *J* 8.0), 3.46 (6H, s). Found: C, 52.95; H, 4.48; N, 17.37. Calc. for $C_{14}H_{14}N_4O_3S$: C, 52.80; H, 4.43; N, 17.60%; APCI-MS: *m*/*z* 319 ($(M + H)^+$).

Derivatization of amino acid with SO_2NHPh/F

To a vial (0.5 ml volume) were added 20 μl of SO_2NHPh/F in CH_3CN (20 mM) and 20 μl of mixed amino acids (8 μM each of alanine, valine, leucine; 1.6 μM of proline) in 0.1 M borate buffer (pH 9.8). The vial was capped and heated at 50 °C for 2 hours. After cooling in ice–water, an aliquot (1 μl) of the reaction solution 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 derivatives was studied on an analytical column, TSK gel ODS-80Ts (150 × 4.6 mm i.d., 5 μm) (TOSOH, Tokyo, Japan). The eluting solvent for SO_2NHPh/amino acids was CH_3CN–H_2O (7 + 12, v/v) containing 0.1% TFA isocratically. The eluate was monitored with fluorescence (excitation at 450 nm, emission at 560 nm) detection.

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