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A Fluorogenic Reagent, 7-Phenylsulfonyl-4-(2,1,3-benzoxadiazolyl) Isocyanate for Alcohols, with Development Based on the Empirical Method for Prediction

Seiichi Uchiyama,[†] Tomofumi Santa,[†] Sachiko Suzuki,[‡] Hirochika Yokosu,[‡] and Kazuhiro Imai*,[†]

Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan, and Tokyo Kasei Kogyo, 6-15-9 Toshima, Kita-ku, Tokyo, 114-0003, Japan

During the course of our studies, we found the relationship between the fluorescence characteristics (the fluorescence intensity and the maximum excitation and emission wavelengths) of benzofurazan compounds and the sum and difference of Hammett substituent constants (*σ*p) at the 4- and 7- positions. This prompted us to design a useful fluorogenic derivatization reagent having the benzofurazan skeleton for alcohols along this line of thought. Accordingly, the fluorogenic derivatization reagents, which have no fluorescence themselves, 7-*N*,*N*-dimethylaminosulfonyl-4-(2,1,3-benzoxadiazolyl) isocyanate (DBD-NCO), 7-phenylsulfonyl-4-(2,1,3-benzoxadiazolyl) isocyanate (PSBD-NCO), and 7-methylsulfonyl-4-(2,1,3-benzoxadiazolyl) isocyanate (MSBD-NCO), were synthesized. Among the derivatives derived from the three reagents, that from PSBD-NCO was most strongly fluorescent. PSBD-NCO reacted with 1-octanol within 4 h in acetonitrile solution in the absence of a catalyst at 60 °C. The derivatives with four alcohols (1-octanol, 1-nonanol, 1-decanol, and 1-undecanol) were separated on a reversed-phase column and detected fluorimetrically at 490 nm with the excitation at 368 nm. The detection limits were at the 10-femtomole level. PSBD-NCO was superior to other fluorescent-labeling reagents with regard to the avoidance of the interfering peaks derived from the reagents themselves and degradation products in the chromatogram. The effectiveness of our approach is disccussed in terms of the development of new fluorogenic reagents.

There have been many reports on the effects of the skeleton and the substituent groups of molecules on the fluorescence characteristics (the fluorescence intensity and the maximum excitation and emission wavelengths), yet general relationships between the fluorescence characteristics and the chemical structure have hitherto not been revealed. Clarification of the relationships between the chemical structure and the fluorescence characteristics is of great value in the fields of the analytical and biological chemistry, since such relationships allow us to design useful fluorescent derivatization reagents and probes.

In the previous research,[1] we investigated the effects of the substituent groups at the 4- and 7-positions on the fluorescence characteristics of the benzofurazan (2,1,3-benzoxadiazole) compounds and found the relationship between the fluorescence characteristics of these compounds and the sum and difference of Hammett substituent constants (*σ*p)[2,3] of the substituent groups at the 4- and 7-positions. Using this relationship, we could predict the fluorescence characteristics from the chemical structures of 4,7-disubstituted benzofurazan compounds.

The present project was focused on the design of useful fluorescent reagents having the benzofurazan skeleton for alcohols, using the relationship obtained. Until now, the following compounds are reported as fluorescent derivatization reagents for alcohols: 3-chloroformyl-7-methoxycoumarin (3CMC),[4] 7-[(chlorocarbonyl)-methoxy]-4-methylcoumarin (CMMC),[5] pyrene-1-carbonylnitrile,[6] 1-anthroylnitrile,[7] 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxo-quinoxaline-2-carbonyl chloride (DMEQ-COCl),[8] 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxo-quinoxaline-2-carbonyl azide (DMEQ-CON_3),[9] 2-(5-chlorocarbonyl-2-oxazolyl)-5,6-methylenedioxybenzofuran (OMB-COCl),[10] 4-(*N*,*N*-dimethylaminosulfonyl)-7-(2-chloroformylpyrrolidin-1-yl)-2,1,3-benzoxadiazole (DBD-Pro-COCl),[11] and 4-(*N*-chloroformylmethyl-*N*-methyl)-amino-7-*N*,*N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole (DBD-COCl).[12] However, these fluorescent reagents are not always satisfactory with respect to the

* To whom correspondence should be addressed. (tel.) 81-3-5841-4760; (fax) 81-3-5841-4885; (e-mail) kimai@mol.f.u-tokyo.ac.jp.

[†] The University of Tokyo.

[‡] Tokyo Kasei Kogyo.

(1) Uchiyama, S.; Santa, T.; Fukushima, T.; Homma, H.; Imai, K. *J. Chem. Soc., Perkin Trans. 2* **1998**, 2165−2173.
(2) Hammett, L. P. *J. Am. Chem. Soc.* **1937**, *59*, 96−103.
(3) Hansch, C.; Leo, A.; Taft, R. W. *Chem. Rev.* **1991**, *91*, 165−195.
(4) Hamada, C.; Iwasaki, M. *J. Chromatogr.* **1985**, *341*, 426−431.
(5) Karlsson, K. E.; Wiesler, D.; Alasandro, M.; Novotny, M. *Anal. Chem.* **1985**, *57*, 229−234.
(6) Goto, J.; Chikai, T.; Nambara, T. *J. Chromatogr.* **1987**, *415*, 45−52.
(7) Goto, J.; Komatsu, S.; Inada, M.; Nambara, T. *Anal. Sci.* **1986**, *2*, 585−586.
(8) Iwata, T.; Yamaguchi, M.; Hara, S.; Nakamura, M.; Ohkura, Y. *J. Chromatogr.* **1986**, *362*, 209−216.
(9) Iwata, T.; Yamaguchi, M.; Nakamura, M. *J. Chromatogr.* **1987**, *421*, 43−50.
(10) Nagaoka, H.; Nohta, H.; Kaetsu, Y.; Saito, M.; Ohkura, Y. *Anal. Sci.* **1989**, *5*, 525−530.
(11) Toyo'oka, T.; Ishibashi, M.; Terao, T.; Imai, K. *Analyst (Cambridge, U.K.)* **1993**, *118*, 759−763.
(12) Imai, K.; Fukushima, T.; Yokosu, H. *Biomed. Chromatogr.* **1994**, *8*, 107−113.

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detectablity of alcohols, since the fluorescence of derivatives is not strong, and the fluorescence of the reagent itself and the degradation byproducts sometimes interfere with the quantification of the small amount of substances. Further, the excitation and emission wavelengths of the derivatives with these reagents are short so that the quantification is often interfered by biomatrixes containing fluorescent substances. Thus useful derivatization reagents for alcohols, to be developed, should ideally have the following characteristics: (1) fluorogenic (reagent itself is not fluorescent), (2) react with alcohols to form the strong fluorescent derivatives, and (3) their derivatives are excited and emit at long wavelengths.

In this article, we develop the fluorogenic derivatization reagents having the benzofurazan skeleton for alcohols based on the relationship obtained and examine the reactivity of the reagents as well as the sensitivity of the derivatives.

EXPERIMENTAL SECTION

Materials. 1-Octanol, 1-nonanol, 1-decanol, and 1-undecanol were of guaranteed grade (Tokyo Kasei, Tokyo, Japan). Silica gel 60 was obtained from Merck (Darmstadt, Germany). Acetonitrile, ethyl acetate, *n*-hexane, dichloromethane, and methanol were of HPLC grade (Kanto Chemicals, Tokyo, Japan). Water was purified using a Milli-Q reagent system (Millipore, Bedford, MA).

Apparatus. Melting points were measured on a Yanagimoto Micro 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, m = multiplet). Mass spectra were measured on a Hitachi M-1200 H mass spectrometer (atmospheric pressure chemical ionization (APCI) system) (Tokyo, Japan). Fluorescence spectra were measured on a Hitachi F-4010 fluorescence spectrometer (Tokyo, Japan).

Design of the Fluorogenic Derivatization Reagents for Alcohols. Figure 1 showed the relationship between the fluorescence intensity of 4,7-disubstituted benzofurazan compounds and the Hammett substituent constants (σp) at the 4- and 7-positions, obtained in our previous research.[1] In this figure, the abscissa (x axis) and the ordinate (y axis) were the sum of σp at the 4- and 7-positions ($x = \sigma p(4) + \sigma p(7)$) and the difference of σp between the 4- and 7-position ($y = |\sigma p(4) - \sigma p(7)|$), respectively. The seventy 4,7-disubstituted benzofurazan compounds were classified into three groups according to their relative fluorescence intensity (RFI; fluorescence intensity of 4-amino-7-*N*,*N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole (DBD-NH_2) was arbitrarily taken as 1.0) (RFI = 0−1, having no or weak fluorescence (○); RFI = 1−5, having moderate fluorescence (▲); RFI > 5, having strong fluorescence (■)). The fluorescent compounds, represented as closed squares and closed triangles, were concentrated in two areas (A and B); in contrast the nonfluorescent compounds scattered out of these two areas. Using this relationship, the compounds with the coordinate ($\sigma p(4) + \sigma p(7)$, $|\sigma p(4) - \sigma p(7)|$) of the substituent groups at the 4- and 7- position in the area A or B were predicted to be fluorescent. On the contrary, the compounds with the coordinate outside the area A and B were predicted to be nonfluorescent.

At first, the isocyanate (NCO; $\sigma p = 0.19$) group was selected as the reaction group at the 4-position. In Figure 1, the 4,7-disubstituted benzofurazan compounds having the NCO group at the 4-position were plotted on the line ①, since there was the relationship $y = |x - 0.38|$. The 4,7-disubstituted benzofurazan compounds having the NHCOOMe group (substituent group after the reaction of NCO with methanol; $\sigma p = -0.17$) at the 4-position were plotted on the line ②, since there was the relationship $y = |x + 0.34|$. In this manner, the compounds having a certain substituent group were plotted on one line. Next, in this graph, the point of intersection of two lines reveals the 4,7-disubstituted benzofurazan compounds having the substituent groups corresponding to the two lines. The line, with which the point of intersection of line ① was out of the fluorescent area (A and B) and the point of intersection of line ② was in the fluorescent area (A or B), was searched to determine the substituent group at the 7-position of the new fluorogenic reagent for alcohols. As a result, the lines in the range of ③ were suited to this criteria, showing that the substituent groups with a σp from 0.25 to 0.75 are appropriate at the 7-position.

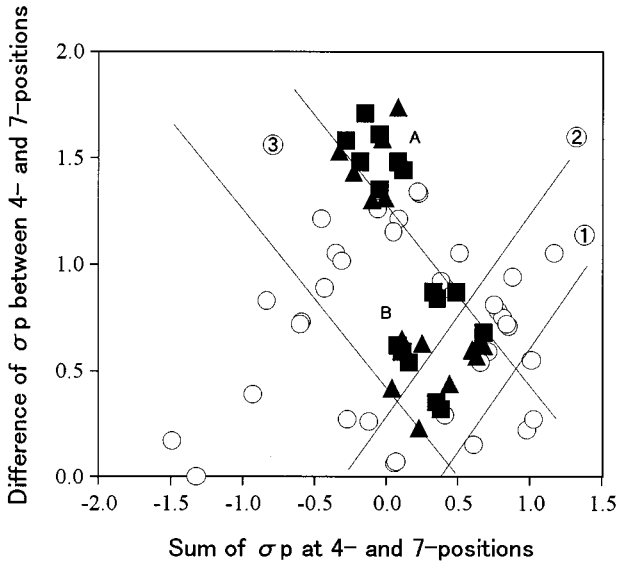

Figure 1. The design of fluorogenic derivatization reagents for alcohols using the relationship between the fluorescence intensity of 4,7-disubstituted benzofurazan compounds and the Hammett substituent constants (σp) at the 4- and 7-positions.

Synthesis. Diphosgene (trichloromethyl chloroformate, TCF)[13,14] and benzofurazan isocyanate should be treated in draft with a safety glove and glass.

7-*N*,*N*-Dimethylaminosulfonyl-4-(2,1,3-benzoxadiazolyl) isocyanate (DBD-NCO). 4-Amino-7-*N*,*N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole (DBD-NH_2)[15] (244 mg, 1.0 mmol) was suspended in 20 mL of dried ethyl acetate. After the addition of 720 μL of diphosgene, the mixture was stirred at 80 °C for 4 h. The completion of the reaction was confirmed using NMR. The reaction mixture was evaporated to dryness under reduced pressure, and DBD-NCO was obtained as yellow powder. Immediately, this powder was dissolved in 20 mL of acetonitrile, and 50 mmol/L of DBD-NCO solution was obtained. δ_H 7.99 (1H, d, J 7.6), 7.11 (1H, d, J 7.6), 2.96 (6H, s).

(13) Kurita, K.; Matsumura, T.; Iwakura, Y. *J. Org. Chem.* **1976**, *41*, 2070−2071.
(14) Nowakowski, J. *J. Prakt. Chem./Chem.-Zty.* **1992**, *334*, 187−189.
(15) Imai, K.; Uzu, S.; Nakashima, K.; Akiyama, S. *Biomed. Chromatogr.* **1993**, *7*, 56−57.

7-Phenylsulfonyl-4-(2,1,3-benzoxadiazolyl) isocyanate (PSBD-NCO). 4-Amino-7-phenylsulfonyl-2,1,3-benzoxadiazole (PSBD-NH_2)[16] (138 mg, 0.5 mmol) was suspended in 10 mL of dried ethyl acetate. After the addition of 360 μL of diphosgene, the mixture was stirred at 80 °C for 4 h. The completion of the reaction was confirmed using NMR. The reaction mixture was evaporated to dryness under reduced pressure, and PSBD-NCO was obtained as yellow powder. Immediately, this powder was dissolved in 10 mL of acetonitrile, and 50 mmol/L of PSBD-NCO solution was obtained. δ_H 8.18−8.25 (3H, m), 7.53−7.64 (3H, m), 7.13 (1H, d, *J* 7.6).

7-Methylsulfonyl-4-(2,1,3-benzoxadiazolyl) isocyanate (MSBD-NCO). 4-Amino-7-methylsulfonyl-2,1,3-benzoxadiazole (MSBD-NH_2)[16] (107 mg, 0.5 mmol) was suspended in 10 mL of dried ethyl acetate. After the addition of 360 μL of diphosgene, the mixture was stirred at 80 °C for 4 h. The completion of the reaction was confirmed using NMR. The reaction mixture was evaporated to dryness under reduced pressure, and MSBD-NCO was obtained as yellow powder. Immediately, this powder was dissolved in 10 mL of acetonitrile and 50 mmol/L of MSBD-NCO solution was obtained. δ_H 8.15 (1H, d, *J* 7.3), 7.18 (1H, d, *J* 7.3), 3.38 (3H, s).

7-*N*,*N*-Dimethylaminosulfonyl-4-methoxyamide-2,1,3-benzoxadiazole (DBD-NHCOOMe). 7-*N*,*N*-dimethylaminosulfonyl-4-methoxythioamide-2,1,3-benzoxadiazole (DBD-NHCSOMe)[17] (10 mg, 0.032 mmol) was dissolved in acetonitrile (2 mL). After the addition of 3% hydrogen peroxide solution in water (5 mL), the mixture was stirred at 50 °C for 30 min. The reaction mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with dichloromethane−methanol (20:1) to afford DBD-NHCOOMe (3.6 mg, 38%) as yellow powder. mp: 210 °C. δ_H 8.07 (1H, d, *J* 8.0), 8.03 (1H, d, *J* 8.0), 7.79 (1H, br), 3.91 (3H, s), 2.92 (6H,s). APCI-MS: *m*/*z* 299-($(M - H)^-$).

7-Phenylsulfonyl-4-methoxyamide-2,1,3-benzoxadiazole (PSBD-NHCOOMe). 7-Phenylsulfonyl-4-(2,1,3-benzoxadiazolyl) isocyanate (PSBD-NCO) solution in acetonitrile (50 mmol/L) (500 μL, 0.025 mmol) was dissolved in methanol (20 mL). The mixture was stirred at room temperature for 10 min and evaporated to dryness under reduced pressure. The residue was chromatographed on silica gel with dichloromethane−methanol (20:1) to afford PSBD-NHCOOMe (4.5 mg, 54%) as yellow powder. mp: 185−186 °C. δ_H 8.27 (1H, d, *J* 8.0), 8.18 (2H, d, *J* 7.6), 8.09 (1H, d, *J* 8.0), 7.78 (1H, br), 7.52−7.62 (3H, m), 3.92 (3H, s). APCI-MS: *m*/*z* 332($(M - H)^-$).

7-Methylsulfonyl-4-methoxyamide-2,1,3-benzoxadiazole (MSBD-NHCOOMe). 7-Methylsulfonyl-4-(2,1,3-benzoxadiazolyl) isocyanate (MSBD-NCS)[16] (15 mg, 0.059 mmol) was dissolved in methanol (10 mL). The mixture was stirred at 50 °C for 30 min and evaporated to dryness under reduced pressure. The residue was chromatographed on silica gel with dichloromethane−methanol (20:1) to afford 7-methylsulfonyl-4-methoxythioamide-2,1,3-benzoxadiazole (MSBD-NHCSOMe) (12 mg, 71%) as yellow powder. mp: 176−178 °C (dec.). δ_H 9.07 (1H, br), 8.48 (1H, br), 8.19 (1H, d, *J* 8.0), 4.22 (3H, s), 3.35 (3H, s). APCI-MS: *m*/*z* 288($(M + H)^+$). MSBD-NHCSOMe (12 mg, 0.042 mmol) was dissolved in acetonitrile (2 mL). After the addition of 3% hydrogen peroxide solution in water (5 mL), the mixture was stirred at 50 °C for 30 min. The reaction mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with dichloromethane−methanol (20:1) to afford MSBD-NHCOOMe (3.6 mg, 33%) as yellow powder. mp: 165 °C. δ_H 8.19 (1H, d, *J* 8.0), 8.13 (1H, d, *J* 8.0), 7.86 (1H, br), 3.92 (3H, s), 3.35 (3H, s). APCI-MS: *m*/*z* 272($(M + H)^+$).

7-Phenylsulfonyl-4-octyloxyamide-2,1,3-benzoxadiazole (PSBD-NHCOOC_8H_{17}). 7-Phenylsulfonyl-4-(2,1,3-benzoxadiazolyl) isocyanate (PSBD-NCO) solution in acetonitrile (50 mmol/L) (0.5 mL, 0.025 mmol) was dissolved in 1-octanol (4 mL). The mixture was stirred at room temperature for 10 min and evaporated to dryness under reduced pressure. The residue was chromatographed on silica gel with ethyl acetate−*n*-hexane (1:2) to afford PSBD-NHCOOC_8H_{17} (9.5 mg, 88%) as yellow powder. mp: 120−121 °C. δ_H 8.27 (1H, d, *J* 8.0), 8.18 (2H, d, *J* 7.6), 8.09 (1H, d, *J* 8.0), 7.77 (1H, br), 7.52−7.62 (3H, m), 4.47 (2H, t), 3.57 (2H, q), 1.27 (13H, m). APCI-MS: *m*/*z* 432($(M + H)^+$).

Measurement of Fluorescence Spectra. The solutions of benzofurazan compounds (5 μM) were used for the measurement of the fluorescence intensity and the maximum excitation and emission wavelengths.

Time Course of the Reaction of PSBD-NCO with 1-Octanol. To a vial (500-μL volume) were added 25 μL of PSBD-NCO in acetonitrile (50 mmol/L), 5μL of 1-octanol in acetonitrile (1 mmol/L), and 470 μL of acetonitrile. The vial was capped and heated at 60 °C for 5 h. At the fixed intervals, an aliquot (2 μL) of the reaction mixture was subjected to HPLC. The reaction yield was determined by comparison with the peak height of authentic PSBD-NHCOOC_8H_{17}.

Calibration Curve for the Derivatives of PSBD-NCO with 1-Octanol. To a vial (500-μL volume) were added 25 μL of PSBD-NCO in acetonitrile (50 mmol/L), 20 μL of 1-octanol in acetonitrile (0.08, 0.4, 2, 10, 50, and 250 μmol/L) and 455 μL of acetonitrile. The vial was capped and heated at 60 °C for 4 h. An aliquot (5 μL) of the 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 derivatives was studied on an analytical column, TSKgel ODS-80Ts (150 × 4.6 mm i.d., 5 μm) (TOSOH, Tokyo, Japan). The column temperature was ambient. The eluent for the derivatives of alcohols with PSBD-NCO was acetonitrile−water (4:1). The eluate was monitored with fluorescence detection (excitation at 368 nm, emission at 490 nm).

Derivatization of Alcohols with PSBD-NCO. To a vial (500-μL volume) were added 25 μL of PSBD-NCO in acetonitrile (50 mmol/L), 20 μL of mixed alcohols (250 μmol/L each of 1-octanol, 1-nonanl, 1-decanol, and 1-undecanol) in acetonitrile, and 455 μL of acetonitrile. The vial was capped and heated at 60 °C for 4 h. After cooling in ice water, an aliquot (1 μL) of the reaction mixture was subjected to HPLC.

RESULTS AND DISCUSSION

Design and Synthesis of New Fluorogenic Reagents for Alcohols. At first, the isocyanate (NCO) group was adopted as the reaction group at the 4-position for alcohols, since the NCO group reacts with alcohols quite easily without catalysts. The

(16) Toriba, A.; Santa, T.; Iida, T.; Imai, K. *Analyst (Cambridge, U.K.)* **1999**, *124*, 43−48.

(17) Uchiyama, S.; Santa, T.; Imai, K. *J. Chem. Soc., Perkin Trans. 2* **1999**, 569−576.

Table 1. Fluorescence Characteristics of Three Derivatives of Methanol with Benzofurazan Isocyanates

	R.F.I.[a] (λ ex(nm), λ em(nm))		
solvents	methanol	acetonitrile	water
DBD-NHCOOMe	4.84(371, 491)	0.59(371, 475)	1.10(369, 517)
PSBD-NHCOOMe	408(371, 489)	451(366, 475)	41.9(372, 508)
MSBD-NHCOOMe	25.3(366, 488)	25.5(363, 473)	2.46(363, 487)
(c.f. NBD-NHMe)	120(461, 528)	192(458, 524)	14.5(478, 541)
(c.f. ABD-SMe)	5.10(384, 513)	26.2(385, 510)	2.40(390, 530)

[a] R.F.I. = Relative fluorescence intensity. Fluorescence intensity of DBD-NH_2 was arbitrarily taken as 1.0.

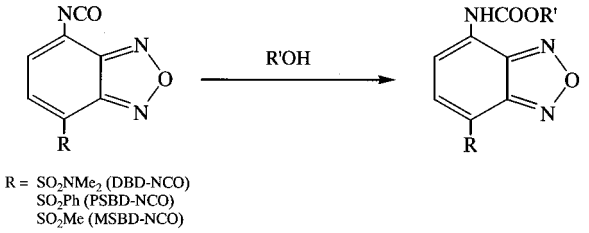

Figure 2. Chemical structures of three benzofurazan isocyanates and their reaction with alcohols.

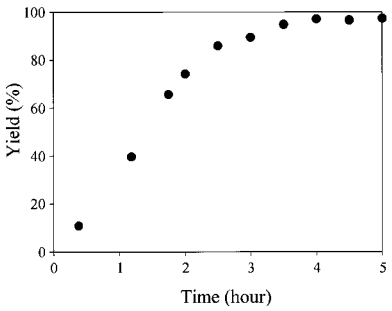

Figure 3. Time course for the formation of a derivative of 1-octanol with PSBD-NCO at 60 °C.

relationship between the fluorescence characteristics and the Hammett substituent constants (σp) of the substituent groups at the 4- and 7-positions suggests that a substituent group with a σp value from 0.25 to 0.75 is required at the 7-position of the fluorogenic reagent for alcohols. Among the substituent groups with a σp value from 0.25 to 0.75, SO_2NMe_2 (σp = 0.65), SO_2Ph (σp = 0.68), and SO_2Me (σp = 0.72) groups were selected, since these substituent groups are strong electron-accepting groups and expected to increase the reactivity for alcohols. Therefore, 7-*N*,*N*-dimethylaminosulfonyl-4-(2,1,3-benzoxadiazolyl) isocyanate (DBD-NCO), 7-phenylsulfonyl-4-(2,1,3-benzoxadiazolyl) isocyanate (PSBD-NCO), and 7-methylsulfonyl-4-(2,1,3-benzoxadiazolyl) isocyanate (MSBD-NCO) were designed as the fluorogenic derivatization reagents for alcohols (Figure 2).

DBD-NCO, PSBD-NCO, and MSBD-NCO were synthesized by the reactions of 4-amino-7-*N*,*N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole (DBD-NH_2), 4-amino-7-phenylsulfonyl-2,1,3-benzoxadiazole (PSBD-NH_2), and 4-amino-7-methylsulfonyl-2,1,3-benzoxadiazole (MSBD-NH_2) with diphosgene, respectively. These reagents, identified by NMR, were stored in dried acetonitrile, since the solids of DBD-NCO, PSBD-NCO, and MSBD-NCO were appreciably hydrolyzed by water in the atmosphere. The NMR spectra showed that these reagents were stable for more than a month in dried acetonitrile at 0 °C in the glass ampule (data not shown). We could not obtain the fluorescence spectra of these reagents because of the interference of a small amount of fluorescent degradation products.

The Fluorescence Characteristics of DBD-NHCOOMe, PSBD-NHCOOMe, and MSBD-NHCOOMe. We synthesized 7-*N*,*N*-dimethylaminosulfonyl-4-methoxyamide-2,1,3-benzoxadiazole (DBD-NHCOOMe), 4-methoxyamide-7-phenylsulfonyl-2,1,3-benzoxadiazole (PSBD-NHCOOMe), and 4-methoxyamide-7-methylsulfonyl-2,1,3-benzoxadiazole (MSBD-NHCOOMe) as derivatives of methanol with the three reagents and measured the fluorescence spectra of these derivatives in methanol, acetonitrile, and water (Table 1). As shown in Table 1, all the three derivatives fluoresced as expected. Especially, PSBD-NHCOOMe fluoresced strongly in comparison with DBD-NHCOOMe and MSBD-NHCOOMe. The fluorescence intensity of PSBD-NHCOOMe was larger than those of 4-methylamino-7-nitro-2,1,3-benzoxadiazole (NBD-NHMe, the derivative of methylamine with NBD-F)[1] and 4-methylthio-7-aminosulfonyl-2,1,3-benzoxadiazole (ABD-SMe, the derivative of methanethiol with ABD-F),[1] the strong fluorescent compounds having the benzofurazan skeleton. These results showed that PSBD-NCO was the most suitable reagent for alcohols with respect to the detectability of the derivative. Therefore, PSBD-NCO was employed in the subsequent study.

Derivatization of 1-Octanol with PSBD-NCO. 1-Octanol was selected as a representative of alcohols. The time course study of the derivatization of 1-octanol (10 μM in acetonitrile) with PSBD-NCO (2.5 mM in acetonitrile) was performed at 60 °C. As shown in Figure 3, the reaction seems to have proceeded completely at 60 °C after 4 h (yield: 97.2%). This result also showed that the derivative of 1-octanol with PSBD-NCO was stable in acetonitrile at 60 °C. The derivatization of alcohols with the conventional reagents proceeded at higher temperature (3CMC[4](100 °C), DMEQ-COCl[8](100 °C), OMB−COCl[10](100 °C), and DBD-COCl[12]-(80 °C)), while the derivatization of 1-octanol with PSBD-NCO proceeded at 60 °C, suggesting the latter's superiority to the other reagents with regard to the avoidance of decomposition of the analytes. The calibration curve for the derivative of PSBD-NCO with 1-octanol was linear over the range from 16 fmol to 50 pmol per injection (r = 0.999).

Separation of the Derivatives of Alcohols with PSBD-NCO on a Reversed-Phase Column. The chromatogram of the derivatives thus obtained is shown in Figure 4. The elution was in the order of the derivatives with 1-octanol, 1-nonanol, 1-decanol, and 1-undecanol. Within five minutes, the two fluorescent by-products were eluted; the major was identified as PSBD-NH_2 (the hydrolyzed product of PSBD-NCO) and the minor was presumed to be a dimer (the reaction product of PSBD-NH_2 with PSBD-

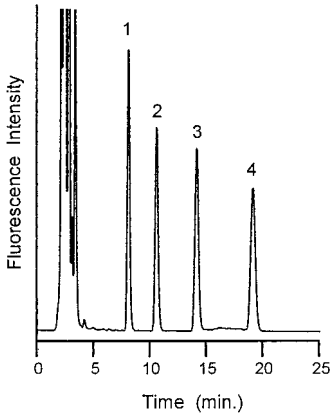

Figure 4. Chromatogram of alcohols derivatized with PSBD-NCO: (1) 1-octanol 10 pmol, (2) 1-nonanol 10 pmol, (3) 1-decanol 10 pmol, (4) 1-undecanol 10 pmol; column, TSK gel ODS-80Ts (150 × 4.6 mm, i.d. 5 μm); eluent, acetonitrile − water (4:1); flow rate, 1.0 mL min^{-1}; detection, excitation 368 nm, emission 490 nm.

NCO). No other interfering peaks were detected. There was no PSBD-NCO peak detected, because PSBD-NCO was presumed to be nonfluorescent or the excess PSBD-NCO was hydrolyzed to PSBD-NH_2. The fluorescence intensity of PSBD-NH_2 was approximately 0.015% that of PSBD-NHCOOMe, and the excitation and emission wavelengths of PSBD-NH_2 (λex/λem = 428 nm/ 544 nm) were different from those of PSBD-NHCOOMe. On the contrary, other reagents (3CMC,[4] pyrene-1-carbonyl nitrile,[6] 1-anthroylnitrile,[7] OMB−COCl[10], and DBD-COCl[12]) produced many fluorescent degradation products with the desired derivatives, since the reagents themselves are fluorescent, and the fluorescence of the excess reagents and the degradation products severely interfered with the quantification of the slight amount of intended derivatives. These facts indicated that PSBD-NCO was superior to the other reagents as a derivatization reagent for alcohols. The detection limits (signal-to-noise ratio = 3) for the respective alcohols' derivatives were 5.6−10.7 fmol, which are equivalent to the derivatives of OMB-COCl (3−8 fmol),[10] 1-anthroylnitrile (20 fmol),[7] and DMEQ-CON_3 (5 fmol)[9] and superior to those of DBD-Pro-COCl (sub-pmol),[11] 3CMC (2−40 pmol),[4] CMMC (1−10 pmol),[5] and DBD-COCl (38 fmol).[12] Furthermore, the excitation and emission wavelengths of PSBD-NHCOOMe (λex/λem = 368 nm/ 490 nm) are longer than those of the alcohols' derivatives with OMB-COCl (357 nm/ 440 nm),[10] 3CMC (355 nm/ 400 nm),[4] CMMC (315 nm/ 400 nm),[5] pyrene-1-carbonyl nitrile (300 nm/ 410 nm),[6] and DMEQ-CON_3 (360 nm/ 440 nm),[9] and the stokes shift of PSBD-NHCOOMe is also larger than those of the derivatives with other reagents. These results showed that PSBD-NCO was prominent with respect to the detectability of the derivatives and the avoidance of interference from the biomatrixes.

In conclusion, considering the relationship between the fluorescence intensity of the 4,7-disubstituted benzofurazan compounds and the Hammett substituent constants (σp) at the 4- and 7-positions, new fluorogenic derivatization reagents for alcohols, DBD-NCO, PSBD-NCO, and MSBD-NCO, were designed. The derivatives of these three reagents fluoresced as expected, with the derivative of PSBD-NCO most strongly fluorescing. PSBD-NCO was superior to other reagents for alcohols with regard to the sensitivity, the reactivity, and the absence of the interfering peaks in the chromatogram. Thus, PSBD-NCO seemed to be useful for the derivatization of a slight amount of biologically important alcohols such as steroids and prostaglandins. These results showed that our approach, employing the relationship between the fluorescence characteristics and the chemical structure, was effective in the development of new fluorogenic reagents. Further theoretical study is in progress for the precise prediction of the fluorescence intensity of 4,7-disubstituted benzofurazan compounds from the chemical structure.

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