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Supporting Information

Design of A Practical Fluorescent Probe for Superoxide Based on Protection-Deprotection Chemistry of Fluoresceins with Benzenesulfonyl Protecting Group

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Experimental Section

General. DMSO (spectrophotometric grade) was purchased from Wako Pure Chemical Industries. Water was purified using a Millipore Milli-Q Gradient-A10 system coupled with an EYELA SA-2100E automatic distillation apparatus. XO, GSH, H_2O_2 (as a 30% aqueous solution), RPMI 1640 medium, and an antibiotic-antimycotic mixed stock solution (10^4 unit/mL penicillin, 10 mg/mL streptomycin, and 25 μg/mL amphotericin B) were obtained from Nacalai Tesque. HPX, SOD, NADPH, NADH, CYP reductase, diaphorase, SIN-1, NOC-5, *t*-BuOOH (as a 70% aqueous solution), and PMA were purchased from Sigma. NaOCl, Tiron, and butyric acid were obtained from Aldrich. Compound **1c** was prepared as previously reported[6] and used without purification. 2,4-DinitroBES, 2-nitroBES, 4-nitroBES, and 4-methoxy-2-nitroBES chlorides were purchased from Aldrich. 4-Methyl-2-nitroBES, 4-ethoxy-2-nitroBES, 4-isopropoxy-2-nitroBES, 4,5-dimethyl-2-nitroBES, 4,6-dimethyl-2-nitroBES, 4,5-dimethoxy-2-nitroBES, and 4,5-diethoxy-2-nitroBES chlorides were prepared from 4-methyl-2-nitroaniline, 4-ethoxy-2-nitroaniline, 4-isopropyloxy-2-nitroaniline,[38] 4,5-dimethyl-2-nitroaniline, 4,6-dimethyl-2-nitroaniline, 4,5-dimethoxy-2-nitroaniline,[39] and 4,5-diethoxy-2-nitroaniline,[39] respectively, according to a known method.[40] Briefly, an aqueous solution of $NaNO_2$ (1 eq) was gradually added to a suspension of a nitroaniline in conc. HCl at 0°C. After stirred for 30 min, the resulting mixture was passed through a glass filter. The filtered solution and an aqueous solution of Na_2SO_3 (0.8 eq) were simultaneously and gradually added to a suspension of $CuSO_4$ (0.15 eq) in conc. HCl at room temperature. After addition of another solution of Na_2SO_3 (0.8 eq), the mixture was stirred for several hours and extracted with CH_2Cl_2. After removal of solvent, the residue was subjected to silica gel chromatography with CH_2Cl_2, to afford the corresponding BES. All other chemicals were used as received without further purification. 96–Well flat bottom microtiter plates were obtained from Iwaki. A Mono–Poly resolving medium was purchased from Dainippon Pharmaceutical. Column chromatography was performed on silica gel (Merck 60, 63~200 μm). ^{1}H NMR (270 MHz) spectra were obtained on a JEOL EX270 spectrometer with

tetramethylsilane (TMS) as an internal standard (δ 0.00). ^{13}C NMR (151 MHz) spectra were recorded on a Varian Unity Inova 600 spectrometer with a solvent as an internal standard. ^{19}F NMR (564 MHz) spectra were also recorded on a Varian Unity Inova 600 spectrometer with hexafluorobenzene as an internal standard (δ 0.00). The splitting patterns are designated as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and bs (broad singlet). Infrared (IR) spectra were taken on a JASCO VALOR-III spectrometer. Fast-atom bombardment (FAB) mass spectra were acquired on a JEOL JMS-700 spectrometer with m-nitrobenzyl alcohol as the matrix. UV-visible spectra were obtained on a Shimadzu UV-2450 spectrophotometer. All melting points were measured on a Yanako MP-S3 micro-melting point apparatus, and are given uncorrected. HPLC analyses were performed on a system equipped with Shimadzu LC-10AD solvent delivery pumps, a Shimadzu DGU-14A degasser, a TOSOH AS-8020 autosampler, a Shimadzu SPD-10AD*vp* UV/vis detector, a Shimadzu CTO-10AD*vp* column oven, a Shimadzu C-R6A Chromatopac recorder, and a ODS column.

Fluorometric Analyses. A JASCO FP-75 fluorescence spectrophotometer equipped with a JASCO ETC-272 Peltier thermostatted cell holder was used for estimating the rate constant for reaction of BESSo with $O_2^{-\bullet}$ as well as determining relative quantum efficiency (Φ_{fl}). Fluorometric measurements with 96-Well microtiter plates were performed with a Molecular Devices SpectraMax GeminiEM fluorescence plate reader with emission, cut-off, and excitation wavelengths at 505, 530, and 544, respectively. Flow cytometry was carried out on FACSCalibur with an argon laser (488 nm), and fluorescence was measured with an emission (515~545) filter in 10^4 cells for each sample on a logarithmic scale of fluorescence over four decades of log. Fluorescence images were observed under an Olympus BX50 epifluorescence microscopy. The microscope was equipped with an Hg lamp, a 20 x UPLANFL objective lens, an excitation filter (450~480 nm), a dichromic mirror (505 nm), and an emission filter (515 nm). Fluorescence images were captured with a Roper Photometrics CoolSnap cooled CCD camera under the same conditions, and were transformed to 1392 x 1040 TIFF images with CoolSnap 1.2.0.

3',6'-O-Bis-(4-nitrobenzenesulfonyl)-2',4',5',7'-tetrafluorofluorescein (**6b**). To a suspension of **1c** (1.0 g, 2.47 mmol) and 4-nitro-benzenesulfonyl chloride (1.45 g, 5.44 mmol) in dry CH_2Cl_2 (20 mL), 2,6-lutidine (5 mL) was added at 0°C. The resulting mixture was stirred at room temperature for 4 h, diluted with CH_2Cl_2 (200 mL), washed with 1 M HCl (200 mL x 2) and brine (200 mL), and dried over $MgSO_4$. The solvent was removed by evaporation, and the residue was subjected to silica gel chromatography with CH_2Cl_2, to afford **6b** (1.69 g, 88%) as a white solid. The purity was determined to be 99.5% by RP-HPLC analysis (YMC-Pack ODS-A column (4.5 x 150 mm, 5 μm), 65:35 CH_3CN/H_2O, flow rate = 1.0 mL/min, 284 nm, 35°C, t_r = 15.0 min). R_f = 0.72 (CH_2Cl_2); m.p. 249-251°C (EtOAc/hexane); ^{1}H NMR ([D$_6$]DMSO) δ = 8.53-8.48 (m, 4H), 8.36-8.31 (m, 4H), 8.04 (d, ^3J (H,H) = 6.4 Hz, 1H), 7.84-7.73 (m, 2H), 7.48 (d, ^3J (H,H) = 7.6 Hz, 1H), 7.07 ppm (dd, ^3J (H,F) = 10.2 Hz, ^5J (H,F) = 2.3 Hz, 2H); ^{13}C NMR ([D$_6$]DMSO) δ: 167.73, 151.55 (2C), 150.98, 150.67 (d, ^1J (C,F) = 250.5 Hz, 2C), 143.98 (d, ^1J (C,F) = 257.2 Hz, 2C), 139.20 (2C), 136.12, 136.03 (d, ^2J (C,F) = 7.3 Hz, 2C), 131.06, 130.13 (4C), 126.52 (dd, ^2J (C,F) = 18.0, 13.1 Hz, 2C), 125.74, 125.24 (4C), 124.67, 124.01, 119.31 (d, ^3J (C,F) = 7.3 Hz, 2C), 110.18 (d, ^2J (C,F) = 22.0 Hz, 2C), 78.11 ppm; IR (KBr) ν bar = 1787 (C=O), 1610 (C=C), 1535 cm^{-1} (NO_2); UV-vis (CH_3CN) λ_{max} (ε): 282.5 nm (9800 mol^{-1} dm^3 cm^{-1}); FABMS calcd for $C_{32}H_{15}F_4N_2O_{13}S_2$ $(MH)^+$: 774.9952, found: 774.9963.

3',6'-O-Bis-(2-nitrobenzenesulfonyl)-2',4',5',7'-tetrafluorofluorescein (**6c**). To a suspension of **1c** (1.0 g, 2.47 mmol) and 2–nitrobenzenesulfonyl chloride (1.45 g, 5.44 mmol) in dry CH_2Cl_2 (20 mL), 2,6-lutidine (5 mL) was added at 0°C. The resulting mixture was stirred at room temperature for 4 h, diluted with CH_2Cl_2 (200 mL), washed with 1 M HCl (200 mL x 2) and brine (200 mL), and dried over $MgSO_4$. The solvent was removed by evaporation, and the residue was subjected to silica gel chromatography with CH_2Cl_2, to afford **6c** (1.74 g, 91%) as a white solid. The purity was determined to be 99.5% by RP-HPLC analysis (YMC-Pack ODS-A column (4.5 x 15 cm, 5 μm), 65:35 CH_3CN/H_2O, flow rate = 1.0 mL/min, 284 nm, 35°C, t_r = 9.2 min). R_f = 0.64 (CH_2Cl_2); m.p. 115-120°C; ^{1}H NMR ([D$_6$]DMSO) δ = 8.28-8.13 (m, 6H), 8.05-7.96 (m, 3H),

7.85-7.73 (m, 2H), 7.46 (d, ^{3}J (H,H) = 7.6 Hz, 1H), 7.10 ppm (dd, ^{3}J (H,F) = 10.2, ^{5}J (H,F) = 2.0 Hz, 2H); ^{13}C NMR ([D_6]DMSO) δ = 167.76, 151.13, 150.67 (d, ^{1}J (C,F) = 248.7 Hz, 2C), 147.70 (2C), 143.97 (d, ^{1}J (C,F) = 257.8 Hz, 2C), 137.81 (2C), 136.13, 135.95 (d, ^{2}J (C,F) = 7.9 Hz, 2C), 133.58 (2C), 131.59 (2C), 131.03, 126.53 (dd, ^{2}J (C,F) = 17.7, 12.8 Hz, 2C), 126.31 (2C), 125.75, 125.68 (2C), 124.60, 123.92, 119.40 (d, ^{3}J (C,F) = 7.9 Hz, 2C), 110.19 (d, ^{2}J (C,F) = 24.4 Hz, 2C), 78.08 ppm; IR (KBr) ν bar = 1787 (C=O), 1610 (C=C), 1535 cm^{-1} (NO_2); UV-vis (CH_3CN) λ_{max} (ε): 283 nm (10000 mol^{-1} dm^3 cm^{-1}); FABMS calcd for $C_{32}H_{15}F_4N_2O_{13}S_2$ $(MH)^+$: 774.9952, found: 774.9950.

3'-O-(4-Nitrobenzenesulfonyl)-2',4',5',7'-tetrafluorofluorescein (**7b**). To a suspension of **1c** (1.0 g, 2.47 mmol) in dry CH_2Cl_2 (20 mL), 2,6-lutidine (5 mL) was added and stirred for 10 min at 0°C. To this mixture, 4-nitrobenzenesulfonyl chloride (0.66 g, 2.97 mmol) was added at 0°C. The resulting mixture was stirred at room temperature for 4 h, diluted with CH_2Cl_2 (200 mL), washed with 1 M HCl (200 mL x 2) and brine (200 mL), and dried over $MgSO_4$. The solvent was removed by evaporation, and the residue was subjected to silica gel chromatography with 20:1 CH_2Cl_2-acetone, to afford a crude product. Further purification of the product by silica gel chromatography with 1:1 hexane/EtOAc gave **7b** (0.31 g, 21%) as a slightly yellow solid. The purity was determined to be 99.0% by RP-HPLC analysis (YMC-Pack ODS-A column (4.5 x 15 cm, 5 μm), 55:45 CH_3CN/H_2O, flow rate = 1.0 mL/min, 284 nm, 35°C, t_r = 12.4 min). R_f = 0.32 or 0.49 (CH_2Cl_2/acetone 20:1 or hexane/EtOAc 1:1, respectively); m.p. 245-250°C (EtOAc/hexane); ^{1}H NMR ([D_6]DMSO) δ = 8.45-8.40 (m, 2H), 8.24-8.20 (m, 2H), 8.02-7.99 (m, 1H), 7.82-7.70 (m, 2H), 7.27-7.23 (m, 1H), 6.64 (dd, ^{3}J (H,F) = 10.1 Hz, ^{5}J (H,F) = 2.3 Hz, 1H), 6.47 ppm (dd, ^{3}J (H,F) = 10.9 Hz, ^{5}J (H,F) = 2.3 Hz, 1H); ^{13}C NMR ([D_6]DMSO) δ = 167.91, 151.51, 151.14, 148.86 (d, ^{1}J (C,F) = 240.1 Hz), 148.75 (d, ^{1}J (C,F) = 216.9 Hz), 144.03 (d, ^{1}J (C,F) = 254.8 Hz), 140.46 (d, ^{1}J (C,F) = 245.0 Hz), 139.26, 136.59–136.03 (m, 3C), 135.96, 130.80, 130.14 (2C), 126.38 (t, ^{2}J (C,F) = 12.8 Hz), 125.44, 125.21 (2C), 125.08, 123.97, 119.60 (d, ^{3}J (C,F) = 7.9 Hz), 110.11 (d, ^{2}J (C,F) = 20.8 Hz), 108.37 (d, ^{3}J (C,F) = 7.3 Hz), 108.27 (d, ^{2}J

(C,F) = 23.2 Hz), 79.13 ppm; IR (KBr) ν bar = 3183 (OH), 1747 (C=O), 1538 cm^{-1} (NO_2); UV-vis (CH_3CN) λ_{max} (ε) = 283 nm (6500 mol^{-1} dm^3 cm^{-1}); FABMS calcd for $C_{26}H_{12}F_4NO_9S$ $(MH)^+$: 590.0169, found: 590.161.

3'-O-(2-Nitrobenzenesulfonyl)-2',4',5',7'-tetrafluorofluorescein (**7c**). To a suspension of **2** (1.0 g, 2.47 mmol) in dry CH_2Cl_2 (20 mL), 2,6-lutidine (5 mL) was added and stirred for 10 min at 0°C. To the mixture, 2-nitrobenzenesulfonyl chloride (0.66 g, 2.97 mmol) was added at 0°C. The resulting mixture was stirred at room temperature for 4 h, diluted with CH_2Cl_2 (200 mL), washed with 1 M HCl (200 mL x 2) and brine (200 mL), and dried over $MgSO_4$. The solvent was removed by evaporation, and the residue was subjected to silica gel chromatography with 20:1 CH_2Cl_2-acetone, to afford a crude product. Further purification of the product by silica gel chromatography with 1:2 hexane/EtOAc gave **7c** (0.33 g, 22%) as a slightly yellow solid. The purity was determined to be 98.5% by RP-HPLC analysis (YMC-Pack ODS-A column (4.5 x 15 cm, 5 μm), 55:45 CH_3CN/H_2O, flow rate = 1.0 mL/min, 284 nm, 35°C, t_r = 9.3 min). R_f = 0.32 or 0.55 (CH_2Cl_2/acetone 20:1 or hexane/EtOAc 1:2, respectively); m.p. 234-246°C (EtOAc/hexane); ^{1}H NMR ([D_6]DMSO) δ = 11.33 (s, 1H), 8.27 (t, ^3J (H,H) = 8.9 Hz, 2H), 8.17 (t, ^3J (H,H) = 7.7 Hz, 1H), 8.05–7.97 (m, 2H), 7.85-7.73 (m, 2H), 7.43 (d, ^3J (H,H) = 7.4 Hz, 1H), 7.02 (d, ^3J (H,F) = 10.1 Hz, 1H), 6.59 ppm (d, ^3J (H,F) = 10.9 Hz, 1H); ^{13}C NMR ([D_6]DMSO) δ = 167.94, 151.21, 150.29 (d, ^3J (C,F) = 248.7 Hz), 148.85 (d, ^3J (C,F) = 240.7 Hz), 147.71, 144.04 (d, ^3J (C,F) = 259.7 Hz), 140.45 (d, ^1J (C,F) = 251.7 Hz), 137.78, 136.53–136.07 (m, 3C), 135.97, 133.56, 131.61, 130.80, 126.55 (d, ^2J (C,F) = 12.2 Hz), 126.35, 125.66 125.46, 125.50, 123.93, 119.72 (d, ^3J (C,F) = 7.3 Hz), 110.13 (d, ^2J (C,F) = 17.1 Hz), 108.39 (d, ^3J (C,F) = 7.9 Hz), 108.28 (d, ^2J (C,F) = 23.8 Hz), 79.11 ppm; IR (KBr) ν bar = 3254 (OH), 1752 (CO), 1553 cm^{-1} (NO_2); UV-vis (CH_3CN) λ_{max} (ε) = 283 nm (8100 mol^{-1} dm^3 cm^{-1}); FABMS calcd for $C_{26}H_{12}F_4NO_9S$ $(MH)^+$: 590.0169; found: 590.0167.

3'-O-(4-Methyl-2-nitrobenzenesulfonyl)-2',4',5',7'-tetrafluorofluorescein (**7d**). To a suspension of **1c** (1.0 g, 2.47 mmol) in dry CH_2Cl_2 (20 mL), 2,6-lutidine (5 mL) was added and stirred for 10 min at 0°C. To the mixture, 4-methyl-2-nitrobenzenesulfonyl chloride (0.70 g, 2.97 mmol) was added at 0°C. The resulting mixture was stirred at room temperature for 4 h, diluted with CH_2Cl_2 (200 mL), washed with 1 M HCl (200 mL x 2) and brine (200 mL), and dried over $MgSO_4$. The solvent was removed by evaporation, and the residue was subjected to silica gel chromatography with 20:1 CH_2Cl_2/acetone, to afford a crude product. Further purification of the product by silica gel chromatography with 1:1 hexane/EtOAc gave **7d** (0.43 g, 29%) as a white solid. The purity was determined to be 99.7% by RP-HPLC analysis (YMC-Pack ODS-A column (4.5 x 15 cm, 5 μm), 55:45 CH_3CN/H_2O, flow rate = 1.0 mL/min, 284 nm, 35°C, t_r = 12.1 min). R_f = 0.35 or 0.31 (CH_2Cl_2/acetone 20:1 or hexane/EtOAc 1:1, respectively); m.p. 204–209°C ($CHCl_3$); ^{1}H NMR ([D_6]DMSO) δ = 11.33 (s, 1H), 8.13 (s, 1H), 8.11 (d, ^3J (H,H) = 8.6 Hz, 1H), 8.03 (d, ^3J (H,H) = 6.9 Hz, 1H), 7.85–7.72 (m, 3H), 7.42 (d, ^3J (H,H) = 8.2 Hz, 1H), 7.00 (dd, ^3J (H,F) = 10.2 Hz, ^5J (H,F) = 2.1 Hz, 1H), 6.58 (dd, ^3J (H,F) = 10.9 Hz, ^5J (H,F) 2.1 Hz, 1H), 2.54 ppm (s, 3H); ^{13}C NMR ([D_6]DMSO) δ = 167.94, 151.22, 150.33 (d, ^1J (C,F) = 248.9 Hz), 149.70, 148.87 (d, ^1J (C,F) = 249.3 Hz), 147.66, 144.08 (d, ^1J (C,F) = 254.2 Hz), 140.45 (d, ^1J (C,F) = 245.0 Hz), 136.60–136.08 (m, 3C), 135.98, 133.63, 131.47, 130.80, 126.54 (dd, ^2J (C,F) = 18.3, 12.8 Hz), 125.87, 125.46, 125.05, 123.93, 123.45, 119.64 (d, ^3J (C,F) = 7.9 Hz), 110.09 (d, ^2J (C,F) = 18.9 Hz), 108.38 (d, ^3J (C,F) = 7.3 Hz), 108.28 (d, ^2J (C,F) = 22.6 Hz), 79.13, 21.01 ppm; IR (KBr) ν bar = 3390 (OH), 1759 (C=O), 1549 cm^{-1} (NO_2); UV-vis (CH_3CN) λ_{max} (ε) = 283.5 nm (7100 mol^{-1} dm^3 cm^{-1}); FABMS calcd for $C_{27}H_{14}F_4NO_9S$ $(MH)^+$: 604.0326, found: 604.0334.

3'-O-(4-methoxy-2-nitrobenzenesulfonyl)-2',4',5',7'-tetrafluorofluorescein (**7e**). To a suspension of **1c** (1.0 g, 2.47 mmol) in dry CH_2Cl_2 (20 mL), 2,6-lutidine (5 mL) was added and stirred for 10 min at 0°C. To the mixture, 4-methoxy-2-nitrobenzenesulfonyl chloride (0.75 g, 2.97 mmol) was added at 0°C. The resulting mixture was stirred at room temperature for 4 h, diluted with CH_2Cl_2 (200 mL), washed with 1 M HCl (200 mL x 2) and brine (200 mL), and dried

over $MgSO_4$. The solvent was removed by evaporation, and the residue was subjected to silica gel chromatography with 20:1 CH_2Cl_2/acetone, to afford a crude product. Further purification of the product by silica gel chromatography with 1:2 hexane/EtOAc gave **7e** (0.23 g, 15%) as a pale yellow solid. The purity was determined to be 98.6% by RP-HPLC analysis (YMC-Pack ODS-A column (4.5 x 15 cm, 5 μm), 55:45 CH_3CN/H_2O, flow rate = 1.0 mL/min, 284 nm, 35°C, t_r = 11.1 min). R_f = 0.3 or 0.58 (CH_2Cl_2/acetone 20:1 or hexane/EtOAc 1:2, respectively); m.p. 111-125°C; ^{1}H NMR ($CDCl_3$) δ = 8.07-8.01 (m, 2H), 7.79–7.68 (m, 2H), 7.36 (s, 2H), 7.20-7.16 (m, 2H), 6.41 (dd, ^3J (H,F) = 9.5 Hz, ^5J (H,F) = 2.2 Hz, 1H), 6.34 (dd, ^3J (H,F) = 10.2 Hz, ^5J (H,F) = 2.0 Hz, 1H), 3.99 ppm (s, 3H); ^{13}C NMR ([D_6]DMSO) δ = 167.95, 165.42, 151.23, 150.40 (d, ^1J (C,F) = 248.1 Hz), 149.74, 148.91 (d, ^1J (C,F) = 251.7 Hz), 143.28 (d, ^1J (C,F) = 257.2 Hz), 140.45 (d, ^1J (C,F) = 242.6 Hz), 136.62–136.03 (m, 3C), 135.98, 135.64, 130.79, 126.58 (dd, ^2J (C,F) = 17.7, 12.8 Hz), 125.46, 125.05, 123.92, 119.55 (d, ^3J (C,F) = 7.9 Hz), 117.62, 116.88, 111.56, 110.05 (d, ^2J (C,F) = 17.7 Hz), 108.36 (d, ^3J (C,F) = 7.3 Hz), 108.27 (d, ^2J (C,F) = 21.4 Hz), 79.16, 57.21 ppm; IR (KBr) ν bar = 3227 (OH), 1755 (C=O), 1552 cm^{-1} (NO_2); UV-vis (CH_3CN) λ_{max} (ε) = 283.5 nm (8700 mol^{-1} dm^3 cm^{-1}); FABMS calcd for $C_{27}H_{14}F_4NO_9S$ $(MH)^+$: 620.0275, found: 620.0280.

3'-O-(4-Ethoxy-2-nitrobenzenesulfonyl)-2',4',5',7'-tetrafluorofluorescein (**7f**). To a suspension of **1c** (1.0 g, 2.47 mmol) in dry CH_2Cl_2 (20 mL), 2,6-lutidine (5 mL) was added and stirred for 10 min at 0°C. To the mixture, 4-ethoxy-2-nitrobenzenesulfonyl chloride (0.79 g, 2.97 mmol) was added at 0°C. The resulting mixture was stirred at room temperature for 4 h, diluted with CH_2Cl_2 (200 mL), washed with 1 M HCl (200 mL x 2) and brine (200 mL), and dried over $MgSO_4$. The solvent was removed by evaporation, and the residue was subjected to silica gel chromatography with 20:1 CH_2Cl_2/acetone, to afford a crude product. Further purification of the product by silica gel chromatography with 1:2 hexane/EtOAc gave **7f** (0.43 g, 27%) as a pale yellow solid. The purity was determined to be 99.8% by RP-HPLC analysis (YMC-Pack ODS-A column (4.5 x 15 cm, 5 μm), 55:45 CH_3CN/H_2O, flow rate = 1.0 mL/min, 284 nm, 35°C, t_r =

15.9 min). R_f = 0.36 or 0.62 (CH_2Cl_2/acetone 20:1 or hexane/EtOAc 1:1, respectively); m.p. 213–217°C ($CHCl_3$). ^{1}H NMR ([D_6]DMSO) δ = 11.32 (bs, 1H), 8.08 (d, ^3J (H,H) = 9.1 Hz, 1H), 8.03 (d, ^3J (H,H) = 7.6 Hz, 1H), 7.84-7.72 (m, 2H), 7.44-7.39 (m, 2H), 6.98 (dd, ^3J (H,F) = 10.2 Hz, ^5J (H,F) = 2.3 Hz, 1H), 6.57 (dd, ^3J (H,F) = 11.1 Hz, ^5J (H,F) = 2.1 Hz, 1H), 4.27 (q, ^3J (H,H) = 6.9 Hz, 2H), 1.38 ppm (t, ^3J (H,H) = 6.9 Hz, 3H); ^{13}C NMR ([D_6]DMSO) δ = 167.95, 164.69, 151.22, 149.78, 148.88 (d, ^1J (C,F) = 240.7 Hz), 148.83 (d, ^1J (C,F) = 234.6 Hz), 144.17 (d, ^1J (C,F) = 257.8 Hz), 140.46 (dd, ^1J (C,F) = 246.2, 6.7 Hz), 136.60–136.05 (m, 3C), 135.97, 133.62, 130.78, 126.57 (dd, ^2J (C,F) = 17.7, 12.8 Hz), 125.45, 125.05, 123.92, 119.54 (d, ^3J (C,F) = 7.9 Hz), 117.84, 116.65, 111.79, 110.05 (d, ^2J (C,F) = 22.0 Hz), 108.38 (d, ^3J (C,F) = 7.9 Hz), 108.28 (d, ^2J (C,F) = 22.6 Hz), 79.15, 65.66, 14.10 ppm; IR (KBr) ν bar = 3366 (OH) 1761 (C=O), 1553 cm^{-1} (NO_2); UV-vis (CH_3CN) λ_{max} (ε) = 283 nm (7800 mol^{-1} dm^3 cm^{-1}); FABMS calcd for $C_{28}H_{16}F_4NO_{10}S$ $(MH)^+$: 634.0431, found: 634.0419.

3'-O-(4-Isopropyloxy-2-nitrobenzenesulfonyl)-2',4',5',7'-tetrafluorofluorescein (**7g**). To a suspension of **1c** (1.0 g, 2.47 mmol) in dry CH_2Cl_2 (20 mL), 2,6-lutidine (5 mL) was added and stirred for 10 min at 0°C. To the mixture, 4-isopropyloxy-2-nitrobenzenesulfonyl chloride (0.83 g, 2.97 mmol) was added at 0°C. The resulting mixture was stirred at room temperature for 4 h, diluted with CH_2Cl_2 (200 mL), washed with 1 M HCl (200 mL x 2) and brine (200 mL), and dried over $MgSO_4$. The solvent was removed by evaporation, and the residue was subjected to silica gel chromatography with 20:1 CH_2Cl_2/acetone, to afford a crude product. Further purification of the product by silica gel chromatography with 1:1 hexane/EtOAc gave **7g** (0.52 g, 33%) as a pale yellow solid. The purity was determined to be 99.9% by RP-HPLC analysis (YMC-Pack ODS-A column (4.5 x 15 cm, 5 μm), 55:45 CH_3CN/H_2O, flow rate = 1.0 mL/min, 284 nm, 35°C, t_r = 21.3 min). R_f = 0.32 or 0.42 (CH_2Cl_2/acetone 20:1 or hexane/EtOAc 1:1, respectively); m.p. 175–194°C ($CHCl_3$); ^{1}H NMR ([D_6]DMSO) δ = 11.33 (bs, 1H), 8.07-8.01 (m, 2H), 7.85-7.72 (m, 3H), 7.43-7.39 (m, 2H), 6.99 (dd, ^3J (H,F) = 10.2 Hz, ^5J (H,F) = 2.1 Hz, 1H), 6.58 (dd, ^3J (H,F) = 10.9 Hz, ^5J (H,F) = 2.1 Hz, 1H), 4.91 (heptet, ^3J (H,H) = 6.1 Hz, 1H), 1.33 ppm (d, ^3J (H,H) = 6.1 Hz,

6H); ^{13}C NMR ([D$_6$]DMSO) δ = 167.94, 163.86, 151.22, 150.42 (d, ^1J (C,F) = 243.2 Hz), 149.94, 148.89 (d, ^1J (C,F) = 241.3 Hz), 144.16 (d, ^1J (C,F) = 253.6 Hz), 140.47 (d, ^1J (C,F) = 238.9 Hz), 136.54–136.05 (m, 3C), 135.97, 133.66, 130.78, 126.56 (dd, ^2J (C,F) = 17.7, 12.8 Hz), 125.45, 125.06, 123.92, 119.53 (d, ^3J (C,F) = 7.3 Hz), 118.37, 116.26, 112.31, 110.05 (d, ^2J (C,F) = 22.0 Hz), 108.36 (d, ^3J (C,F) = 7.9 Hz), 108.26 (d, ^1J (C,F) = 20.2 Hz), 79.15, 72.33, 21.28, 21.26 ppm; IR (KBr) ν bar = 3375 (OH), 1759 (C=O), 1553 cm^{-1} (NO_2); UV-vis (CH_3CN) λ_{max} (ε): 283.5 nm (7900 mol^{-1} dm^3 cm^{-1}); FABMS calcd for $C_{29}H_{18}F_4NO_{10}S$ $(MH)^+$: 648.0588, found: 648.0613.

3'-O-(4,5-Dimethyl-2-nitrobenzenesulfonyl)-2',4',5',7'-tetrafluorofluorescein (**7h**). To a suspension of **1c** (1.0 g, 2.47 mmol) in dry CH_2Cl_2 (20 mL), 2,6-lutidine (5 mL) was added and stirred for 10 min at 0°C. To the mixture, 4,5-dimethyl-2-nitrobenzenesulfonyl chloride (0.74 g, 2.97 mmol) was added at 0°C. The resulting mixture was stirred at room temperature for 4 h, diluted with CH_2Cl_2 (200 mL), washed with 1 M HCl (200 mL x 2) and brine (200 mL), and dried over $MgSO_4$. The solvent was removed by evaporation, and the residue was subjected to silica gel chromatography with 20:1 CH_2Cl_2/acetone, to afford a crude product. Further purification of the product by silica gel chromatography with 1:1 hexane/EtOAc gave **7h** (0.48 g, 31%) as a pale yellow solid. The purity was determined to be 99.6% by RP-HPLC analysis (YMC-Pack ODS-A column (4.5 x 15 cm, 5 μm), 55:45 CH_3CN/H_2O, flow rate = 1.0 mL/min, 284 nm, 35°C, t_r = 15.0 min). R_f = 0.41 or 0.36 (CH_2Cl_2/acetone 20:1 or hexane/EtOAc 1:1, respectively); m.p. 227–233°C ($CHCl_3$); ^{1}H NMR ([D$_6$]DMSO) δ = 11.31 (bs, 1H), 8.09 (s, 1H), 8.02 (d, ^3J (H,H) = 7.6 Hz, 1H), 8.01 (s, 1H), 7.84-7.72 (m, 2H), 7.42 (d, ^3J (H,H) = 7.1 Hz, 1H), 6.98 (dd, ^3J (H,F) = 10.2 Hz, ^5J (H,F) = 2.3 Hz, 1H), 6.57 (dd, ^3J (H,F) = 10.9 Hz, ^5J (H,F) = 2.1 Hz, 1H), 2.43 (s, 3H), 2.39 ppm (s, 3H); ^{13}C NMR ([D$_6$]DMSO) δ = 167.94, 151.23, 150.35 (d, ^1J (C,F) = 250.5 Hz), 148.87 (d, ^1J (C,F) = 245.0 Hz), 147.96, 145.54, 144.12 (d, ^1J (C,F) = 256.0 Hz), 143.40, 140.48 (d, ^1J (C,F) = 249.0 Hz), 136.59–136.03 (m, 3C), 135.96, 131.47, 130.79, 126.63 (dd, ^2J (C,F) = 18.3, 12.8 Hz), 126.21, 125.45, 125.05, 123.93, 123.69, 119.55 (d, ^3J (C,F) = 7.3 Hz),

110.03 (d, ^2J (C,F) = 21.4 Hz), 108.64 (d, ^3J (C,F) = 7.3 Hz), 108.29 (d, ^2J (C,F) = 22.0 Hz), 79.16, 19.49, 19.04 ppm; IR (KBr) ν bar = 3447 (OH), 1757 (C=O), 1541 cm^{-1} (NO_2); UV-vis (CH_3CN) λ_{max} (ε) = 282.5 nm (8300 mol^{-1} dm^3 cm^{-1}); FABMS calcd for $C_{28}H_{16}F_4NO_9S$ $(MH)^+$: 618.0482, found: 618.0504.

3'-O-(4,6-Dimethyl-2-nitrobenzenesulfonyl)-2',4',5',7'-tetrafluorofluorescein (**7i**). To a suspension of **1c** (1.0 g, 2.47 mmol) in dry CH_2Cl_2 (20 mL), 2,6-lutidine (5 mL) was added and stirred for 10 min at 0°C. To the mixture, 4,6-dimethyl-2-nitrobenzenesulfonyl chloride (0.74 g, 2.97 mmol) was added at 0°C. The resulting mixture was stirred at room temperature for 4 h, diluted with CH_2Cl_2 (200 mL), washed with 1 M HCl (200 mL x 2) and brine (200 mL), and dried over $MgSO_4$. The solvent was removed by evaporation, and the residue was subjected to silica gel chromatography with 20:1 CH_2Cl_2/acetone, to afford **7i** (0.42 g, 27%) as a pale yellow solid. The purity was determined to be 99.7% by RP-HPLC analysis (YMC-Pack ODS-A column (4.5 x 15 cm, 5 μm), 55:45 CH_3CN/H_2O, flow rate = 1.0 mL/min, 284 nm, 35°C, t_r = 15.7 min). R_f = 0.45 or 0.36 (CH_2Cl_2/acetone 20:1 or hexane/EtOAc 1:1, respectively); m.p. 208–220°C ($CHCl_3$); ^{1}H NMR ([D_6]DMSO) δ = 11.31 (bs, 1H), 8.02 (d, ^3J (H,H) = 7.3 Hz, 1H), 7.83-7.71 (m, 4H), 7.37 (d, ^3J (H,H) = 7.9 Hz, 1H), 7.01 (dd, ^3J (H,F) = 10.2 Hz, ^5J (H,H) =1.8 Hz, 1H), 6.58 (dd, ^3J (H,F) = 10.9 Hz, ^5J (H,F) = 1.8 Hz, 1H), 2.76 (s, 3H), 2.45 ppm (s, 3H); ^{13}C NMR ([D_6]DMSO) δ =167.96, 151.38, 150.47 (d, ^2J (C,F) = 249.3 Hz), 150.01, 148.89 (d, ^2J (C,F) = 238.9 Hz), 148.55, 144.23 (d, ^2J (C,F) = 257.2 Hz), 141.93, 140.46 (dd, ^1J (C,F) = 245.3 Hz, 3^2J (C,F) = 6.4 Hz), 136.59–136.28 (m, 3C), 136.12, 135.97, 130.76, 126.14 (dd, ^2J (C,F) = 18.0, 12.5 Hz), 125.48, 124.92, 123.76, 122.51, 121.27, 119.75 (d, ^3J (C,F) = 7.9 Hz), 110.07 (d, ^2J (C,F) = 21.4 Hz), 108.32 (d, ^3J (C,F) = 7.9 Hz), 108.23 (d, ^2J (C,F) = 21.4 Hz), 79.04, 20.91, 20.77 ppm; IR (KBr) ν bar = 3379 (OH), 1762 (CO), 1553 cm^{-1} (NO_2); UV-vis (CH_3CN) λ_{max} (ε) = 283.5 nm (6500 mol^{-1} dm^3 cm^{-1}); FABMS calcd for $C_{28}H_{16}F_4NO_9S$ $(MH)^+$: 618.0482, found: 618.0468.

3'-O-(4,5-Dimethoxy-2-nitrobenzenesulfonyl)-2',4',5',7'-tetrafluorofluorescein (**7j**)(BESSo). To a suspension of **1c** (1.0 g, 2.47 mmol) in dry CH_2Cl_2 (20 mL), 2,6-lutidine (5 mL) was added and stirred for 10 min at 0°C. To the mixture, 4,5-dimethoxy-2-nitrobenzenesulfonyl chloride (0.83 g, 2.97 mmol) was added at 0°C. The resulting mixture was stirred at room temperature for 4 h, diluted with CH_2Cl_2 (200 mL), washed with 1 M HCl (200 mL x 2) and brine (200 mL), and dried over $MgSO_4$. The solvent was removed by evaporation, and the residue was subjected to silica gel chromatography with 20:1 CH_2Cl_2/acetone, to afford a crude product. Further purification of the product by silica gel chromatography with 1:2 hexane/EtOAc gave **7j** (0.42 g, 24%) as a pale yellow solid. The purity was determined to be 99.9% by RP-HPLC analysis (YMC-Pack ODS-A column (4.5 x 15 cm, 5 μm), 55:45 CH_3CN/H_2O, flow rate = 1.0 mL/min, 284 nm, 35°C, t_r = 10.5 min). R_f = 0.32 or 0.29 (CH_2Cl_2/acetone 20:1 or hexane/EtOAc 1:1, respectively); m.p. 233–237°C ($CHCl_3$); ^{1}H NMR ([D_6]DMS) δ = 11.32 (bs, 1H), 8.02 (d, ^3J (H,H) = 7.3 Hz, 1H), 7.87 (s, 1H), 7.84-7.72 (m, 2H), 7.42 (s, 1H), 7.41 (d, ^3J (H,H) = 7.8 Hz, 1H), 6.98 (dd, ^3J (H,F) = 10.3 Hz, ^5J (H,H) = 1.9 Hz, 1H), 6.58 (dd, ^3J (H,H) = 10.9 Hz, ^5J (H,H) = 1.8 Hz, 1H), 3.98 (s, 3H), 3.86 ppm (s, 3H); ^{13}C NMR ([D_6]DMSO) δ = 167.96, 154.09, 151.33, 150.57, 150.36 (d, ^1J (C,F) = 250.5 Hz), 148.86 (d, ^1J (C,F) = 243.2 Hz), 144.23 (d, ^1J (C,F) = 259.4 Hz), 142.52, 140.47 (d, ^1J (C,F) = 245.6 Hz), 136.59–136.05 (m, 3C), 135.95, 130.78, 126.63 (dd, ^2J (C,F) = 17.7, 12.8 Hz), 125.44, 125.02, 123.90, 119.51 (d, ^3J (C,F) = 7.9 Hz), 118.00, 112.20, 109.97 (d, ^2J (C,F) = 24.4 Hz), 108.37 (d, ^3J (C,F) = 7.3 Hz), 108.28 (d, ^2J (C,F) = 21.4 Hz), 79.15, 57.21, 56.92 ppm; ^{19}F NMR ([D_6]DMSO) δ = 32.69 (d, ^3J (F,H) = 9.8 Hz, 1F), 27.91 (d, ^3J (F,H) = 10.3 Hz, 0.5F), 27.89 (d, ^3J (F,H) = 9.8 Hz, 0.5F), 20.23 (s, 1F), 9.57 ppm (bs, 1F); IR (KBr) ν bar = 3354 (OH), 1763 (C=O), 1543 cm^{-1} (NO_2); UV-vis (CH_3CN) λ_{max} (ε): 284.5 nm (9800 mol^{-1} dm^3 cm^{-1}); FABMS calcd for $C_{28}H_{16}F_4NO_{11}S$ $(MH)^+$: 650.0380, found: 650.0380.

3'-O-(4,5-Diethoxy-2-nitrobenzenesulfonyl)-2',4',5',7'-tetrafluorofluorescein (**7k**). To a suspension of **1c** (1.0 g, 2.47 mmol) in dry CH_2Cl_2 (20 mL), 2,6-lutidine (5 mL) was added and

stirred for 10 min at 0°C. To the mixture, 4,5-diethoxy-2-nitrobenzenesulfonyl chloride (0.83 g, 2.97 mmol) was added at 0°C. The resulting mixture was stirred at room temperature for 4 h, diluted with CH_2Cl_2 (200 mL), washed with 1 M HCl (200 mL x 2) and brine (200 mL), and dried over $MgSO_4$. The solvent was removed by evaporation, and the residue was subjected to silica gel chromatography with 20:1 CH_2Cl_2/acetone, to afford a crude product. Further purification of the product by silica gel chromatography with 1:2 hexane/EtOAc gave **7k** (0.69 g, 41%) as a pale yellow solid. The purity was determined to be 98.0% by RP-HPLC analysis (YMC-Pack ODS-A column (4.5 x 15 cm, 5 μm), 55:45 CH_3CN/H_2O, flow rate = 1.0 mL/min, 284 nm, 35°C, t_r = 17.8 min). R_f = 0.33 or 0.68 (20:1 CH_2Cl_2/acetone or 1:1 hexane/EtOAc, respectively); m.p. 233–237°C ($CHCl_3$); ^{1}H NMR ($CDCl_3$) δ = 8.05 (d, ^3J (H,H) = 7.1 Hz, 1H), 7.80-7.68 (m, 2H), 7.47 (s, 1H), 7.45 (s, 1H), 7.21 (d, ^3J (H,H) = 7.6 Hz, 1H), 6.41 (dd, ^3J (H,F) = 9.4 Hz, ^5J (H,F) = 2.0 Hz, 1H), 6.31 (dd, ^3J (H,F) = 10.2 Hz, ^5J (H,F) = 1.8 Hz, 1H), 4.25 (q, ^3J (H,H) = 6.9 Hz, 2H), 4.18 (q, ^3J (H,H) = 6.9 Hz, 2H), 1.54 (t, ^3J (H,H) = 6.9 Hz, 3H), 1.47 ppm (t, ^3J (H,H) = 6.9 Hz, 3H); ^{13}C NMR ([D_6]DMS) δ = 167.96, 153.46 151.29, 150.37 (d, ^1J (C,F) = 248.7 Hz), 149.67, 148.86 (d, ^1J (C,F) = 245.6 Hz), 144.24 (d, ^1J (C,F) = 260.9 Hz), 142.44, 140.47 (dd, ^1J (C,F) = 245.0 Hz, ^3J (C,F) 6.7 Hz), 136.59–136.06 (m, 3C), 135.93, 130.79, 126.63 (dd, ^2J (C,F) = 17.7, 12.8 Hz), 125.45, 125.05, 123.90, 119.54 (d, ^3J (C,F) = 7.3 Hz), 117.59, 113.04, 110.00 (d, ^2J (C,F) = 23.2 Hz), 109.49, 108.42 (d, ^3J (C,F) = 7.3 Hz), 108.30 (d, ^2J (C,F) = 28.1 Hz), 79.16, 65.75, 65.34, 14.15, 14.03 ppm; IR (KBr) ν bar 3354 (OH), 1763 (CO), 1543 cm^{-1} (NO_2); UV-vis (CH_3CN) λ_{max} (ε) = 284.5 (10200 mol^{-1} dm^3 cm^{-1}); FABMS calcd for $C_{30}H_{20}F_4NO_{11}S$ $(MH)^+$: 678.0693, found: 678.0710.

3'-O-(4,5-Dimethoxy-2-nitrobenzenesulfonyl)-6'-O-acetoxymethyl-2',4',5',7'-tetrafluorofluorescein (**BESSo-AM**). To a suspension of **1c** (0.5 g, 1.24 mmol) in dry CH_2Cl_2 (20 mL), DIEA (0.26 mL, 1.48 mmol) was added and stirred for 10 min at 0°C. To the mixture, acetoxymethyl bromide (0.15 g, 1.48 mmol) was added at 0°C. The resulting mixture was stirred at room temperature for 24 h, diluted with EtOAc (100 mL), washed with 1 M HCl (100 mL x 2)

and brine (100 mL), and dried over $MgSO_4$. The solvent was removed by evaporation, and the residue was subjected to silica gel chromatography with 20:1 CH_2Cl_2/acetone, to afford 3'-O-acetoxymethyl-2',4',5',7'-tetrafluorofluorescein (0.18 g, 31%), of which purity was determined to be 96.1% by RP-HPLC analysis (YMC-Pack ODS-A column (4.5 x 15 cm, 5 μm), 55:45 CH_3CN/H_2O, flow rate = 1.0 mL/min, 284 nm, 35°C, t_r = 5.8 min). To a suspension of this acetoxymethyl derivative (0.18g, 0.37 mmol) in CH_2Cl_2 (5 mL), triethylamine (61 μL, 0.44 mmol), was added and stirred for 10 min at 0°C. To the mixture, 4,5-dimethoxy-2-nitrobenzenesulfonyl chloride (124 mg, 0.44 mmol) was added at 0°C. The resulting mixture was stirred at room temperature for 2 h, diluted with CH_2Cl_2 (50 mL), washed with 1 M HCl (50 mL) and brine (50 mL), and dried over $MgSO_4$. The solvent was removed by evaporation, and the residue was subjected to silica gel chromatography with 1:1 hexane/EtOAc, to afford a crude product. Further purification of the product by silica gel chromatography with 30:1 CH_2Cl_2/acetone gave BESSo-AM (0.27 g, 100%) as a slightly yellow solid. The purity was determined to be 99.9% by RP-HPLC analysis (YMC-Pack ODS-A column (4.5 x 15 cm, 5 μm), 55:45 CH_3CN/H_2O, flow rate = 1.0 mL/min, 284 nm, 35°C, t_r = 21.5 min). R_f = 0.68 or 0.39 (CH_2Cl_2/acetone 30:1 or hexane/EtOAc 1:1, respectively); m.p. 169–172°C (EtOAc/hexane); ^{1}H NMR ($CDCl_3$) δ = 8.07 (d, ^3J (H,H) = 6.8Hz, 1H), 7.80-7.69 (m, 2H), 7.50 (s, 1H), 7.49 (s, 1H), 7.21 (d, ^3J (H,H) = 6.8 Hz, 1H), 6.45 (dd, ^3J (H,F) = 9.5 Hz, ^5J (H,F) = 2.4 Hz, 1H), 6.40 (dd, ^3J (H,F) = 10.1 Hz, ^3J (H,F) = 2.4 Hz, 1H), 5.71 (s, 2H), 4.05 (s, 3H), 3.99 (s, 3H), 2.13 ppm (s, 3H); ^{13}C NMR ($CDCl_3$) δ = 169.69, 167.97, 153.60, 152.04 (d, ^1J (C,F) = 248.7 Hz), 151.41, 151.39 (d, ^1J (C,F) = 253.6 Hz), 151.04, 145.05 (d, ^1J (C,F) = 258.4 Hz), 144.97 (dd, ^1J (C,F) = 258.4 Hz, ^3J (C,F) = 4.9 Hz), 142.38, 136.93 (d, ^2J (C,F) = 10.4 Hz), 136.71 (d, ^2J (C,F) = 11.0 Hz), 136.11 135.44 (dd, ^2J (C,F) = 16.5 Hz, 11.6 Hz), 131.11, 128.06 (dd, ^2J (C,F) = 17.7, 12.8 Hz), 125.95, 125.28, 123.72, 121.59, 118.99 (d, ^3J (C,F) = 7.3 Hz), 115.29 (d, ^3J (C,F) = 7.9 Hz), 112.80, 108.92 (d, ^2J (C,F) = 22.0 Hz), 108.54, 108.50 (d, ^2J (C,F) = 22.0 Hz), 88.25, 79.39, 57.08 (2C), 20.68 ppm; ^{19}F NMR ($CDCl_3$) δ = 34.55 (d, J = 9.6 Hz, 1F), 31.21 (dd, J = 10.2, 3.8

Hz, 1F), 21.99 (s, 1F), 16.59 ppm (s, 1F); IR (KBr) ν bar = 1771 (C=O), 1544 cm^{-1} (NO_2); UV-vis (CH_3CN) λ_{max} (ε) = 284.5 nm (11500 mol^{-1} dm^3 cm^{-1}). FABMS calcd for $C_{31}H_{19}F_4NO_{13}S$ $(MH)^+$: 722.0592, found: 722.0593.

Sample Preparation. Probe solutions (25 μM) were prepared immediately before use by diluting a stock solution of a BES derivative (5 mM) in DMSO 200 times with pH 7.4, 10 mM HEPES buffer. Solutions of XO (0.26 U/mL for evaluating specificity and 0.52 U/ml for obtaining calibration curves), HPX (1.0 mM), SOD (1000 U/mL), GSH (1.0 mM), H_2O_2 (1.0 mM), $Fe(ClO_4)_2$ (5.0 mM), NaClO (1.0 mM), *t*-BuOOH (1.0 mM), SIN-1 (1.0 mM), CYP reductase (1.36 U/mL), NADPH (1.0 mM), diaphorase (1.3 U / mL), NADH (1.0 mM) were prepared with H_2O. NOC-5 was used as a solution (1.0 mM) in aqueous 10 mM NaOH.

Determination of Relative Quantum Efficiencies (Φ_{fl}). According to a reported procedure, Φ_{fl} values were determined by comparing the area under the corrected emission spectra obtained for solutions (25 μM, 3.0 mL) of BES derivatives in pH 7.4, 10 mM HEPES buffer at 37°C with emission wavelength at 492 nm with that for a solution (5.0 μM, 3.0 mL) of fluorescein in aqueous 0.1 M NaOH, whish has a quantum efficiency of 0.85.

Evaluation of Reactivity of BES Derivatives. To examine reactivity of BES derivatives toward $O_2^{-\bullet}$ in the absence or the presence of SOD, other ROS, GSH, Fe^{2+}, and enzyme systems such as CYP reductase/NADPH and diaphorase/NADH, each of reactions was carried out as follows. According to Table 1S, aqueous reagent solutions and/or blank solution were added to a probe solution (25 μM, 170 μL) in each well of a 96–well flat bottom microtiter plate. After mixing mechanically for 10 sec in the plate reader, the resulting mixture was incubated at 37°C for 10 min. Fluorescent augmentation for each well was measured immediately after incubation with excitation, cut-off, and emission wavelengths at 505, 530, and 544 nm, respectively. Each of reported data is the mean value of fluorescent responses obtained from the same reaction performed in 8 wells.

Table S1. Reagent Solutions Used for Evaluating Reactivity of BES Derivatives Toward $O_2^{-\bullet}$ in the absence or the presence of SOD, other ROS, GSH, Fe^{2+}, and Enzyme Systems

ROS or enzyme systems	reagent solutions added to a probe solution in this order
control	H_2O (30 μL)
$O_2^{-\bullet}$	H_2O (10 μL) + XO (10 μL) + HPX (10 μL)
$O_2^{-\bullet}$ + SOD	XO (10 μL) + SOD (10 μL) + HPX (10 μL)
GSH	H_2O (20 μL) + GSH (10 μL)
H_2O_2	H_2O (20 μL) + H_2O_2 (10 μL)
t-BuOOH	H_2O (20 μL) + *t*-BuOOH (10 μL)
NaOCl	H_2O (20 μL) + NaOCl (10 μL)
$^1O_2$	H_2O (10 μL) + H_2O_2 (10 μL) + NaOCl (10 μL)
HO•	H_2O (10 μL) + H_2O_2 (10 μL) + $Fe(ClO_4)_2$ (10 μL)
Fe^{2+}	H_2O (20 μL) + $Fe(ClO_4)_2$ (10 μL)
NO•	H_2O (20 μL) + NOC-5 (10 μL)
$ONOO^-$	H_2O (20 μL) + SIN-1 (10 μL)
CYP reductase	H_2O (10 μL) + CYP reductase (10 μL) + NADPH (10 μL)
diaphorase	H_2O (10 μL) + diaphorase (10 μL) + NADH (10 μL)

Assay of $O_2^{-\bullet}$. Solutions of XO (0.52 U/mL, 10 μL) and HPX (10 nM~100 μM, 10 μL) were added in this order to a probe solution of BESSo (25 μM, 180 μL) in each well of a flat bottom microtiter plate. After mixing mechanically for 10 sec in the plate reader, the resulting mixture was incubated at 37°C for 10 min. Fluorescent augmentation for each well was measured immediately after incubation with excitation, cut-off, and emission wavelengths at 505, 530, and 544 nm. Fluorescent responses were obtained as the mean values ± SD by performing the same reaction in 8 wells.

Product Analysis of Reaction of BESSo with KO_2. To a solution in BESSo (32.5 mg, 50 μmol) in pH 7.4 HEPES buffer (20 mL) containing 1 mL DMSO, KO_2 (7.2 mg, 100 μmol) was

added all at once at room temperature. After stirred for 10 min, the mixture was transferred to 100 mL volumetric flask and diluted to the mark with 1:1 MeOH-H_2O containing 0.05% CF_3CO_2H. The resulting solution was diluted 20 times with 1:1 MeOH-H_2O containing 0.05% CF_3CO_2H, and was subjected to a HPLC analysis. The conditions were as follows: detection wavelength, 270 nm; mobile phase, 60:40 MeOH-H_2O containing 0.05% CF_3CO_2H; flow rate, 1.0 mL/min; column, YMC-Pack ODS-A column (4.5 x 15 cm, 5 μm); column temperature, 35°C; injection volume, 10 μL. Standard solutions of **1c** and **8** for the HPLC analysis were prepared as follows. To a solution diacetyl tetrafluorofluorescein (24.6 mg, 50 μmol) in CH_3CN-H_2O (1:1)(20 mL), 28% aqueous NH_3 (68 μL, 1 mmol) was added. The resulting solution was stirred at room temperature for 2 h, transferred to 100 mL volumetric flask, and diluted to the mark with 1:1 MeOH-H_2O containing 0.05% CF_3CO_2H. The resulting solution was further diluted 20 time with 1:1 MeOH-H_2O containing 0.05% CF_3CO_2H, providing a standard solution of **1c**. To a solution 2-nitro-4,5-dimethoxyBES chloride (14.0 mg, 50 μmol) in CH_3CN (10 mL), NaOH (20 mg, 500 μmol) in H_2O (10mL) was added. The resulting solution was stirred at room temperature for 2 h, transferred to 100 mL volumetric flask, and diluted to the mark with 1:1 MeOH-H_2O containing 0.05% CF_3CO_2H. The resulting solution was further diluted 20 time with 1:1 MeOH-H_2O containing 0.05% CF_3CO_2H, providing a standard solution of **8**. A standard solution of BESSo was prepared by diluting a solution of BESSo (6.64 mg, 10 μmol) in CH_3CN (20 mL) 20 times with 1:1 MeOH-H_2O containing 0.05% CF_3CO_2H.

Assay of $O_2^{-\bullet}$ Released from Neutrophils. Heparinized whole blood (3.5 mL) from healthy volunteers was centrifuged on a Mono–Poly resolving medium (3.0 mL) at 400 g and 20°C. After separation, cells were washed twice with Ca, Mg-free phosphate buffered saline [PBS (–)] by centrifugation for 10 min at 250 g and 4°C, resuspended in PBS containing 0.9 mM $CaCl_2$ and 0.4 mM $MgCl_2$ [PBS (+)] to yield a final concentration of 1 x 10^6 cells/mL, and stored on ice until later use. A cell suspension (100 μL), a probe solution [25 μM in PBS (+), 50 μL], an SOD solution [10 μL] or a blank solution [PBS (+), 10 μL], and a PMA solution [0.64 μM in PBS (+),

50 μL] or a blank solution [PBS (+), 50 μL] were added to each well of a 96–well flat bottom microtiter plate. A zero time measurement was taken, and the plate was incubated at 37°C. During incubation, fluorescence of the cells was measured every 5 min. Each of reported data is the mean value ± SD of fluorescent responses obtained from experiments performed in 8 wells under each condition.

Measurements of intracellular $O_2^{-\bullet}$ in human Jurkat T cells. RPMI 1640 medium containing 10% fetal bovine albumin and 1% antibiotic-antimycotic mixed stock solution was used throughout. Cells were cultured in RMPI 1640 medium at 37°C in a 5% CO_2/95% air incubator. The cultured cells were centrifuged at 180 g and 4°C for 5 min, and suspended in RPMI 1640 medium. Thus obtained cell suspension (2 x 10^6 cells/mL, 500 μL), RPMI 1640 medium (250 μL) or a Tiron solution (20 mM, 250 μL) in RPMI 1640 medium, and a DMSO solution of BESSo-AM (5 mM, 5 μL) were added to each well of 24 well tissue culture plate, and incubated at 37°C in a 5% CO_2/95% air incubator. After 1h, RPMI 1640 medium (250 μL) or a butyric acid solution in RPMI 1640 medium (20 mM, 250 μL) was added to each well and incubated for another 1 h. The cells were centrifuged at 180 g and 4°C for 5 min, and washed twice with ice-cold PBS (–). The cells from each well were resuspended with PBS (–)(1.0 mL for flow cytometry and 100 μL for fluorescence microcopy), and were subjected to flow cytometry or fluorescence microscopy. All experiments were repeated at least twice.