Supporting Information

"Mechanism-Based Molecular Design of Highly Selective Fluorescence Probes for Nitrative Stress"

Tasuku Ueno,[†,‡] Yasuteru Urano,[†,§] Hirotatsu Kojima,[†,‡] and Tetsuo Nagano[†,‡,*]

[†]*Graduate School of Pharmaceutical Sciences, The University of Tokyo*

Hongo, Bunkyo-ku, Tokyo,113-0033, Japan.

[‡]*CREST, JST and* [§]*Presto, JST, 4-8-1 Honcho, Kawaguchi, Saitama, 332-0012, Japan*

* To whom correspondence should be addressed. E-mail: tlong@mol.f.u-tokyo.ac.jp

Abbreviations

HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital; PeT, photoinduced electron transfer; d-PeT, donor excited photoinduced electron transfer: electron transfer from excited electron donor to electron acceptor; a-PeT, acceptor excited photoinduced electron transfer: electron transfer from electron donor to excited acceptor; ΔG_{eT}, free energy change of electron transfer process; Φ_{fl}, fluorescence quantum efficiency; nitroBODIPY, nitro-substituted BODIPY derivatives; DAMBO, 8-(3,4-diaminophenyl)-1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene and its derivatives; HPLC, high-performance liquid chromatography; NO_2BF_4, nitronium tetrafluoroborate; CH_3CN, acetonitrile; DMF, *N,N*-dimethylformamide; MeOH, methanol; $ONOO^-$, peroxynitrite; H_2O_2, hydrogen peroxide; ^-OCl, hypochlorite; NO, nitric oxide; E_{ox}, oxidation potential; E_{red}, reduction potential; $\Delta E_{0,0}$, excitation energy; w_p, work term; TBAP, tetrabutylammonium perchlorate; NOC-13: 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-methyl-1- triazene; $^1O_2$, singlet oxygen; SCE, saturated calomel electrode; PPTS, pyridinium *p*-toluenesulfonate; TFA, trifluoroacetic acid; DDQ, 2,3-dichloro-5,6-dicyano-*p*-benzoquinone; MeMgBr, methyl magnesium bromide; THF, tetrahydrofuran; AcOH, acetic acid: EtOH, ethanol; NaOEt, sodium ethoxide; AcOEt, ethyl acetate: BF_3•OEt_2, boron trifluoride diethyl etherate; DIEA, diisopropylethylamine

Figures and Tables

Table S1. Photophysical properties of nitroBODIPY derivatives.

	reduction potential of benzene moiety (V vs. SCE)[a]	oxidation potential of BODIPY moiety (V vs SCE)[a]	absorption maximum (nm)[b]	emission maximum (nm)[b]	calcd $\Delta E_{0,0}$ (eV)[c]	calculated relative free energy change (eV)[d]	Φ_{fl}[e]
3	-1.15	1.12	503	508	2.45	0	0.001
4	-1.15	1.12	503	511	2.45	0	0.004
5	-1.28	1.12	506	515	2.43	+0.15	0.023
6	-1.28	1.36	511	523	2.40	+0.42	0.529
NiSPY-1 N[f]	-1.42	1.63	503	514	2.44	+0.79	0.687

[a] Measured in acetonitrile containing 0.1 M TBAP as a supporting electrolyte (see Figure S1). [b] Measured in methanol. [c] $\Delta E_{0,0}$ values were determined from the average wave numbers of absorption maximum and emission maximum. [d] Relative ΔG_{eT} values were calculated with respect to the value for **1** as a standard. [e] Calculated by using fluorescein as a fluorescence standard (0.850). [f] All data were measured in acetonitrile.

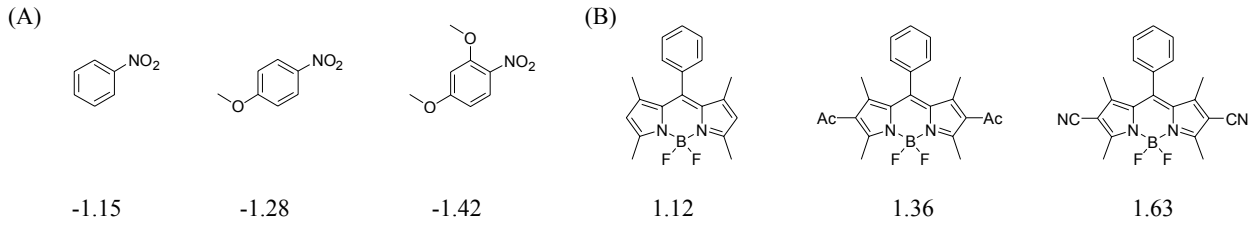


Figure S1. (A) Reduction potentials of the benzene moiety and (B) Oxidation potentials of the BODIPY fluorophore of nitroBODIPY derivatives in acetonitrile (V vs SCE).

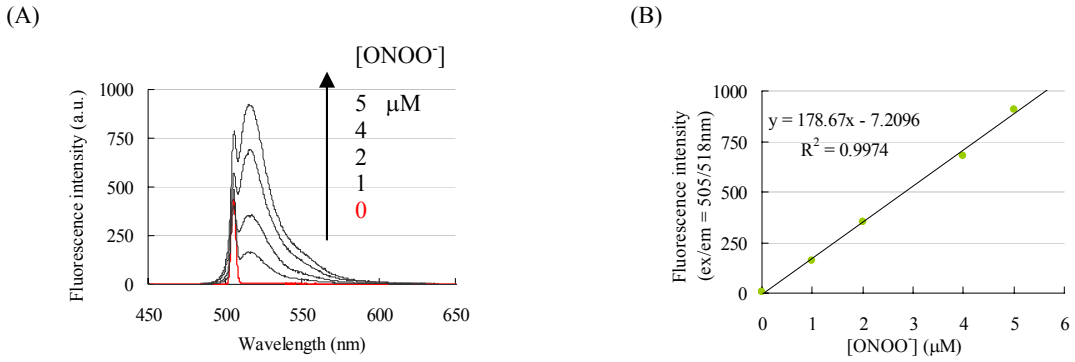


Figure S2. Detection of peroxynitrite using NiSPY-2. (A) Fluorescence spectra (excited at 505 nm) of NiSPY-2 (5 μM; 0.2% DMF as a cosolvent) in 0.1 M sodium phosphate buffer (pH 7.4) upon addition of peroxynitrite solution (final 0, 1, 2, 4 and 5 μM). (B) Relation between the final concentration of added peroxynitrite and the fluorescence intensity determined at 518 nm (excited at 505 nm).

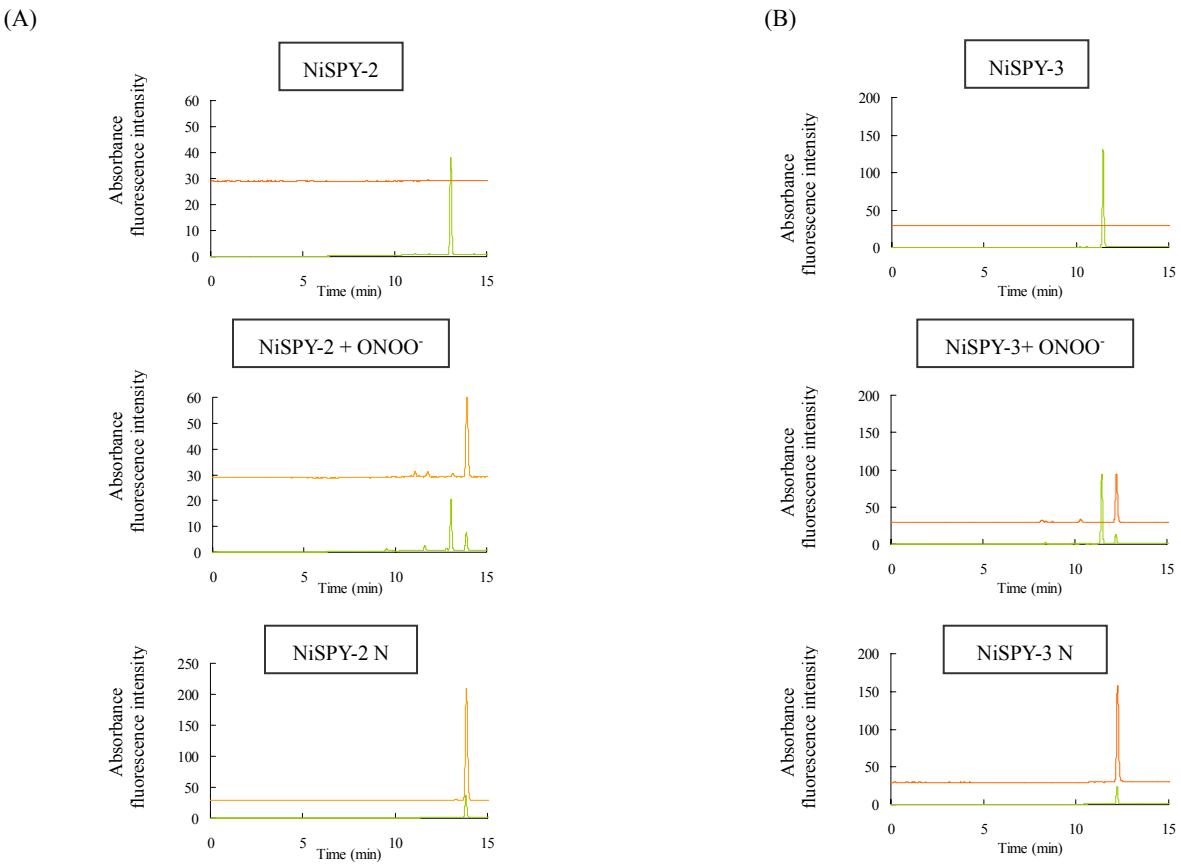


Figure S3. HPLC chromatograms during the reaction of (A) NiSPY-2 and (B) NiSPY-3 with peroxynitrite. The reaction of NiSPY-2 (5 μM, 0.1% DMF) with peroxynitrite (25 μM) was carried out in 0.1 M sodium phosphate buffer (pH 7.4). The reaction of NiSPY-3 (10 μM, 0.1% DMF) with peroxynitrite (30 μM) was carried out in 0.1 M sodium phosphate buffer (pH 7.4). The product was detected using HPLC with a linear gradient (eluent, 0 min, 32% CH_3CN/0.1% TFA aq. ~ 10 min, 80% CH_3CN/0.1% TFA aq.; flow rate = 1.0 mL/min; detection wavelength, 500 nm (absorbance, green solid line) and 500/510 nm (fluorescence, orange solid line)).

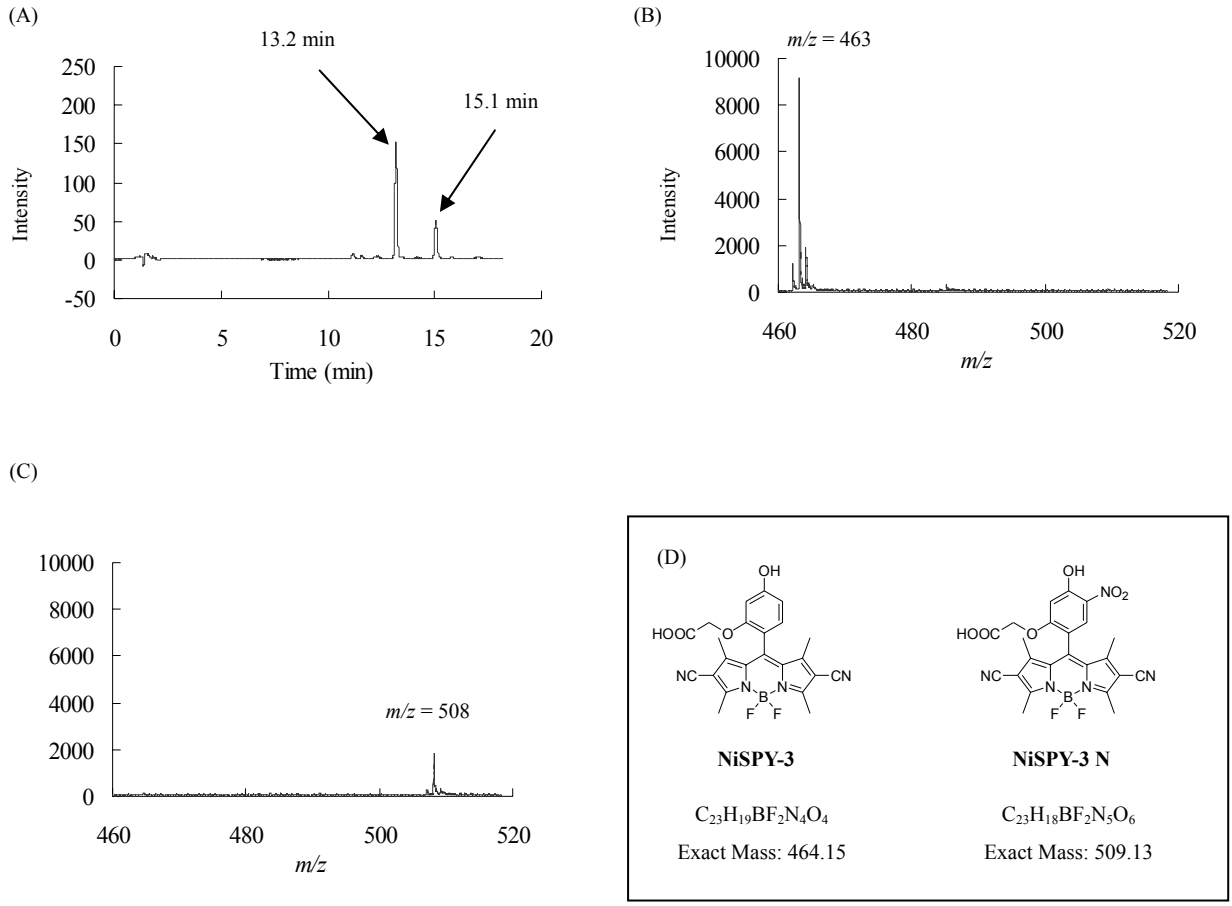


Figure S4. LC-MS analysis in the reaction of NiSPY-3 with peroxynitrite. The reaction of NiSPY-3 (10 μM, 0.1% DMF) with peroxynitrite (10 μM) was carried out in 0.1 M sodium phosphate buffer (pH 7.4) at ambient temperature. The product was detected using HPLC with a linear gradient (eluent, 0 min, 32% CH_3CN/0.1% HCOOH aq. ~ 10 min, 80% CH_3CN/0.1% HCOOH aq; flow rate, 0.2 mL/min; detection wavelength, 500 nm (absorbance)). (A) HPLC chromatogram in the reaction of NiSPY-3 with peroxynitrite. Mass spectrum of the peak at (B) 13.2 minutes and (C) 15.1 minutes. The corresponding *m/z* values were analyzed by mass spectrometry in the negative ion mode. (D) Calculated molecular weights of NiSPY-3 and NiSPY-3 N.

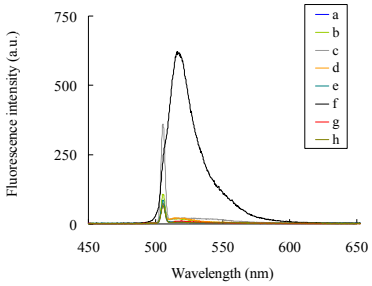


Figure S5. Fluorescence response of NiSPY-3 in various reactive oxygen species generation systems. The dye (10 mM, 0.1% DMF as a cosolvent) was added to 0.1 M sodium phosphate buffer (pH 7.4). The fluorescence spectra of NiSPY-3 were measured with 505 nm excitation after stirring for 3 min (30 min for NO and $O_2^{\bullet-}$) at room temperature (37 °C for NO). [a] •OH: Ferrous perchlorate (final, 100 μM) and H_2O_2 (final, 1 mM) were added at room temperature. [b] $^1O_2$: H_2O_2 (final, 1 mM) and NaClO (final, 100 μM) were added at room temperature. [c] H_2O_2: H_2O_2 (final, 1 mM) was added at room temperature. [d] $^-$OCl: NaClO (final, 10 μM) was added at room temperature. [e] NO: 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-methyl-1-triazene (100 μM) was added and the mixture were stirred at 37 °C for 30 min. [f] $ONOO^-$: Sodium peroxynitrite (final 10 μM) was added at room temperature. [g] $O_2^{\bullet-}$: xanthine/xanthine oxidase system. [h] Blank.

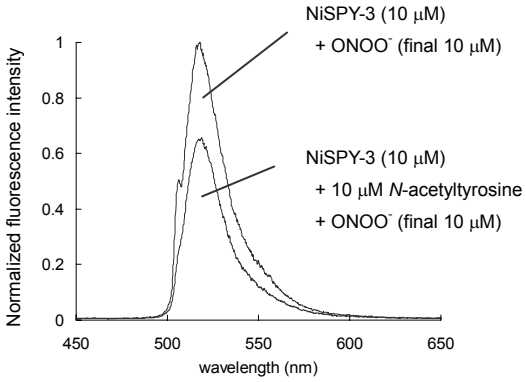


Figure S6. Fluorescence measurement for comparison of the relative reaction rate constants. To a mixture of NiSPY-3 (10 μM) and *N*-acetyltyrosine (0 or 10 μM) was added sodium peroxynitrite (final 10 μM). The fluorescence spectra of NiSPY-3 were measured with excitation at 505 nm after stirring for 1 min at room temperature. The fluorescence response of NiSPY-3 was decreased to approximately 0.6 times by addition of the same concentration of *N*-acetyltyrosine. This result showed that the reaction rate constants of these molecules are of the same order of magnitude.

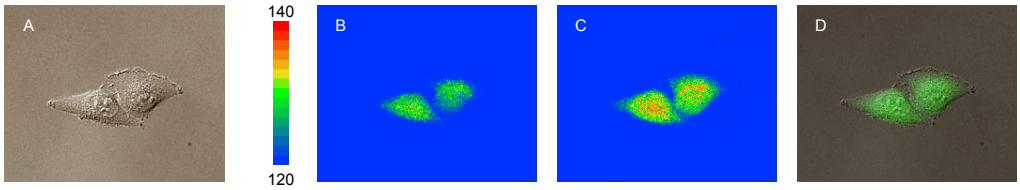


Figure S7. Differential interference contrast (A), pseudo-color fluorescence (B, C) and merged images (D) of HeLa cells loaded with NiSPY-3 in Hank's balanced salts solution. Pseudo-color images were obtained before (B) and after (C) addition of peroxynitrite. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 μg/mL) in a humidified incubator containing 5% CO_2 gas. For fluorescence microscopy, HeLa cells were plated in a 35-mm glass-bottom dish (MatTek Corporation) and cultured overnight in DMEM. The medium was removed after 24 hr, and the cells were washed with HBSS (2 x 1 mL). HBSS (2 mL) solution of 2 μM NiSPY-3 (0.01% DMF, 0.01% Cremophor EL (SIGMA) as a cosolvent) was then added, and the cells were incubated for 20 min at room temperature. The cells were then washed with 2 x 1 mL HBSS. Fluorescence images excited to 470-490 nm were obtained before and after addition of peroxynitrite.

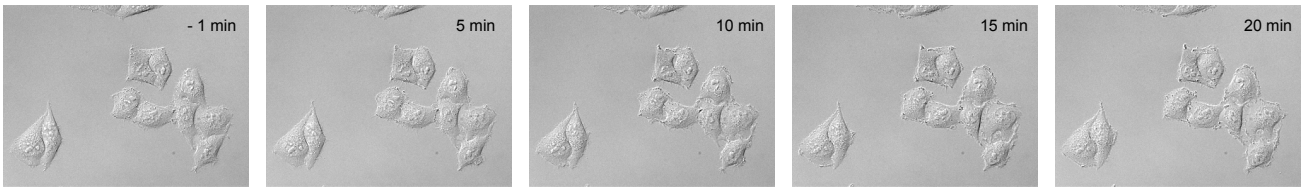


Figure S8. Differential interference contrast images of NiSPY-3 loaded HeLa cells in HBSS to evaluate the toxicity of NiSYP-3. HeLa cells were exposed to the same experimental conditions as used for fluorescence imaging. No apparent cytotoxicity was observed under these conditions.

Experimental Section

Materials. General chemicals were of the best grade available, supplied by Tokyo Chemical Industries, Wako Pure Chemical, Aldrich Chemical Co., Acros Organics and Lancaster Synthesis, and used without further purification. Special chemicals were DMF (fluorometric grade, Dojindo) and TBAP, (electrochemical grade, Fluka). All solvents were used after appropriate distillation or purification.

Instruments. NMR spectra were recorded on a JEOL JNM-LA300 instrument at 300 MHz for ^{1}H NMR and at 75 MHz for ^{13}C NMR. Mass spectra (MS) were measured with a JEOL SX-102A for FAB and a JEOL JMS-T100LC AccuToF for ESI. UV-visible spectra were obtained on a Shimadzu UV-1600. Fluorescence spectroscopic studies were performed on a Hitachi F4500.

Fluorometric Analysis. The slit width was 2.5 nm for both excitation and emission. The photon multiplier voltage was 700 V. Relative fluorescence quantum efficiency of BODIPY derivatives was obtained by comparing the area under the emission spectrum of the test sample excited at 490 nm with that of a solution of fluorescein in 0.1 N NaOH, which has a quantum efficiency of 0.850 according to the literature.[1]

In the experiment to measure the fluorescence response of NiSPYs, the slit width was 2.5 nm for excitation and 2.5 nm for emission, and the photomultiplier voltage was 900 V. Compounds were dissolved in DMF to make a stock solution, which was diluted to the required concentration for measurement.

Calculation of ΔG_{eT} value. ΔG_{eT} values were calculated from the Rehm-Weller equation: $\Delta G_{eT} = E_{ox} - E_{red} - \Delta E_{0,0} - w_p$, where E_{ox} and E_{red} are oxidation and reduction potentials of electron donor and acceptor, respectively, $\Delta E_{0,0}$ is the excited energy, and w_p is the work term for the charge separation.[2] Relative ΔG_{eT} values were calculated with respect to the value for **1** as a standard. w_p values of each compounds were assumed to be the same, due to the similarity of their structures.

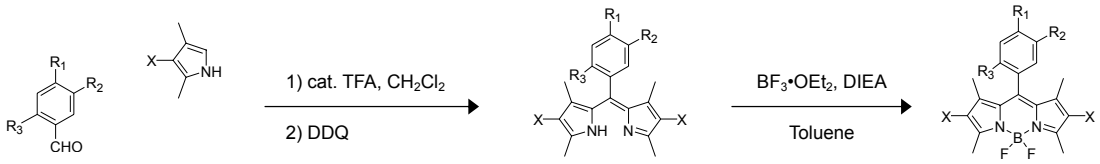
Cyclic voltammetry. Cyclic voltammetry was performed on a ALS 600 A electrochemical analyzer. A three-electrode arrangement in a single cell was used for the measurements: a Pt wire as the auxiliary electrode, a Pt electrode and a GC electrode as the working electrode, and a Ag/Ag^{+} electrode as the reference electrode. The sample solutions contained 1.0×10^{-3} M sample and 0.1 M TBAP as a supporting electrolyte in acetonitrile, and argon was bubbled for 2 min before each measurement. The scan rate was 0.1 V s^{-1}. Obtained potentials (vs Ag/Ag^{+}) were converted to those vs a saturated calomel electrode (SCE) by adding 0.25 V.

HPLC analysis. HPLC analysis were performed on an Inertsil ODS-3 (4.6 x 250 mm) column using an HPLC system composed of a pump (G1312A, Agilent) and a detector (G1315B or G1321A, Agilent).

In the LCMS experiment to analyze the reaction mixture of NiSPY-3 and peroxynitrite, LC analysis was performed an Intertsil ODS-3 (2.1 x 150 mm) column using an HPLC system composed of a pump (G1312A, Agilent) and a detector (G1314A, Agilent).

Synthesis and characterization of compounds 3 – 6.

Scheme S1. Synthetic scheme of **3** – **6**.



R_1	R_2	R_3	X	**7-10**	**3-6**
NO_2	H	H	H	**7** (crude)	**3**. y. 24% (2 step)
H	NO_2	H	H	**8** (crude)	**4**. y. 26% (2 step)
H	NO_2	OMe	H	**9** (crude)	**5**. y. 41% (2 step)
H	NO_2	OMe	Ac	**10** (crude)	**6**. y. 9% (2 step)

NitroBODIPY derivatives (**3**, **4**, **5** and **6**) were synthesized from commercially available compounds (4-nitrobenzaldehyde, 3-nitrobenzaldehyde and 2,4-dimethylpyrrole, TCI; 3-acetyl-2,4-dimethylpyrrole and 2-methoxy-5-nitrobenzaldehyde, Aldrich) according to the following procedures.

Synthesis of 8-(4-nitrophenyl)-1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (**3**). 4-Nitrobenzaldehyde (302 mg, 2 mmol) and 2,4-dimethylpyrrole (380 mg, 4 mmol) were dissolved in 300 mL of CH_2Cl_2. A catalytic amount of TFA was added, and the solution was stirred overnight at ambient temperature. When TLC monitoring showed complete consumption of the aldehyde, a solution of DDQ (590 mg, 2.6 mmol) was added, and stirring was continued for 10 min. The reaction mixture was washed with water (3 x 100 mL). The combined organic layers were washed with brine (1 x 100 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated. The crude product was purified by alumina column chromatography (aluminium oxide 90, Merck), and dried under reduced pressure to constant weight to afford **6** as a crude red amorphous solid. The crude dipyrromethene **6** and DIEA (4 mL, 23.0 mmol) were dissolved in 100 mL of toluene (100 mL) and stirred at ambient temperature for 5 min. BF_3•OEt_2 (3 mL, 23.7 mmol) was added, and stirring was continued for 10 min. The reaction mixture was washed with water (3 x 80 mL), and brine (1 x 100 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated. The crude compound was purified by silica gel column chromatography (silica gel 60N, Kanto Chemical) to afford **3** as a red powder (177 mg, yield 24%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.30 (s, 6H); 2.57 (s, 6H); 6.02 (s, 2H); 7.54 (d, J = 8.6 Hz, 2H); 8.41 (d, J = 8.6 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.6, 121.8, 124.3, 129.6, 130.6, 138.3, 141.9, 142.5, 148.3, 156.6. HRMS (ESI$^-$): calcd for $[M-H]^-$, 368.1382; found, 368.1384. Anal. Calcd for $C_{19}H_{18}BF_2N_3O_2$: N, 11.38; C, 61.81; H, 4.91. Found: N, 11.26; C; 61.73; H, 5.04.

4 and **5** were similarly prepared from 3-nitrobenzaldehyde (**4**) and 2-methoxy-5-nitrobenzaldehyde (**5**) in 26% yield and 41% yield, respectively.

4. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.36 (s, 6H); 2.57 (s, 6H); 6.02 (s, 2H); 7.69 (m, 1H); 7.73 (m, 1H); 8.23 (m, 1H); 8.37 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.6, 14.8, 121.9, 123.5, 124.0, 130.4, 131.0, 134.5, 136.7, 137.8, 142.4, 148.6, 156.6. HRMS (ESI^-): calcd for $[M-H]^-$, 368.1382; found, 368.1405.

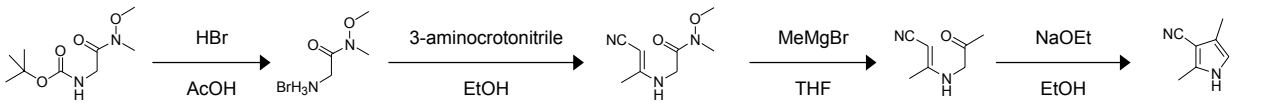
5. ^{1}H NMR (300 MHz, $CDCl_3$): 1.43 (s, 6H); 2.57 (s, 6H); 3.91 (s, 3H); 6.00 (s, 2H); 7.08 (d, J = 9.2 Hz, 1H); 8.14 (d, J = 2.9 Hz, 1H); 8.40 (dd, J = 2.9, 9.2 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.1, 14.5, 56.6, 111.0, 121.4, 124.7, 125.8, 126.9, 131.0, 141.8, 141.9, 156.0, 161.7. HRMS (ESI^+): calcd for $[M+Na]^+$, 422.1464; found, 422.1436.

6 was similarly prepared from 2-methoxy-5-nitrobenzaldehyde and 3-acetyl-2,4-dimethylpyrrole in 9% yield.

^{1}H NMR (300 MHz, CDCl3): δ 1.65 (s, 6H); 2.45 (s, 6H); 2.79 (s, 6H); 3.95 (s, 3H,); 7.14 (d, J = 9.2 Hz, 1H); 8.11 (d, J = 2.9 Hz, 1H); 8.49 (dd, J = 2.9, 9.2 Hz, 1H). HRMS (FAB+): calcd for $[M+H]^+$, 484.1856; found, 484.1844.

Synthesis and characterization of fluorescence probes for nitrative stress, NiSPYs.

Scheme S2. Synthetic scheme of 3-cyano-2,4-dimethyopyrrole



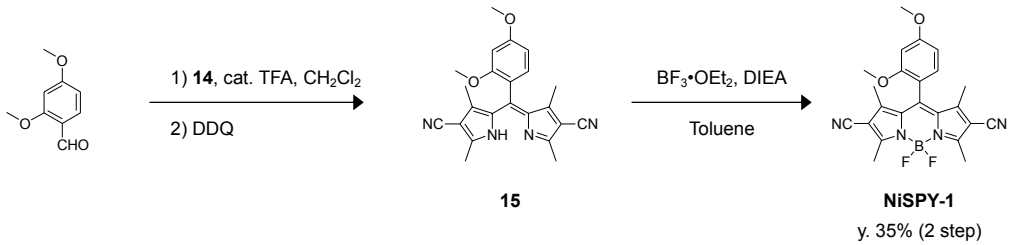
3-Cyano-2,4-dimethylpyrrole (**14**) was prepared from commercially available compounds, (*N*-tert-butoxycarbonyl-*N*-methoxy-*N*-methyl-glycinamide, Aldrich; 3-aminocrotonitrile and MeMgBr, TCI), according to the following procedure.

Synthesis of 3-cyano-2,4-dimethylpyrrole (**14**). **13** was prepared according to the literature.[3] **13** (3.4 g, 18.6 mmol) was dissolved in absolute THF (200 mL) under an argon atmosphere. To a vigorously magnetically stirred solution of **13** was added dropwise MeMgBr (3 M/diethyl ether, 18 mL, 54 mmol) at –10 °C. At the end of the reaction (monitored by TLC), the mixture was concentrated in vacuo to approximately 20 mL, and the residue was poured into water (200 mL). The aqueous solution was extracted with AcOEt (3 x 150 mL). The combined organic layers were washed with brine (1 x 100 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated to afford crude **14**. **14** was suspended in absolute EtOH (100 mL). After addition of NaOEt (200 mg) under argon atmosphere at 0 °C, the reaction mixture was stirred for 1 h, then pour into water (250 mL). The aqueous solution was extracted with AcOEt (3 x 150 mL) and brine (1 x 100 mL), dried over anhydrous Na_2SO_4, filtrated, and evaporated. The crude compound was purified by silica gel column chromatography (silica gel 60N, Kanto Chemical) to afford **14** as a white solid (1.3 g, yield 59% in 2step).

13. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.99 (s, 3H); 2.08 (s, 3H); 2.83 (s, 3H); 6.37 (m, 1H), 8.08 (br, 1H).

14. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.18 (d, *J* = 5.9 Hz, 3H); 2.37 (s, 3H); 6.37 (m, 1H), 8.08 (br, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 10.7, 12.4, 92.9, 114.6, 116.9, 121.9, 136.6.

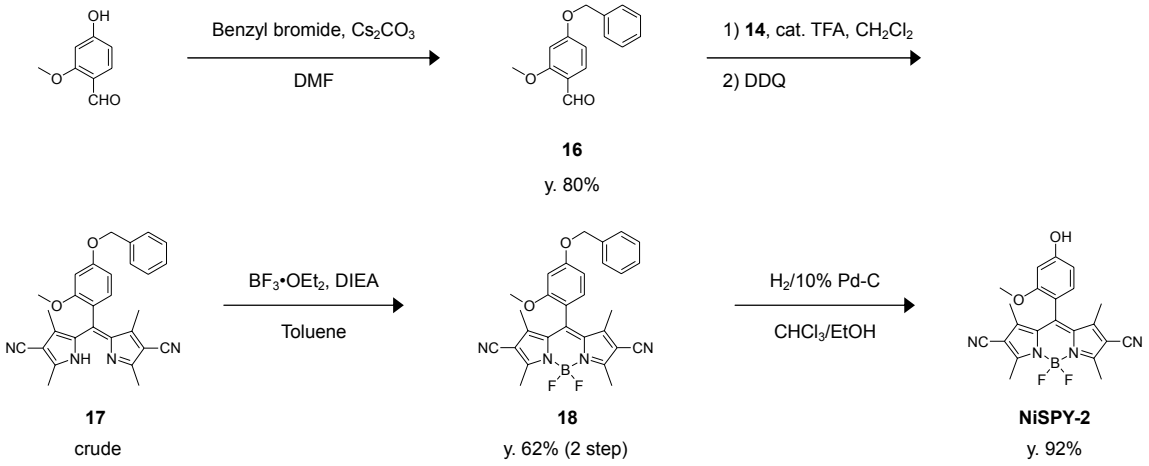
Scheme S3. Synthetic scheme of NiSPY-1



NiSPY-1 was synthesized from **14** and 2,4-dimethoxybenzaldehyde (TCI) according to the following procedure.

Synthesis of NiSPY-1. 2,4-Dimethoxybenzaldehyde (116 mg, 1.0 mmol) and 3-cyano-2,4-dimethylpyrrole (252 mg, 2.1 mmol) were dissolved in 200 mL of CH_2Cl_2. TFA (1 mL) was added dropwise into the vigorously stirred solution, and the reaction mixture was stirred overnight at ambient temperature. When TLC monitoring showed complete consumption of the aldehyde, DDQ (295 mg, 1.3 mmol) was added and stirring was continued for 20 min. The reaction mixture was washed with water (3 x 100 mL). The combined organic layers were washed with brine (1 x 100 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated. The crude product was purified by alumina column chromatography (alminium oxide 90, Merck), and dried under reduced pressure to constant weight to afford **15** as a crude amorphous solid. The crude dipyrromethene and DIEA (4 mL, 17.3 mmol) were dissolved in 100 mL of toluene (100 mL) and stirred at ambient temperature for 5 min. BF_3•OEt_2 (2 mL, 15.8 mmol) was added, and stirring was continued for 10 min. The reaction mixture was washed with water (3 x 80 mL), and brine (1 x 100 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated. The crude compound was purified by silica gel column chromatography (silica gel 60N, Kanto Chemical) to afford NiSPY-1 as an orange solid (152 mg, yield 35%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.70 (s, 6H); 2.71 (s, 6H); 3.76 (s, 3H); 3.90 (s, 3H); 6.60 (d, *J* = 2.1 Hz, 1H); 6.68 (dd, *J* = 2.1, 8.4 Hz, 1H); 6.95 (d, *J* = 8.4 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 13.5, 13.8, 55.6, 55.8, 99.5, 105.9, 106.1, 113.6, 113.8, 129.2, 132.2, 145.0, 149.0, 156.9, 159.0, 163.1. HRMS (ESI^-): calcd for [M^--1], 433.1647; found, 433.1634.

Scheme S4. Synthetic scheme of NiSPY-2



NiSPY-2 was synthesized from 5-hydroxy-2-methoxybenzaldehyde (Acros Organics) according to the following procedure.

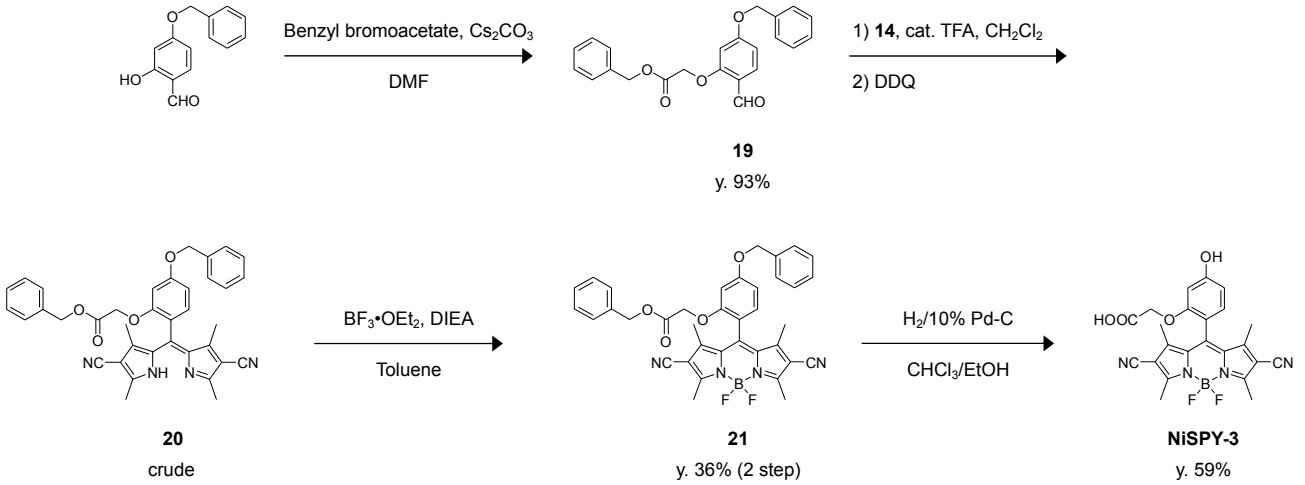
Synthesis of **16**. 4-Hydroxy-2-methoxybenzaldehyde (650 mg, 4.28 mmol) was dissolved in DMF (10 mL), and benzyl bromide (878 mg, 5.14 mmol) and cesium carbonate (1.67 g, 5.14 mmol) were added. The reaction mixture was stirred overnight at ambient temperature, water (150 mL) was added, and the reaction mixture was extracted with AcOEt (3 x 100 mL). The combined organic layers were washed with water (5 x 50 mL) and brine (1 x 100 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated to afford **17** as a crude amorphous solid. The crude product was purified by silica gel column chromatography and recrystallized from n-hexane to afford **16** as a white powder (823 mg, yield 80%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.86 (s, 3H): 5.11 (s, 2H); 6.52 (d, J = 2.2 Hz, 1H); 6.61 (dd, J = 2.2, 8.6 Hz, 1H); 7.39 (m, 5H); 7.80 (d, J = 8.6 Hz, 1H); 10.28 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 55.5, 70.3, 98.8, 106.4, 119.1, 127.4, 128.3, 128.7, 130.6, 135.8, 163.5, 165.2, 188.2. Mp: 94.0 – 94.6 °C. Anal. Calcd for $C_{15}H_{14}O_3$: N, 0; C, 74.36; H, 5.82. Found: N, 0; C; 74.28; H, 5.92.

18 were prepared according to a similar synthetic procedure (see "Synthesis of NiSPY-1") from 4-benzyloxy-2-methoxybenzaldehyde (300 mg, 1.24 mmol), 3-cyano-2,4-dimethylpyrrole (354 mg, 2.6 mmol) and DDQ (366 mg, 1.61 mg) in 62% yield (395 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.69 (s, 6H); 2.71 (s, 6H); 3.74 (s, 3H); 5.14 (s, 2H); 6.69 (d, J = 2.2 Hz, 1H); 6.75 (dd, J = 2.2, 8.3 Hz, 1H); 6.95 (d, J = 8.3 Hz, 1H); 7.42 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 13.4, 13.8, 55.8, 70.5, 100.4, 105.9, 107.1, 113.7, 113.9, 127.6, 128.4, 128.7, 129.2, 132.1, 135.9, 144.9, 148.9, 156.9, 159.0, 162.1. HRMS (ESI$^-$): calcd for $[M-H]^-$, 509.1960; found, 509.1915. Anal. Calcd for $C_{29}H_{25}BF_2N_4O_5$•0.25 H_2O: N, 10.79; C, 67.07; H, 5.05. Found: N, 10.61; C; 67.38; H, 5.08.

Synthesis of NiSPY-2. **18** (53 mg, 0.10 mmol) was dissolved in $CHCl_3$ (20 mL). The solution was diluted with EtOH 80 mL. After the addition of 10% Pd-C (10 mg), the mixture was vigorously stirred overnight under a H_2 atmosphere. The Pd-C was filtered off, and then filtrate was concentrated under reduced pressure to constant weight. The crude

compound was purified by silica gel column chromatography (silica gel 60, Kanto chemical) to afford NiSPY-2 as an orange powder (40 mg, 92%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.71 (s, 6H); 2.72 (s, 6H); 3.75 (s, 3H); 6.02 (br, 1H); 6.59 (d, J = 2.2 Hz, 1H); 6.63 (dd, J = 2.2, 8.0 Hz, 1H); 6.88 (d, J = 8.0 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 13.4, 13.8, 55.8, 100.0, 105.7, 109.0, 113.2, 113.8, 129.2, 132.2, 145.1, 149.0, 157.1, 159.0, 159.6. HRMS (ESI^-): calcd for $[M-H]^-$, 419.1491; found, 419.1464. Anal. Calcd for $C_{22}H_{19}BF_2N_4O_2$•CH_2Cl_2: N, 11.09; C, 54.69; H, 4.19. Found: N, 11.00; C; 54.89; H, 4.38.

Scheme S5. Synthetic scheme of NiSPY-3



NiSPY-3 was synthesized from 4-benzyloxy-2-hydroxybenzaldehyde (Lancaster Synthesis) according to the following procedure.

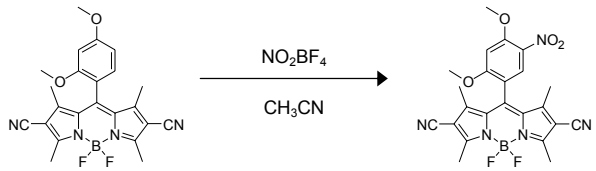
Synthesis of **19**. 4-Benzyloxy-2-hydroxybenzaldehyde (1 g, 4.39 mmol) and benzyl bromoacetate (1.1 g, 4.82 mmol) were dissolved in absolute DMF (8 mL), then cesium carbonate (1.57 g, 4.83 mmol) were added. The reaction mixture was stirred overnight at ambient temperature, water (150 mL) was added, and the reaction mixture was extracted with AcOEt (3 x 100 mL). The combined organic layers were washed with water (5 x 50 mL) and brine (1 x 100 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated. The crude product was purified by silica gel column chromatography over neutral silica gel and recrystallized from n-hexane to afford **19** as a white powder (1.54 g, yield 93%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.73 (s, 2H); 5.03 (s, 2H); 5.23 (s, 2H); 6.35 (d, J = 2.0 Hz, 1H); 6.66 (dd, J = 2.0, 8.8 Hz, 1H); 7.33-7.40 (m, 10H); 7.94 (d, J = 8.8 Hz, 1H); 10.38 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 65.4, 67.1, 70.3, 99.6, 107.6, 119.4, 127.5, 128.3, 128.4, 128.6, 128.7, 130.7, 134.8, 135.6, 161.6, 164.9, 167.8, 188.0. MS (ESI^+): 399 $[M+Na]^+$. Mp: 84.4 – 85.7 °C. Anal. Calcd for $C_{23}H_{20}O_5$: N, 0; C, 73.39; H, 5.36. Found: N, 0; C; 73.18; H, 5.62.

21 was prepared according to a similar synthetic procedure (see "Synthesis of NiSPY-1") from 4-benzyloxy-2-methoxycarbonylmethoxybenzaldehyde (400 mg, 1.06 mmol), 3-cyano-2,4-dimethylpyrrole (262 mg, 2.18 mmol) and DDQ (314 mg, 1.38 mg) in 36% yield (245 mg, orange powder). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.54 (s, 6H); 2.70 (s, 6H); 4.61 (s, 2H); 5.05 (s, 2H); 5.17 (s, 2H); 6.42 (d, J = 2.0 Hz, 1H); 6.78 (dd, J = 2.0, 8.4 Hz, 1H); 7.00 (d,

J = 8.4 Hz, 1H); 7.31-7.43 (m, 10H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 13.4, 13.8, 64.7, 67.4, 70.4, 100.6, 106.0, 108.0, 113.8, 113.9, 127.7, 128.5, 128.6, 128.7, 128.8, 129.4, 132.1, 134.6, 135.7, 144.1, 149.4, 155.0, 159.1, 161.8, 167.7. HRMS (ESI^-): calcd for $[M-H]^-$, 643.2328; found, 463.2334. Anal. Calcd for $C_{37}H_{31}BF_2N_4O_4$: N, 8.69; C, 68.95; H, 4.85. Found: N, 8.39; C, 68.71; H, 5.02.

Synthesis of NiSPY-3. **21** (35 mg, 0.05 mmol) was dissolved in $CHCl_3$ (10 mL). The solution was diluted with EtOH 40 mL. After the addition of 10% Pd-C (7 mg), the mixture was stirred vigorously under a H_2 atmosphere overnight. The Pd-C was filtered off, and the filtrate was concentrated under reduced pressure to constant weight. The crude compound was purified by silica gel column chromatography (silica gel 60, Kanto Chemical) to afford NiSPY-3 as an orange powder (15 mg, 59%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.75 (s, 6H); 2.71 (s, 6H); 4.61 (s, 2H); 6.44 (d, *J* = 1.6 Hz, 1H); 6.68 (dd, *J* = 1.6, 8.2 Hz, 1H); 6.93 (d, *J* = 8.2 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 13.0, 13.5, 64.3, 100.3, 105.0, 109.4, 110.7, 113.6, 129.2, 131.6, 149.1, 155.0, 158.4, 160.7, 169.9. HRMS (ESI^-): calcd for $[M-H]^-$, 463.1389; Found, 463.1342.

Scheme S6. Synthetic scheme of NiSPY-1 N

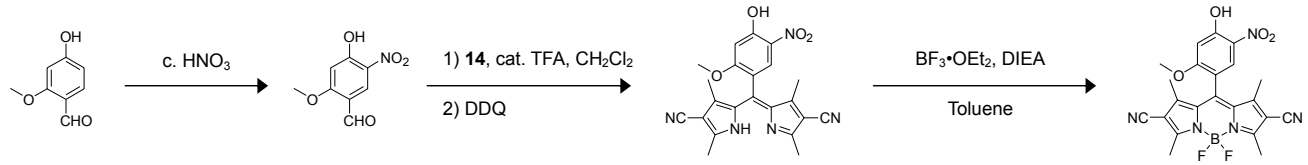


NiSPY-1 **NiSPY-1 N**

NiSPY-1 N was synthesized from NiSPY-1 according to the following procedure.

Synthesis of **NiSPY-1 N**: NiSPY-1 was dissolved in absolute CH_3CN (dried over molecular sieve). After addition of nitronium tetrafluoroborate (30 mg), the reaction mixture was stirred at ambient temperature for 5 min, and then saturated $NaHCO_3$ (200 mL) was added. The aqueous mixture was extracted with CH_2Cl_2 (3 x 100 mL). The combined organic layers were washed with water (2 x 100 mL) and brine (1 x 100 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated. The crude product was purified by chromatography on a preparative silica gel plate to afford NiSPY-1 N as an orange powder. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.74 (s, 6H); 2.73 (s, 6H); 3.93 (s, 3H); 4.12 (s, 3H); 6.69 (s, 1H); 7.88 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): 13.9, 56.9, 60.0, 96.7, 106.7, 113.1, 113.4, 127.7, 131.8, 133.3, 140.7, 148.4, 157.4, 160.1, 160.8. HRMS (ESI^-): calcd for $[M-H]^-$, 478.1498; found, 478.1468.

Scheme S7. Synthetic scheme of NiSPY-2 N

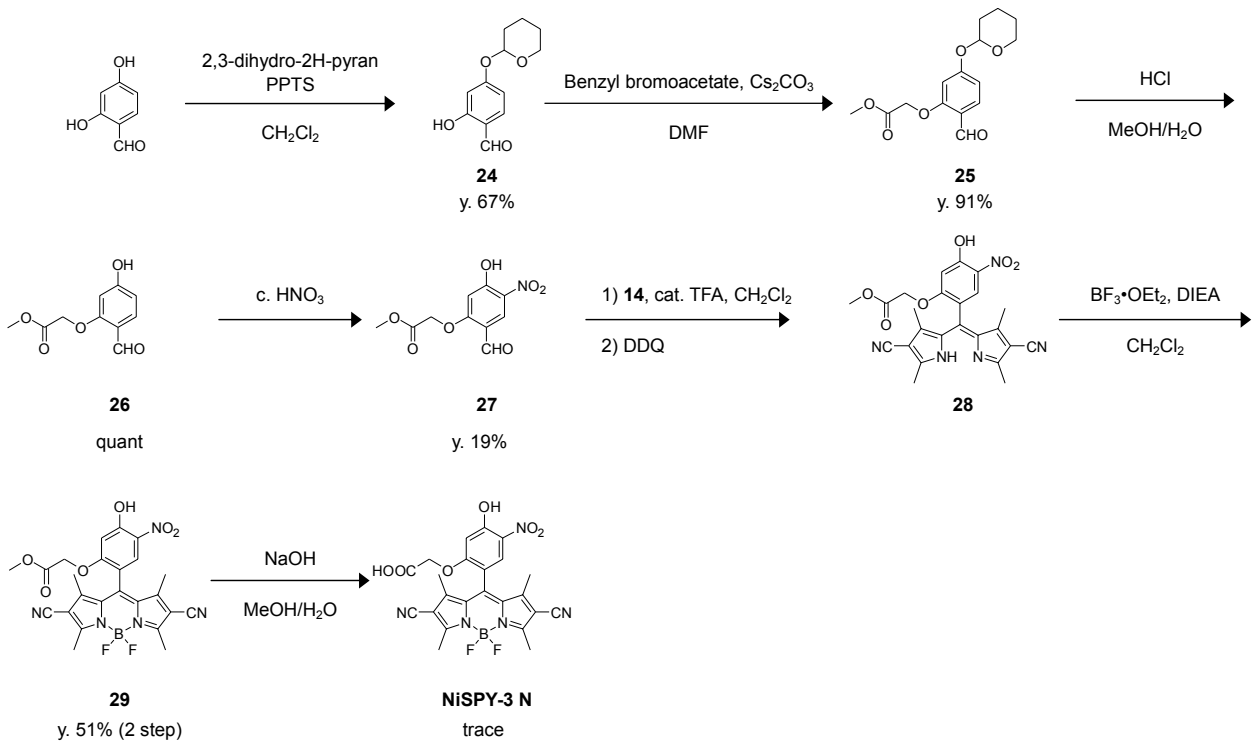


NiSPY-2 N was synthesized from 4-hydroxy-2-methoxybenzaldehyde according to the following procedure.

Synthesis of **22**. Conc. nitric acid (5 mL) was carefully added dropwise to 4-hydroxy-2-methoxybenzaldehyde (800 mg, 5.26 mmol) at 0 °C. The reaction mixture was stirred at ambient temperature for 20 min, then diluted with water (150 mL). The aqueous mixture was extracted with CH_2Cl_2 (3 x 50 mL). The combined organic layers were washed with water (1 x 100 mL) and brine (2 x 50 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated. The crude product was purified by silica gel column chromatography (silica gel 60, Kanto Chemical) to afford **22** as a pale yellow powder (274 mg, yield 26%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.03 (s, 3H); 6.63 (s, 1H); 8.67 (s, 1H); 10.27 (s, 1H); 11.29 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 56.8, 100.9, 119.1, 127.7, 128.1, 161.0, 167.3, 186.3. Mp: 145.6 – 146.4 °C. Anal. Calcd for $C_8H_7NO_5$: N, 7.10; C, 48.74; H, 3.58. Found: N, 7.01; C, 48.92; H, 3.75.

Synthesis of NiSPY-2 N. **22** (100 mg, 0.51 mmol) and 3-cyano-2,4-dimethylpyrrole (145 mg, 1.07 mmol) were dissolved in 200 mL of CH_2Cl_2. TFA (1.5 mL) was added dropwise into the vigorously stirred solution, and the reaction mixture was stirred overnight at ambient temperature. When TLC monitoring showed complete consumption of the aldehyde, DDQ (189 mg, 0.58 mmol) was added and stirring was continued for 10 min. The reaction mixture was washed with water (3 x 100 mL). The organic layers were washed with brine (1 x 100 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated. The crude product was purified by alumina column chromatography (aluminium oxide 90 active neutral, Merck) to afford **23** as a crude red amorphous solid. The crude dipyrromethene **23** and DIEA (4 mL, 17.3 mmol) were dissolved in 100 mL of absolute toluene (100 mL) and stirred at ambient temperature for 5 min. BF_3•OEt_2 (2 mL, 15.8 mmol) was added, and stirring was continued for 10 min. The reaction mixture was washed with water (3 x 80 mL), and brine (1 x 100 mL), dried over anhydrous Na_2SO_4, filtrated, and evaporated. The crude compound was purified by silica gel column chromatography (silica gel 60, Kanto Chemical) to afford NiSPY-2 N as an orange powder (36 mg, yield 15%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.76 (s, 6H); 2.73 (s, 6H); 3.91 (s, 3H); 6.78 (s, 1H); 7.97 (s, 1H); 11.16 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 13.9, 14.0, 57.3, 101.7, 106.8, 113.2, 115.0, 126.3, 128.1, 131.8, 140.2, 148.2, 159.1, 160.2, 163.1. HRMS (ESI$^-$): calcd for $[M-H]^-$, 464.1342; found, 464.1331.

Scheme S8. Synthetic scheme of NiSPY-3 N



NiSPY-3 N was synthesized from 2,4-dihydroxybenzaldehyde (TCI) according to the following procedure.

Synthesis of **24**. 2,4-Dihydroxybenzaldehyde (2.0 g, 14.50 mmol) and 2,4-dihydro-2*H*-pyran (1.92 g, 22.78 mmol) were dissolved in absolute CH_2Cl_2 (150 mL). After the addition of pyridinium para-toluenesulfonate (PPTS, 1.8 g, 14.50 mmol), the reaction mixture was stirred at ambient temperature for 4 h. The reaction mixture was washed with sodium phosphate buffer pH 6.8 (3 x 100 mL) and brine (1 x 100 mL). The organic layers was dried over anhydrous Na_2SO_4, filtered, and evaporated. The crude product was purified by silica gel column chromatography (silica gel 60N, Kanto Chemical) to afford **24** as an oil (2.16 g, yield 67%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.61-2.04 (m, 6H); 3.61-3.87 (m, 2H); 5.51 (m, 1H); 6.64 (m, 2H); 7.45 (d, J = 8.6 Hz, 1H); 9.73 (s, 1H); 11.36 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 18.4, 24.9, 29.9, 62.1, 96.2, 103.6, 109.3, 115.7, 135.2, 164.1, 164.3, 194.5. HRMS (ESI^-): calcd for $[M-H]^-$, 221.0814; found, 221.0801.

Synthesis of **25**. **24**. (2.16 g, 9.73 mmol) and methyl bromoacetate (1.64 g, 10.70 mmol) were dissolved in absolute DMF (20 mL). Cesium carbonate (4.1, 12.65 mmol) was added, then the reaction mixture was stirred overnight at ambient temperature, water (150 mL) was added, and the reaction mixture was extracted with AcOEt (3 x 100 mL). The combined organic layers were washed with water (4 x 50 mL) and brine (1 x 100 mL), dried over anhydrous Na_2SO_4, filtrated, and evaporated. The crude product was purified by silica gel column chromatography (silica gel 60N, Kanto Chemical) and recrystallized from n-hexane to afford **25** as a white powder (2.60 g, yield 91%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.60-1.90 (m, 6H); 3.64 (m, 1H); 3.78 (m. 1H); 3.82 (s, 3H); 4.74 (s, 2H); 5.49 (t, J = 2.9 Hz, 1H); 6.50 (d, J =

2.2 Hz, 1H); 6.77 (m, 1H); 7.83 (d, J = 8.8 Hz, 1H); 10.39 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 18.2, 24.8, 29.9, 52.3, 61.9, 65.4, 96.2, 100.5, 109.5, 119.7, 130.2, 161.6, 163.4, 168.4, 188.1. HRMS (ESI^+): calcd for $[M+Na]^+$, 317.1001; found, 317.1001.

Synthesis of **26**. **25** (1.5 g, 5.10 mmol) was dissolved in MeOH (40 mL). After addition of 1 N HCl aq. (10 mL), the reaction mixture was stirred at ambient temperature for 5 h. The reaction mixture was extracted with AcOEt (3 x 100 mL). The combined organic layers were washed with brine (1 x 100 mL), dried over anhydrous Na_2SO_4, filtrated, and evaporated. The crude product was purified by silica gel column chromatography (silica gel 60, Kanto Chemical) to afford **26** as a white powder (quant.). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.83 (s, 3H); 4.74 (s, 2H); 6.10 (br, 1H); 6.30 (d, J = 2.2 Hz, 1H); 6.53 (m, 1H); 7.80 (d, J = 8.6 Hz, 1H); 10.36 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 52.0, 65.0, 100.0, 109.4, 117.3, 129.9, 162.1, 165.0, 168.8, 186.9. HRMS (ESI^+): calcd for $[M+Na]^+$, 233.0426; found, 233.0383.

Synthesis of **27**. Conc. nitric acid (10 mL) was carefully added dropwise to **26** (1.05 g, 5.02 mmol) at 0 °C. The reaction mixture was stirred at ambient temperature for 20 min, then diluted with water (150 mL). The aqueous mixture was extracted with CH_2Cl_2 (3 x 50 mL). The combined organic layers were washed with water (1 x 100 mL) and brine (2 x 50 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated. The crude product was purified by silica gel column chromatography (silica gel 60, Kanto Chemical) to afford **27** as a pale yellow powder (243 mg, yield 19%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.86 (s, 3H); 4.84 (s, 2H); 6.49 (s, 1H); 8.70 (s, 1H); 10.35 (s, 1H); 11.24 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 52.8, 65.6, 101.7, 119.2, 127.7, 128.8, 160.6, 165.3, 167.1, 186.1. HRMS (ESI^+): calcd for $[M+Na]^+$, 278.0277; found, 278.0271.

Synthesis of **28.** **27** (135 mg, 0.53 mmol) and 3-cyano-2,4-dimethylpyrrole (134 mg, 1.11 mmol) were dissolved in 200 mL of CH_2Cl_2. TFA (1 mL) was added dropwise into vigorously stirred solution, and the reaction mixture was stirred at ambient temperature overnight. When TLC monitoring showed complete consumption of the aldehyde, DDQ (189 mg, 0.58 mmol) was added and stirring was continued for 10 min. The reaction mixture was washed with water (3 x 100 mL). The organic layers were washed with brine (1 x 100 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated. The crude product was purified by alumina column chromatography over alminium oxide 90 active neutral (Merck) to afford **28** as a red powder. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.57 (s, 6H); 2.46 (s, 6H); 3.79 (s, 3H); 4.71 (s, 2H); 6.51 (s, 1H); 7.91 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 13.9, 15.2, 52.7, 65.2, 101.8, 104.9, 115.0, 118.1, 127.2, 128.4, 135.5, 136.8, 147.1, 155.1, 158.2, 162.0, 167.0. HRMS (ESI^+): calcd for $[M+H]^+$, 476.1570; found, 476.1535.

Synthesis of **29**. **28** and DIEA (4 mL, 17.3 mmol) were dissolved in 100 mL of absolute CH_2Cl_2 (100 mL) and the solution was stirred at ambient temperature for 5 min. BF_3•OEt_2 (2 mL, 15.8 mmol) was added, and stirring was

continued for 10 min. The reaction mixture was washed with 1 N HCl aq. (3 x 80 mL) and brine (1 x 100 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated. The crude compound was purified by silica gel column chromatography (silica gel 60, Kanto Chemical) to afford **29** as an orange powder (140 mg, yield 51%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.84 (s, 6H); 2.73 (s, 6H); 3.82 (s, 3H); 4.75 (s, 2H); 6.55 (s, 1H); 8.01 (s, 1H); 11.10 (br, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 13.9, 52.8, 65.1, 102.3, 106.7, 113.3, 115.0, 126.4, 128.6, 131.7, 139.6, 148.8, 158.5, 160.2, 161.1, 166.9. HRMS (ESI^-): calcd for $[M-H]^-$, 522.1397; found, 522.1342.

Synthesis of NiSPY-3 N. **29** was dissolved in 20 mL of THF, then 1 N NaOH was added, and the mixture was stirred at 0 °C for 3 h. The reaction mixture was neutralized with 1 N HCl, and extracted with CH_2Cl_2 (3 x 100 mL). The combined organic extracts were dried over Na_2SO_4, filtered, and evaporated. The crude compound was purified by silica gel column chromatography (silica gel 60, Kanto Chemical) to afford NiSPY-3 N as an orange-red solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.83 (s, 6H); 2.73 (s, 6H); 4.79 (s, 2H); 6.61 (s, 1H); 8.02 (s, 1H). HRMS (ESI^-): calcd for $[M-H]^-$, 508.1240; found, 508.1205.

References

(1) Miura, T.; Urano, Y.; Tanaka, K.; Nagano, T.; Ohkubo, K.; Fukuzumi, S. *J. Am. Chem. Soc.* **2003**, *125*, 8666-8671.

(2) Rehm, D.; Weller,; *Isr. J. Chem.* **1970**, *8*, 259-271.

(3) Mendelson, W. L.; Homes, M; Dougherty, U. *Synth.* **1996**, *26*, 593-601