Supporting Information

for

Detection of Enzymatically Generated Hydrogen Peroxide by Metal-Based Fluorescent Probe

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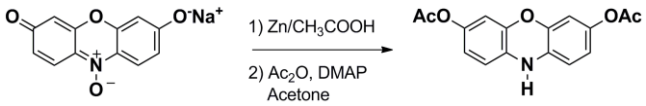
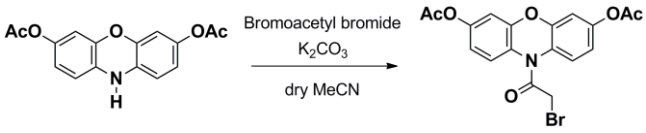
General: The reagents and the solvents used in this study, except the ligand and the iron complex, were commercial products of the highest available purity and were further purified by the standard methods, if necessary. FT-IR spectra were recorded on a Shimazu FTIR-8400 spectrometer. ^{1}H-NMR spectra were recorded on a JMN-A 500 spectrometer. Mass spectra were recorded on a JEOL JMS-DX 300 Tandem MS-station mass spectrometer. Electrospray ionization mass spectroscopy (ESI-MS) was performed on a JEOL JMS-T100CS spectrometer. Elemental analyses were recorded with a Perkin-Elmer Elemental Analyzer (2400 Series II). Catalase (# 038K7005) and horseradish peroxidase (# 058K7410) were purchased from Aldrich, and glucose oxidase (# 041-00595) and *β*-D-glucose (# 074-02401) were purchased from Wako Pure Chemicals. Resorufin was purchased from Tokyo Chemical Industry (# R0012).

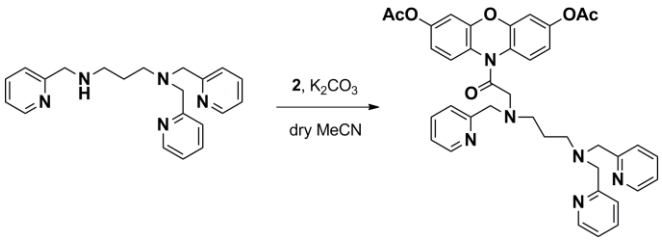
Synthesis.



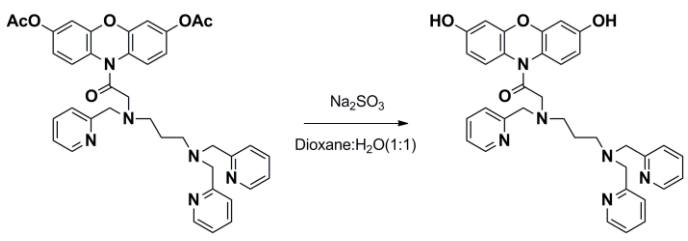
3,7-diacetoxy-10*H*-phenoxazine (1): A solution of resazurin sodium salt (1.50 g, 5.97 mmol), zinc powder (1.8 g, 29.6 mmol), and glacial acetic acid (45 mL) were stirred vigorously at room temperature for 2 h under an atmosphere of N_2. After evaporation of the solvent under N_2 atmosphere, the resulting brown material was dissolved in acetone (25 mL). To the solution was *N*,*N*-dimethyl-4-aminopyridine (0.73 g, 5.97 mmol) added, and then, acetic anhydride (1.1 mL, 11.9 mmol) was added dropwise to the stirring solution. The reaction was monitored by TLC. Evaporation of the solvents under N_2 atmosphere gave a yellow material, from which desired product was isolated as a pale yellow powder by silica-gel column chromatography (eluent: hexane:AcOEt = 5:1) in 62% yield (1.1 g); ^{1}H NMR ($CDCl_3$, 500 MHz): δ (ppm) = 2.25 (6H, s, -COC*H*$_3$), 5.26 (1 H, s, N*H*), 6.28 (2 H, d, *J* = 8.5 Hz, Ar_{H-1}), 6.41 (2 H, d, *J* = 2.3 Hz, Ar_{H-4}), 6.47 (2 H, dd, *J* = 8.3, 2.6 Hz, Ar_{H-2}); ^{13}C NMR ($CDCl_3$, 125 MHz): δ (ppm) = 22.3 (2 C, $OCOCH_3$), 110.4 (2 C, Ar), 113.2 (2 C, Ar), 116.5 (2 C, Ar), 129.4 (2 C, Ar), 143.1 (2 C, Ar), 144.9 (2 C, Ar), 169.9 ($OCOCH_3$); FT-IR (KBr) 1755 cm^{-1} (C=O), 3379 cm^{-1} (NH); MS (FAB, pos) *m/z* = 300.1, calcd for $C_{16}H_{14}NO_5$ = 300.3.



10-*N*-bromoacetyl-3,7-diacetoxyphenoxazine: To an acetonitrile solution (40 ml) containing **1** (1.0 g, 3.34 mmol) and Na_2CO_3 (0.425 g, 4.07 mmol) was added bromoacetyl bromide (318 μL, 3.68 mmol) at 0 °C under an atmosphere of N_2. The reaction mixture was stirred for 2 h at room temperature. After removal of Na_2CO_3 by Celite filtration, the filtrate was concentrated to give the product as a greenish powder in 94% yield (1.3 g); ^{1}H NMR ($CDCl_3$, 500 MHz): δ (ppm) = 2.31 (6H, -COC*H*$_3$), 4.12 (2 H, s, -C*H*$_2$Br), 6.93 (2 H, d, *J* = 8.6Hz, Ar_{H-1}), 6.95 (2 H, d, *J* = 2.3 Hz, Ar_{H-4}), 7.62 (2 H, d, *J* = 8.6 Hz, Ar_{H-2}); ^{13}C NMR ($CDCl_3$, 125 MHz): δ (ppm) = 21.2 (-CH_2Br), 26.7 (2 C, -$COCH_3$), 111.2 (2 C, Ar), 117.2 (2 C, Ar), 124.5 (2 C, Ar), 126.3 (2 C, Ar), 149.5 (2 C, Ar), 151.8 (2 C, Ar), 165.9 (-$COCH_2$Br), 169.2 (-$COCH_3$); FT-IR (KBr) 1676.0 cm^{-1} (C=O), 1762.8 cm^{-1} (C=O).



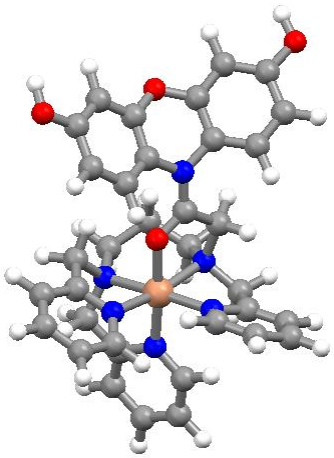
***N*-(2-(*N*'-3,7-diacetoxy-10-*N*'-phenoxazinylacetoamide))-*N*,*N*'',*N*''-tris(2-pyridylmethyl)-1,3-diaminopropane :** To an acetonitrile solution (25 mL) containing *N,N,N'*-tris(2-pyridyl-methyl)propane-1,3-diamine[1] (226 mg, 0.65 mmol) and K_2CO_3 (99 mg, 0.94 mmol) was added 10-*N*-bromoacetyl-3,7-diacetoxyphenoxazine (302 mg, 0.72 mmol), and the mixture was stirred for 24 h under an atmosphere of N_2. After removal of K_2CO_3 by Celite filtration, the filtrate was concentrated to give the product as a yellow solid in 71% yield (349 mg); ^{1}H NMR ($CDCl_3$, 500 MHz): δ (ppm) = 1.68 (2 H, m, *J* = 8.0, 7.5, 6.9 Hz, -CH_2-C*H*$_2$-CH_2-), 2.52 (2 H, t, *J* = 7.4, 6.9 Hz, -C*H*$_2$-CH_2-CH_2-), 2.69 (2 H, t, *J* = 7.1 Hz, -CH_2-CH_2-C*H*$_2$-), 3.61 (2 H, s, -C*H*$_2$-Py'), 3.75 (4 H, s, -C*H*$_2$-Py), 3.86 (2 H, s, -C*H*$_2$CO-), 6.81 (2 H, d, *J* = 8.6 Hz, Ar_{H-4}), 6.88 (2 H, d, *J* = 2.3 Hz, Ar_{H-2}), 7.09 (2H, dd, *J* = 8.0 Hz, Ar_{H-1}), 7.42(8 H, m, $Py_{H-3,5}$, $Py'_{H-3,5}$), 7.54 (1 H, ddd, *J* = 7.4, 5.7 Hz, Py'_{H-4}), 7.56 (2 H, ddd, *J* = 9.1, 6.3 Hz, Py_{H-4}), 8.47 (3 H, dd, *J* = 5.7, 5.1 Hz, Py_{H-6}, Py'_{H-6}); ^{13}C NMR ($CDCl_3$, 125 MHz): δ (ppm) = 21.1 (2 C, OCH_3), 25.3 (2 C, $CH_2CH_2CH_2$), 52.4 (4 C, $CH_2CH_2CH_2$), 55.4 (2 C, NCH_2CO), 59.7 (2 C, CH_2Py'), 60.4 (4 C, CH_2Py), 110.9 (2 C, Ar), 116.8 (2 C, Ar), 122.0 (2 C, Ar), 123.0 (3 C, Py, Py'), 125.2 (2 C, Ar), 126.6 (2 C, Ar), 126.5 (3 C, Py, Py'), 136.5 (2 C, Ar), 149.0 (3 C, Py, Py'), 151.0 (3 C, Py, Py'), 159.8 (3 C, Py, Py'), 169.0 (CO), 169.5 (CO); MS (ESI, pos) *m/z* = 687.3, calcd for $C_{39}H_{39}N_6O_6$ = 687.8.



***N*-(2-(*N*'-3,7-dihydroxy-10-*N*'-phenoxazinylacetoamide))-*N*,*N*'',*N*''-tris(2-pyridylmethyl)-1,3-diaminopropane (2):** A solution of *N*-(2-(*N*'-3,7-diacetoxy-10-*N*'-phenoxazinylacetoamide))-*N*,*N*'',*N*''-tris(2-pyridylmethyl)-1,3-diaminopropane (300 mg, 0.44 mmol), sodium sulfite (111 mg, 0.88 mmol) in 1,4-dioxane/distilled water (1/1) (16 mL) was stirred at 60°C for 24 h under an atmosphere of N_2. After cooling to room temperature, acetic acid was added (0.5 ml) and the mixture was stirred for 30 min. After evaporation of the solvents, the resulting solid residue was dissolved in methanol (10 mL) and the precipitate was filtered off. Evaporation of the filtrate afforded **2** as a red solid in 82% yield (217 mg); ^{1}H NMR (CD_3OD, 500 MHz): δ (ppm) = 1.61 (2 H, m, *J* = 6.9 Hz, -CH_2-C*H*$_2$-CH_2-), 2.50 (2 H, t, *J* = 7.4, 6.3 Hz, -C*H*$_2$-CH_2-CH_2-), 2.59 (2 H, t, *J* = 6.9 Hz, -CH_2-CH_2-C*H*$_2$-), 3.64 (2 H, s, -C*H*$_2$Py'), 8.39 (2 H, d, *J* = 5.2 Hz, $Py_{H\text{-}6}$), 3.73 (4 H, s, -C*H*$_2$Py), 3.81 (2 H, s, -C*H*$_2$CO-), 6.51 (2 H, d, *J* = 2.9 Hz, $Ar_{H\text{-}2}$), 6.53 (2 H, d, *J* = 2.3 Hz, $Ar_{H\text{-}3}$), 7.24 (8 H, m, $Py_{H\text{-}3,5}$, $Py'_{H\text{-}3,5}$), 7.47 (2H, d, *J* = 8.0 Hz, $Ar_{H\text{-}1}$), 7.67 (1 H, ddd, *J* = 7.4, 5.7 Hz, $Py'_{H\text{-}4}$), 7.72 (2 H, ddd, *J* = 9.1, 6.3 Hz, $Py_{H\text{-}4}$), 8.35 (1 H, d, *J* = 5.1 Hz, $Py'_{H\text{-}4}$); ^{13}C NMR (CD_3OD, 125 MHz): δ (ppm) = 22.6 (6 C, $CH_2CH_2CH_2$), 53.5 (2 C, CH_2Py'), 59.8 (4 C, CH_2Py), 68.1 (2 C, CH_2CO), 104.5 (2 C, Ar), 111.2 (2 C, Ar), 122.9 (3 C, Py, Py'), 123.8 (2 C, Ar), 124.9 (2 C, Ar), 126.5 (3 C, Py, Py'), 138.7 (2 C, Ar), 149.5 (2 C, Ar), 153.2 (3 C, Py, Py'), 158.0 (3 C, Py, Py'), 160.1 (3 C, Py, Py'), 178.0 (CO); MS (ESI, pos) *m/z* = 603.3, calcd for $C_{35}H_{35}N_6O_4$ = 603.7.

Synthesis of Iron Complexes.

[Fe^{III}(2)](OTf)$_3$ (MBFh1). Ligand **2** (60 mg, 0.1 mmol) was treated with $Fe(OTf)_2{\cdot}2CH_3CN$ (46 mg, 0.1 mmol) in methanol/acetonitrile (v/v = 1:4, 2 mL) for 1 h at room temperature. Addition of ether (2 mL) to the solution gave brown powder after standing overnight. The supernatant was removed by decantation, and the remained brown solid was washed with ether and dried *in vacuo* to give MBFh1 as a brown solid in 35 % yield (38 mg); MS (ESI, pos) *m/z* 329.1 $\{[Fe^{III}(\mathbf{2}) + e^-]\}^{2+}$; Anal. Calcd for $[Fe^{III}(\mathbf{2})](OTf)_3{\cdot}H_2O{\cdot}2CH_3CN$ ($C_{42}H_{42}F_9FeN_8O_{14}S_3$): C, 40.37; H, 3.97; N, 6.89. Found: C, 40.25; H, 3.76; N, 6.66. The observed MS signals at 329.1 correspond to Fe(II) species $[Fe^{II}(\mathbf{2})]^{2+}$, although the elementary analyses indicates that MBFh1 has an iron(III) center. The ferric oxidation states are also supported by the absorption spectra indicated in Figure S1. Iron(II) complexes supported by polypyridyl ligands show iron(II)-to-pyridyl charge transfer bands at around 400 nm, but MBFh1 does not show such bands assignable to the MLCT bands.[2] It has been reported that iron(III) and copper(II) complexes are often reduced to the corresponding iron(II) and copper(I) complexes under ESI-MS conditions.[3] Taken together, we judged that MBFh1 has an iron(III) center.



Scheme S1. Computationally optimized structure of MBFh1. The geometry was fully optimized at the BP level of theory with TZV (Fe) and SV (rest) basis sets using by using the ORCA 2.8 program.[4]

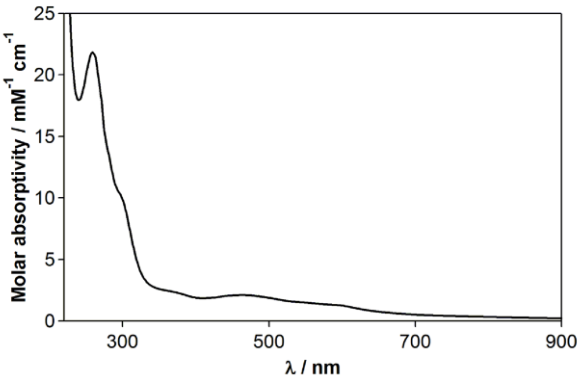


Figure S1. UV-vis spectrum of MBFh1 in MeCN.

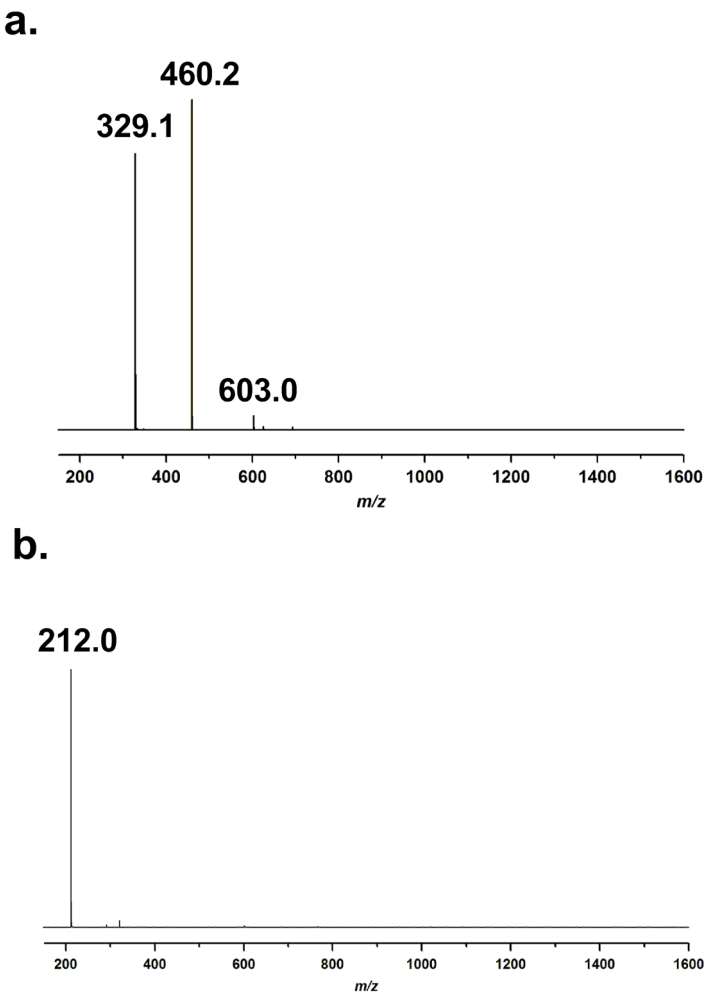


Figure S2. ESI-MS spectra of the reaction mixture. (a) positive mode. *m*/*z* 329.1 $\{[Fe^{III}(\mathbf{2})]^{3+} + e^{-}\}^{2+}$, *m*/*z* 460.2 $\{[Fe^{III}(\mathbf{3})]^{2+} + e^{-}\}^{+}$, and *m*/*z* 603.0 unknown product. (b) negative mode. *m*/*z* 212.0 $\{\text{resorufin} - H^{+}\}^{-}$.

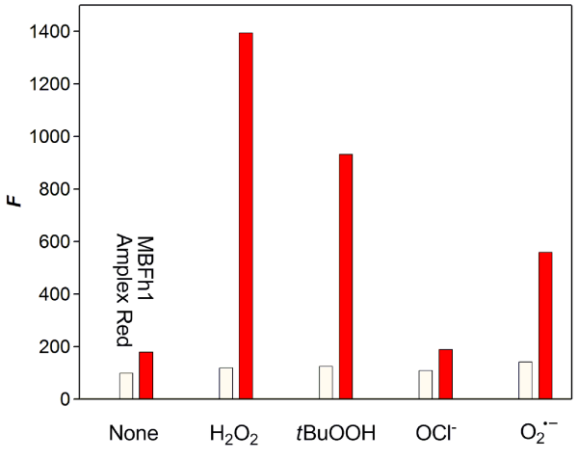


Figure S3. Reactivity of MBFh1 and AmplexRed against oxidants. Fluorescence intensities were measured in 20 mM MOPS (pH 7.2, 2.5% DMSO) at 25 °C. [MBFh1] = 5 μM. [AmplexRed] = 5 μM. Collected emission was 590 nm (Excitation at 570 nm). OCl^-: NaOCl (50 μM) was added and the mixture was stirred at 25°C for 500 sec. $O_2^{\bullet-}$: KO_2 (50 μM) was added and the mixture (containing catalase) was stirred at 25 °C for 500 sec. H_2O_2: H_2O_2 (50 μM) was added and the mixture was stirred at 25 °C for 500 sec. *t*BuOOH: *t*BuOOH (50 μM) was added and the mixture was stirred at 25°C for 500 sec.

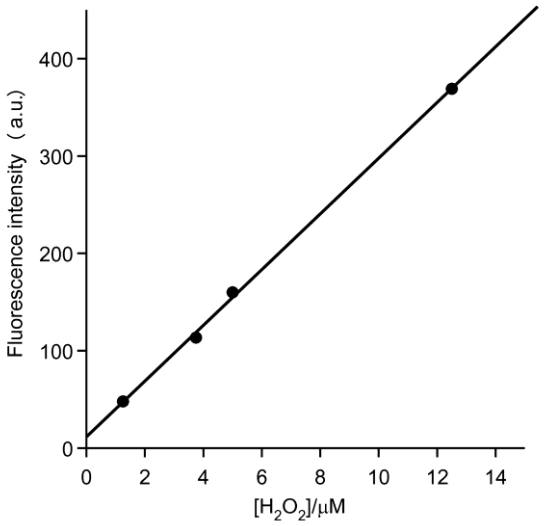


Figure S4. Calibration plot to evaluate the limit of detection.

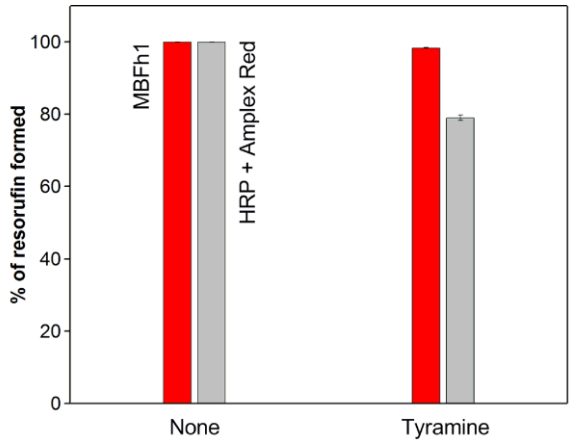


Figure S5. The inhibitory effect of tyramine on the fluorometirc detection of enzymatically generated H_2O_2 by using the MBFh1 and HRP/Amplex Red methods. Red bars: An aliquot of glucose oxidase (8 μg/mL) was added to a 5 μM solution of MBFh1 containing D-glucose (2 to 125 μM) and tyramine (none or 100 μM) in 20 mM MOPS (pH 7.2, 2.5% DMSO) at 25°C. Gray bars: A 5 μM solution of Amplex Red containing HRP (22 nM) was used instead of the solution of BFh1.

References

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2 Hitomi, Y.; Furukawa, S.; Higuchi, M.; Shishido, T.; Tanaka, T. *J. Mol. Cat. A: Chem.* **2008**, *288*, 83–86 and references therein.

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