

## Supporting information

### A new class of high-contrast Fe(II) selective fluorescent probes based on spirocyclized scaffolds for visualization of transferrin-delivered intracellular Fe(II) ion.

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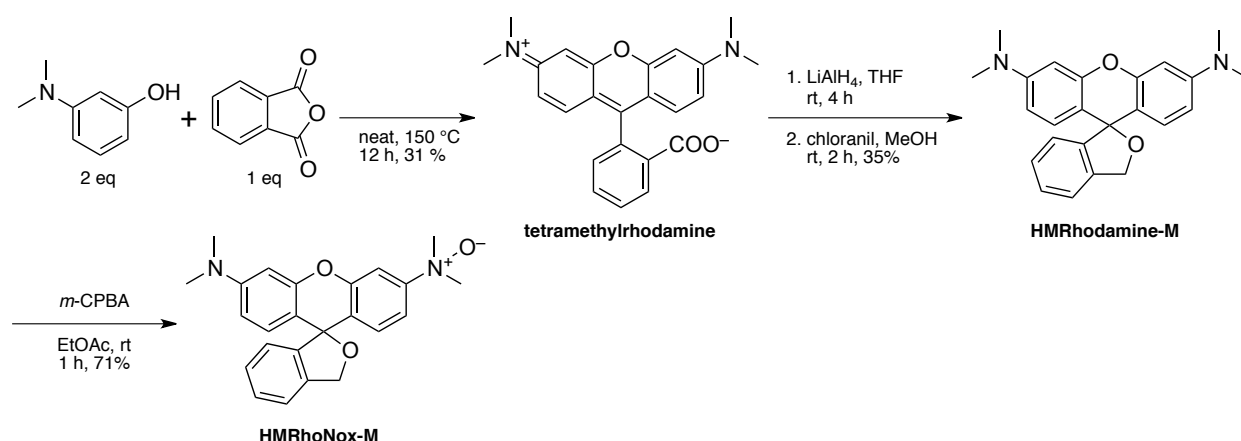
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**General:** All chemicals used in this study were commercial products of the highest available purity and were further purified by the standard methods, if necessary.  $^1\text{H-NMR}$  spectra were obtained on a JEOL ECA-500 spectrometer at 500 MHz and JEOL JNM AL-400 spectrometer at 400 MHz.  $^{13}\text{C-NMR}$  spectra were obtained on a JEOL ECA-500 spectrometer at 125 MHz and JEOL AL-400 spectrometer at 100 MHz. Chemical shifts of  $^1\text{H-NMR}$  are referenced to tetramethylsilane (TMS). Chemical shifts of  $^{13}\text{C-NMR}$  are referenced to  $\text{CDCl}_3$  (77.0) or  $\text{CD}_3\text{OD}$  (49.0). Chemical shifts and coupling constants were recorded in units of ppm and Hz, respectively. ESI-mass spectra were measured on a JEOL JMS-T100TD mass spectrometer. High-resolution mass spectra (HRMS) were measured on a JEOL JMS-T100TD by using polyethyleneglycol (PEG) as an internal standard. Anhydrous organic solvents were prepared by distillation with appropriate drying reagents before every use. Reactions were monitored by silica gel TLC (Merck Silica gel 60 PF<sub>254</sub>) with visualization of components by UV light (254 nm) or with visual observation of the dye spots. Products were purified on a silica gel column chromatography (Taiko-shoji AP-300S) or alumina column chromatography (Wako Pure Chemicals, activated, basic (pH  $\approx$  9), 200 mesh).

### 1. Synthesis of HMRhoNox-M



**Scheme 1.** Synthetic route of HMRhoNox-M

#### Tetramethylrhodamine<sup>1</sup>

A mixture of 3-dimethylaminophenol (100 mg, 0.73 mmol) and phthalic anhydride (55 mg, 0.37 mmol) was heated at 150 °C for 12 h. After cooling to room temperature, the residue was purified by silica gel column chromatography ( $\text{CHCl}_3$  : MeOH = 10 : 1 to 5 : 1) to afford the desired compound as a purple solid (45 mg, 31%).  $^1\text{H-NMR}$  (500 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  : 7.99 (dd,  $J$  = 7.5 Hz, 1.2 Hz, 1H), 7.56–7.47 (m, 2H), 7.11 (m, 3H), 6.79 (dd,  $J$  = 9.7 Hz, 2.4 Hz, 2H), 6.65 (d,  $J$  = 2.4 Hz, 2H), 3.07 (s, 12H).

#### HMRhodamine-M<sup>1,2</sup>

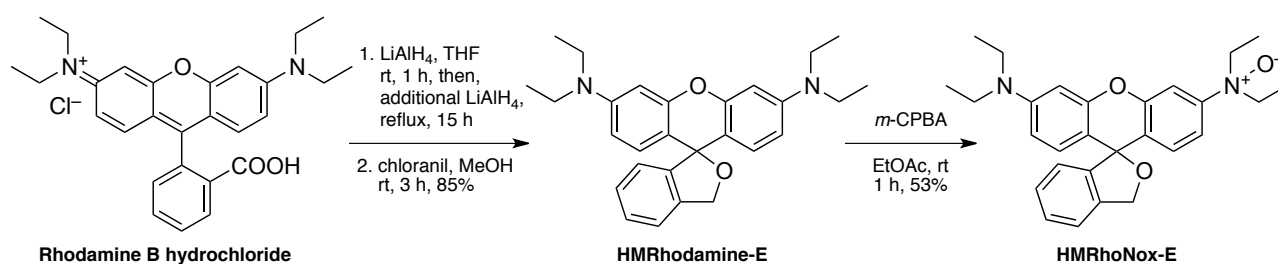
To a solution of tetramethylrhodamine (1.00 g, 2.60 mmol) in dry THF (120 mL) was slowly added  $\text{LiAlH}_4$  (296 mg, 7.8 mmol) at 0 °C. After stirring at room temperature for 30 min, an additional portion of  $\text{LiAlH}_4$  (296 mg, 7.8 mmol) at 0 °C was added. This process was repeated additional 3 times, and total 1.48 g (39 mmol) of  $\text{LiAlH}_4$  was added. Then, after stirring at room temperature for 4 h, the reaction was quenched by a sequential addition of

H<sub>2</sub>O (1.5 mL), 1 M aq. NaOH (1.5 mL), and H<sub>2</sub>O (4.5 mL), and then the mixture was stirred for 1 h. The resulting mixture was filtered with a pad of celite to remove insoluble materials. After evaporation of the filtrate, the residue was re-dissolved in CH<sub>2</sub>Cl<sub>2</sub> (300 mL) and washed with brine (200 mL). The organic phase was dried over anhydrous magnesium sulfate and then evaporated to afford purple solid (976 mg). The residue was dissolved in MeOH (200 mL), and chloranil (3.93 g, 16.0 mmol) was added. The mixture was stirred at room temperature for 2 h. Insoluble materials were removed by filtration with a pad of celite, and then the filtrate was evaporated. The residue was purified by silica gel column chromatography twice (1st, CHCl<sub>3</sub> : MeOH = 20 : 1 to 10 : 1, and 2nd, EtOAc : hexane = 1 : 2 to 1 : 1) to afford the desired compound as a purple powder (336 mg, 35%). <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>) δ : 7.34 (d, *J* = 3.9 Hz, 2H), 7.28–7.23 (m, 1H), 6.93 (d, *J* = 7.7 Hz, 1H), 6.77 (d, *J* = 8.7 Hz, 2H), 6.48 (d, *J* = 2.4 Hz, 2H), 6.42 (dd, *J* = 8.7 Hz, 2.4 Hz, 2H), 5.24 (s, 2H), 2.95 (s, 12H).

### HMRhoNox-M

To a solution of HMRhodamine-M (90 mg, 0.24 mmol) in EtOAc (18 mL) was slowly added *m*-chloroperoxybenzoic acid (83 mg, 0.48 mmol) at 0 °C. The mixture was warmed to room temperature and stirred for 1 h. The reaction mixture was evaporated, and then the residue was purified by alumina column chromatography (CHCl<sub>3</sub> : MeOH = 50 : 1 to 20 : 1) to afford HMRhoNox-M as colorless powder (64 mg, 71%). <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>) δ : 7.93 (d, *J* = 2.3 Hz, 1H), 7.45 (dd, *J* = 8.6 Hz, 2.3 Hz, 1H), 7.39–7.38 (m, 2H), 7.29–7.26 (m, 1H), 7.05 (d, *J* = 8.6 Hz, 1H), 6.91 (d, *J* = 7.4 Hz, 1H), 6.81 (d, *J* = 8.6 Hz, 1H), 6.48–6.46 (m, 2H), 5.30 (dd, *J* = 20.0 Hz, 12.6 Hz, 2H), 3.58 (m, 6H), 2.98 (s, 6H); <sup>13</sup>C-NMR (125 MHz, CDCl<sub>3</sub>) δ : 154.5, 151.5, 151.3, 150.9, 144.5, 139.1, 129.9, 129.2, 128.4, 128.2, 128.2, 125.7, 123.8, 120.7, 114.2, 111.7, 109.2, 109.0, 98.5, 83.3, 72.1, 63.2, 63.1, 40.3 ; HRMS (ESI+): *m/z* calculated for C<sub>24</sub>H<sub>25</sub>N<sub>2</sub>O<sub>3</sub><sup>+</sup> : 389.1860, found 389.1871.

### 1. Synthesis of HMRhoNox-E



**Scheme 2.** Synthetic route of **HMRhoNox-E**

### HMRhodamine-E<sup>3</sup>

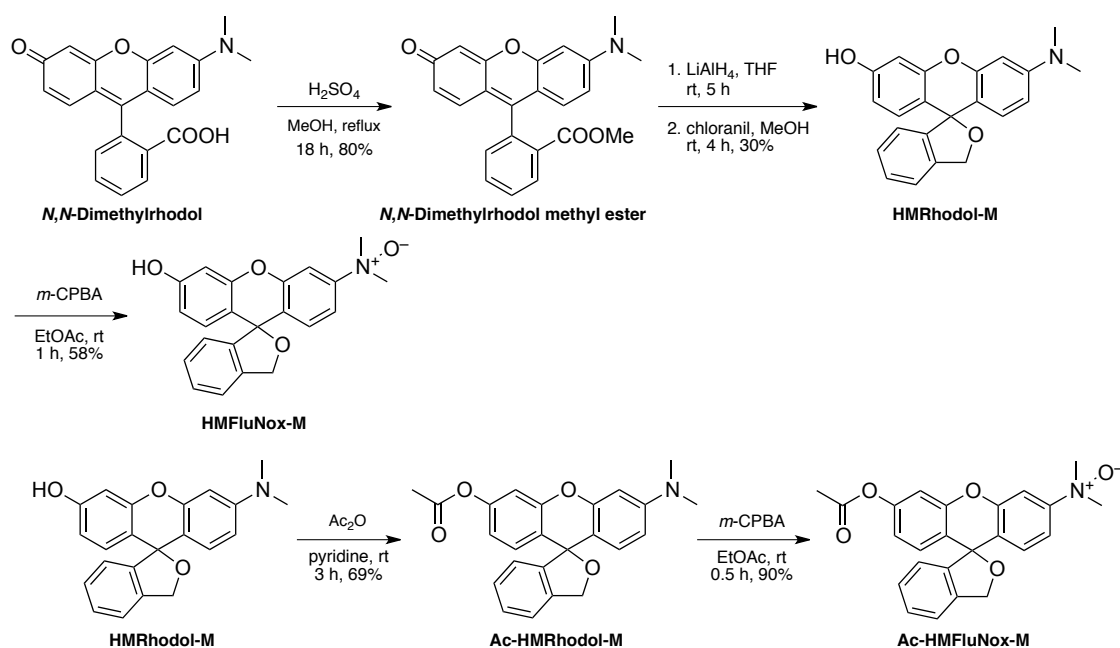
To a solution of rhodamine B hydrochloride (1.00 g, 2.09 mmol) in dry THF (50 mL) was slowly added LiAlH<sub>4</sub> (317 mg, 8.35 mmol) at 0 °C. The mixture was warmed to room temperature and then stirred for 1 h under N<sub>2</sub> atmosphere. Additional portion of LiAlH<sub>4</sub> (634 mg, 16.7 mmol) was added to the reaction mixture at 0 °C, and then refluxed for 15 h. The reaction was quenched by sequential addition of H<sub>2</sub>O (950 μL), 25 % aq. NaOH (950 μL), and H<sub>2</sub>O (2.85 mL), and the mixture was stirred for 1 h. The resulting mixture was filtered with a pad of celite to remove insoluble materials. After evaporation of the filtrate, the residue was re-dissolved in CH<sub>2</sub>Cl<sub>2</sub> (50 mL) and washed with water (40 mL), brine (40 mL), dried over anhydrous magnesium sulfate, and evaporated to

afford purple solid (848 mg). Then, this residue was dissolved in MeOH (50 mL), and chloranil (1.54 g, 6.27 mmol) was added. The mixture was stirred at room temperature for 3 h, and then filtered with a pad of celite to remove insoluble materials. After evaporation of the filtrate, the residue was purified by silica gel column chromatography (EtOAc : hexane = 1 : 1, then CHCl<sub>3</sub> : MeOH = 10 : 1) to afford the desired compound as a black purple solid (947 mg). Then, the residue was dissolved in 1 M aq. NaOH (100 mL), extracted with EtOAc (50 mL, three times), dried over anhydrous magnesium sulfate, and evaporated to afford desired compound as a purple powder. (764 mg, 85%). <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>) δ: 7.33 (d, *J* = 5.7 Hz, 1H), 7.25–7.24 (m, 2H), 6.97 (d, *J* = 7.5 Hz, 1H), 6.71 (d, *J* = 8.6 Hz, 2H), 6.40 (d, *J* = 2.9 Hz, 2H), 6.34 (dd, *J* = 8.6, 2.9 Hz, 2H), 5.20 (s, 2H), 3.33 (q, *J* = 6.9 Hz, 8H), 1.15 (t, *J* = 6.9 Hz, 12H).

### HMRhoNox-E

To a solution of HMRhodamine-E (267 mg, 0.62 mmol) in EtOAc (20 mL) was slowly added *m*-chloroperoxybenzoic acid (214 mg, 1.24 mmol) at 0 °C. The mixture was warmed to room temperature and stirred for 1 h, then, evaporated and the residue was purified by silica gel column chromatography (CHCl<sub>3</sub> : MeOH = 20 : 1 to 10 : 1) to afford HMRhoNox-E as a pale purple solid (145 mg, 53%). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ: 7.78 (d, *J* = 1.9 Hz, 1H), 7.38–7.37 (m, 2H), 7.29–7.25 (m, 2H), 7.03 (d, *J* = 8.7 Hz, 1H), 6.92 (d, *J* = 7.7 Hz, 1H), 6.77 (d, *J* = 8.7 Hz, 1H), 6.43 (d, *J* = 2.4 Hz, 1H), 6.41 (dd, *J* = 8.7 Hz, 2.7 Hz, 1H), 5.29 (dd, *J* = 16.2 Hz, 12.3 Hz, 2H), 3.73–3.65 (m, 4H), 3.38 (q, *J* = 7.2 Hz, 4H), 1.18–1.13 (m, 12H); <sup>13</sup>C-NMR (125 MHz, CDCl<sub>3</sub>) δ : 151.7, 151.0, 149.0, 148.8, 144.4, 139.2, 129.6, 129.4, 128.3, 128.1, 125.5, 123.8, 120.6, 115.5, 111.1, 110.7, 108.5, 97.5, 83.3, 71.9, 66.9, 44.4, 12.5, 8.3; HRMS (ESI<sup>+</sup>): *m/z* calculated for C<sub>28</sub>H<sub>33</sub>N<sub>2</sub>O<sub>3</sub><sup>+</sup>: 445.2486, found 445.2501.

### 3. Synthesis of HMFluNox-M and Ac-HMFluNox-M



Scheme 3. Synthetic route of HMFluNox-M and Ac-HMFluNox-M

### ***N,N*-Dimethylrhodol methyl ester<sup>3</sup>**

To a solution of *N,N*-dimethylrhodol<sup>4</sup> (602 mg, 1.32 mmol) in MeOH (30 mL) was added conc. H<sub>2</sub>SO<sub>4</sub> (1.5 mL). The mixture was refluxed for 18 h, then neutralized with sodium bicarbonate. Insoluble materials were removed by filtration with a pad of celite, and then the filtrate was evaporated. The residue was purified by silica gel column chromatography (CHCl<sub>3</sub> : MeOH= 20 : 1) to afford red solid (393 mg, 80%). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) δ : 8.21 (dd, *J* = 7.7 Hz, 1.0 Hz, 1H), 7.71 (td, *J* = 7.5 Hz, 1.4 Hz, 1H), 7.65 (td, *J* = 7.5 Hz, 1.4 Hz, 1H), 7.31 (dd, *J* = 7.5 Hz, 1.2 Hz, 1H), 6.83–6.80 (m, 2H), 6.63 (d, *J* = 2.4 Hz, 1H), 6.55–6.50 (m, 2H), 6.45 (d, *J* = 1.9 Hz, 1H), 3.62, (s, 3H), 3.12 (s, 6H).

### **HMRhodol-M<sup>3</sup>**

To a solution of *N,N*-dimethylrhodol methyl ester (393 mg, 1.05 mmol) in dry THF (30 mL) was slowly added LiAlH<sub>4</sub> (80 mg, 2.10 mmol) at 0 °C. The mixture was warmed to room temperature and then stirred for 2.5 h under N<sub>2</sub> atmosphere. Then, LiAlH<sub>4</sub> (80 mg, 2.10 mmol) was added slowly at 0 °C. After stirring at room temperature for 2.5 h under N<sub>2</sub> atmosphere, the reaction was quenched by sequential addition of H<sub>2</sub>O (160 μL), 15% aq. NaOH (160 μL), and H<sub>2</sub>O (480 μL), and the mixture was stirred for 2 h. The resulting mixture was filtered with a pad of celite to remove insoluble materials. After evaporation of the filtrate, the residue was re-dissolved in CH<sub>2</sub>Cl<sub>2</sub> (50 mL). The mixture was washed with brine (50 mL), and the organic phase was dried over anhydrous magnesium sulfate and then evaporated to afford purple solid (336 mg). The residue was dissolved in MeOH (30 mL), and chloranil (716 mg, 2.91 mmol) was added. The mixture was stirred at room temperature for 4 h. Insoluble materials were removed by filtration with a pad of celite, and then the filtrate was evaporated. The residue was purified by silica gel column chromatography (EtOAc : hexane = 1 : 2 to 1 : 1) to afford the title compound as a red solid (98 mg, 30%). <sup>1</sup>H-NMR (400 MHz, CD<sub>3</sub>OD) δ : 7.74–7.68 (m, 2H), 7.59–7.58 (m, 1H), 7.33–7.30 (m, 4H), 7.17 (d, *J* = 2.4 Hz, 1H), 7.12 (d, *J* = 2.4 Hz, 1H), 7.03 (dd, *J* = 9.6 Hz, 2.0 Hz, 1H), 4.34 (s, 2H), 3.41 (s, 6H); <sup>13</sup>C-NMR (100 MHz, CD<sub>3</sub>OD) δ : 169.3, 161.3, 160.7, 160.4, 158.7, 141.2, 133.6, 133.2, 131.9, 131.6, 130.5, 129.7, 128.9, 118.5, 118.2, 117.5, 116.6, 103.4, 97.6, 63.1, 28.2; HRMS (ESI+): *m/z* calculated for C<sub>22</sub>H<sub>20</sub>NO<sub>3</sub><sup>+</sup>: 346.1438, found 346.1451.

### **HMFluNox-M**

To a solution of HMRhodol-M (34 mg, 0.098 mmol) in EtOAc (4 mL) was slowly added *m*-chloroperoxybenzoic acid (34 mg, 0.20 mmol) at 0 °C. The mixture was warmed to room temperature and stirred for 1 h. Then, an additional portion of *m*-chloroperoxybenzoic acid (34 mg, 0.20mmol) was added at 0 °C. After stirring at room temperature for 1 h, the mixture was evaporated. The residue was purified by silica gel column chromatography (CHCl<sub>3</sub> : MeOH= 10 : 1 to 8 : 1) to afford HMFluNox-M as a white powder (21 mg, 58%). <sup>1</sup>H-NMR (500 MHz, CD<sub>3</sub>OD) δ : 7.83 (d, *J* = 2.9 Hz, 1H), 7.47 (dd, *J* = 8.6 Hz, 2.3 Hz, 1H), 7.35 (d, *J* = 7.4 Hz, 1H), 7.30 (t, *J* = 7.2 Hz, 1H), 7.17 (t, *J* = 7.4 Hz, 1H), 7.03 (d, *J* = 9.2 Hz, 1H), 6.73 (d, *J* = 8.6 Hz, 1H), 6.69 (d, *J* = 7.4 Hz, 1H), 6.55 (d, *J* = 2.3 Hz, 1H), 6.46 (dd, *J* = 8.6 Hz, 2.3 Hz, 1H) 5.24 (s, 2H), 3.50 (s, 3H), 3.49 (s, 3H); <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>) δ : 159.8, 153.7, 151.5, 151.0, 144.4, 139.0, 129.9, 129.4, 128.4, 128.2, 126.1, 124.0, 120.6, 113.7, 113.4, 112.8, 110.5, 103.1, 83.3, 72.1, 63.1, 62.2; HRMS (ESI+): *m/z* calculated for C<sub>22</sub>H<sub>20</sub>NO<sub>4</sub><sup>+</sup>: 362.1387, found 362.1389.

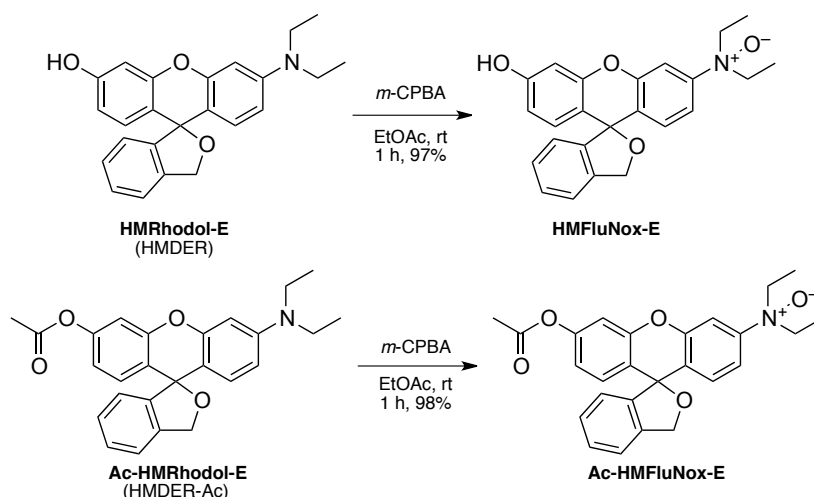
### Ac-HMRhodol-M

To a solution of HMRhodol-M (51 mg, 0.15 mmol) in pyridine (10 mL) was added acetic anhydride (312  $\mu$ L, 3.3 mmol). After stirring at room temperature for 3 h, the resulting mixture was evaporated. The residue was purified by silica gel column chromatography ( $\text{CHCl}_3$  : MeOH = 100 : 1) to afford the desired compound as a red solid (40 mg, 69%).  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  : 7.35 (m, 2H), 7.28–7.25 (m, 1H), 6.97–6.91 (m, 3H), 6.80 (d,  $J$  = 8.2 Hz, 1H), 6.74 (dd,  $J$  = 8.7 Hz, 2.4 Hz, 1H), 6.48–6.45 (m, 2H), 5.28 (s, 2H), 2.96 (s, 6H), 2.29 (s, 3H).  $^{13}\text{C-NMR}$  (125 MHz,  $\text{CDCl}_3$ )  $\delta$  : 169.1, 151.5, 151.4, 151.3, 150.7, 144.8, 139.3, 129.8, 129.3, 128.3, 128.0, 122.6, 124.1, 112.3, 120.6, 116.5, 109.6, 109.1, 98.7, 85.6, 71.9, 40.4, 21.3; HRMS (ESI<sup>+</sup>):  $m/z$  calculated for  $\text{C}_{24}\text{H}_{22}\text{NO}_4$ <sup>+</sup>: 388.1543, found 388.1552.

### Ac-HMFluNox-M

To a solution of Ac-HMRhodol-M (37 mg, 0.15 mmol) in EtOAc (3 mL), was added *m*-chloroperoxybenzoic acid (33 mg, 0.19 mmol) at 0  $^\circ\text{C}$ . After stirring at room temperature for 0.5 h, the mixture was evaporated. The residue was purified by silica gel column chromatography ( $\text{CHCl}_3$  : MeOH = 20 : 1 to 10 : 1) to afford Ac-HMFluNox-M as a white powder (35 mg, 90%).  $^1\text{H-NMR}$  (500 MHz,  $\text{CDCl}_3$ )  $\delta$  : 7.95 (d,  $J$  = 2.3 Hz, 1H), 7.52 (dd,  $J$  = 8.6 Hz, 1.7 Hz, 1H), 7.39–7.38 (m, 2H), 7.27 (m, 1H), 7.09 (d,  $J$  = 8.6 Hz, 1H), 7.00 (m, 2H), 6.87 (d,  $J$  = 8.0 Hz, 1H), 6.81 (dd,  $J$  = 8.6 Hz, 2.3 Hz, 1H) 5.35 (s, 2H), 3.61 (m, 6H), 2.30 (s, 3H);  $^{13}\text{C-NMR}$  (125 MHz,  $\text{CDCl}_3$ )  $\delta$  : 169.1, 151.2, 150.6, 150.4, 144.2, 138.5, 130.0, 129.7, 128.72, 128.69, 125.6, 123.8, 121.9, 120.9, 117.6, 114.9, 109.9, 109.2, 82.9, 72.9, 63.0, 62.9, 21.1; HRMS (ESI<sup>+</sup>):  $m/z$  calculated for  $\text{C}_{24}\text{H}_{22}\text{NO}_5$ <sup>+</sup>: 404.1493, found 404.1503.

## 4. Synthesis of Ac-HMFluNox-E and Ac-HMFluNox-E



Scheme 4. Synthetic route of HMFluNox-E and Ac-HMFluNox-E

### HMFluNox-E

To a solution of HMRhodol-E (HMDER)<sup>3</sup> (100 mg, 0.27 mmol) in EtOAc (10 mL) was slowly added *m*-chloroperoxybenzoic acid (93 mg, 0.54 mmol) at 0  $^\circ\text{C}$ . The mixture was warmed to room temperature and stirred for 1 h. Then, *m*-chloroperoxybenzoic acid (93 mg, 0.54 mmol) was added at 0  $^\circ\text{C}$ . After stirring at room

temperature for 1 h, the mixture was evaporated. The residue was purified by silica gel column chromatography (CHCl<sub>3</sub> : MeOH= 20 : 1 to 10 :1) to afford HMFluNox-E as a white powder (102 mg, 97%). <sup>1</sup>H-NMR (500 MHz, CD<sub>3</sub>OD) δ : 7.81 (d, *J* = 2.3 Hz, 1H), 7.42–7.39 (m, 3H), 7.27 (m, 1H), 7.12 (d, *J* = 9.2 Hz, 1H), 6.81 (d, *J* = 8.0 Hz, 2H), 6.65 (d, *J* = 2.3 Hz, 1H), 6.55 (dd, *J* = 8.9 Hz, 2.6 Hz, 1H), 5.33 (s, 2H), 3.88–3.84 (m, 2H), 3.66–3.62 (m, 2H), 1.11 (q, *J* = 6.7 Hz, 6H); <sup>13</sup>C-NMR (125 MHz, CD<sub>3</sub>OD) δ : 160.1, 152.4, 152.0, 149.7, 146.0, 140.0, 131.3, 131.1, 129.7, 129.6, 129.3, 124.5, 122.1, 117.2, 116.6, 113.6, 112.2, 103.0, 84.6, 73.5, 67.7, 8.6; HRMS (ESI<sup>+</sup>): *m/z* calculated for C<sub>24</sub>H<sub>24</sub>NO<sub>4</sub><sup>+</sup>: 390.1700, found 390.1695.

### Ac-HMFluNox-E

To a solution of Ac-HMRhodol-E (HMDER-Ac)<sup>3</sup> (100 mg, 0.24 mmol) in EtOAc (7 mL) was slowly added *m*-chloroperoxybenzoic acid (83 mg, 0.48 mmol) at 0 °C. After stirring at room temperature for 1 h, the mixture was evaporated. The residue was purified by silica gel column chromatography (CHCl<sub>3</sub> : MeOH= 30 : 1 to 10 :1) to afford Ac-HMFluNox-E as a colorless powder (102 mg, 98%). <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>) δ: 7.89 (d, *J* = 2.3 Hz, 1H), 7.39 (m, 2H), 7.36 (d, *J* = 8.0 Hz, 1H), 7.28 (m, 1H), 7.09 (d, *J* = 8.6 Hz, 1H), 7.03–7.01 (m, 2H), 6.89 (d, *J* = 8.0 Hz, 1H), 6.82 (dd, *J* = 8.6 Hz, 2.3 Hz, 1H), 5.37 (s, 2H), 3.74–3.65 (m, 4H), 2.30 (s, 3H), 7.17–7.14 (m, 6H); <sup>13</sup>C-NMR (125 MHz, CDCl<sub>3</sub>) δ: 169.0, 151.0, 150.5, 150.3, 144.2, 138.4, 129.6, 129.5, 129.0, 128.6, 128.5, 128.1, 123.7, 121.9, 120.8, 117.4, 116.2, 111.5, 109.9, 82.9, 72.8, 67.2, 67.1, 21.1, 8.4; HRMS (ESI<sup>+</sup>): *m/z* calculated for C<sub>26</sub>H<sub>26</sub>NO<sub>5</sub><sup>+</sup>: 432.1801, found 432.1819.

## 5. Steady-state absorption and fluorescence spectroscopy

The UV-vis absorption spectra were recorded on Agilent 8453 photodiode array spectrometer equipped with a Unisoku thermo-static cell holder (USP-203). Fluorescence spectra were recorded using a JASCO FP6600 with a slit width of 5 nm and 6 nm for excitation and emission, respectively. The path length was 1 cm with a cell volume of 3.0 mL. For determination of p*K*<sub>a1</sub> and p*K*<sub>a2</sub>, absorption spectra were measured in 200 mM sodium phosphate buffer at various pH values. The pH profiles of the absorbance at each maximal wavelength were fitted to the following equations (eq. 1) or (eq. 2)<sup>3</sup>

$$A = \frac{A_0 + A_1 \times 10^{(pH - pK_{a1})}}{1 + 10^{(pH - pK_{a1})}} \quad (\text{eq. 1})$$

$$A = \frac{A_0 + A_1 \times 10^{(pH - pK_{a1})} + A_2 \times 10^{(pH - pK_{a1} - pK_{a2})}}{1 + 10^{(pH - pK_{a1})} + 10^{(pH - pK_{a1} - pK_{a2})}} \quad (\text{eq. 2})$$

where p*K*<sub>a1</sub> and p*K*<sub>a2</sub> are the acid dissociation constants of probe; *A*<sub>0</sub> is the initial absorbance at each wavelength at pH = 1.60; and *A*<sub>1</sub> and *A*<sub>2</sub> are the maximum absorbance at each wavelength associated with the corresponding p*K*<sub>a</sub> values.

For all the fluorescence measurements, probes were used at a final concentration of 2 μM (from 1 mM stock solution in DMF) in 50 mM HEPES (3 mL, pH 7.4). For measurements with various concentrations of Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>•6H<sub>2</sub>O (from 10 mM stock solution in water) was added to 2 μM each probe solution in HEPES

buffer. After incubation at 25 °C for 1 h, fluorescence spectra were measured.

Fluorescence responses of the probes to various metal ions were measured as follows. An aqueous solution of transition metal ion species (stock solutions: 10 mM for MnSO<sub>4</sub>, CoSO<sub>4</sub>, NiSO<sub>4</sub>, FeSO<sub>4</sub>, FeCl<sub>3</sub>, CuSO<sub>4</sub>, and ZnSO<sub>4</sub>; 100 mM for NaCl, KCl, MgCl<sub>2</sub>, and CaCl<sub>2</sub>) or [Cu(MeCN)<sub>4</sub>]PF<sub>4</sub> (from 10 mM stock solution in MeCN) was added to give the final concentrations of 1 mM for Na<sup>+</sup>, K<sup>+</sup>, Mg<sup>2+</sup>, and Ca<sup>2+</sup> and 20 μM for other metal ion species. The mixtures were incubated for 1 h at room temperature, and then fluorescence spectra were measured. Selectivity for reductants and reactive oxygen species, and effect of chelator were tested under the conditions as follows.

Reductants and reactive nitrogen species

Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> : 100 μM from 100 mM stock solution in water

Sodium ascorbate : 1 mM from 100 mM stock solution in water

Cysteine : 1 mM from 100 mM stock solution in water

Glutathione : 1 mM from 100 mM stock solution in HEPES buffer (pH was adjusted to 7.4)

NaNO<sub>2</sub> : 100 μM from 100 mM stock solution in water

Reactive oxygen species

O<sub>2</sub><sup>•-</sup> : 100 μM from saturated KO<sub>2</sub> solution in DMSO (ca. 1 mM)<sup>5</sup>

H<sub>2</sub>O<sub>2</sub> : 100 μM from 100 mM stock solution in water

•OH : 200 μM H<sub>2</sub>O<sub>2</sub> and 20 μM FeSO<sub>4</sub>

NaOCl : 100 μM from 100 mM stock solution in water

NO : 100 μM NOC-5 from 10 mM stock solution in 0.1 M NaOH aq.

Chelator

2,2'-Bipyridyl : 100 μM 2,2'-bipyridyl from 100 mM in stock solution in DMSO and 20 μM

Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>•6H<sub>2</sub>O.

Probe (2 μM) was incubated under the each condition in 50 mM HEPES buffer (pH 7.4) for 1 h, and then fluorescence spectra were measured.

## **6. Product analysis by LC-MS**

To a solution of probe (50 μM) in 50 mM HEPES buffer (pH 7.4) was added a solution of Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>•6H<sub>2</sub>O (final, 200 μM). The mixture was kept for 1 h under ambient conditions. The resulting mixtures were analyzed with a LC-MS (HP 1100 LC system equipped with Agilent 1946B ESI-mass system, Waters symmetry C18 column, 3.5 μm, 4.6 × 75 mm) eluted with H<sub>2</sub>O/MeCN (80:20 to 50:50 or 90:10 to 70:30 over 20 min) containing 0.05% formic acid. The retention times were compared with those of each authentic sample in 50 mM HEPES buffer (pH 7.4).

## **7. Cell culture experiments**

Human hepatocellular carcinoma (HepG2) cells were cultured in modified essential medium (MEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco), 1% Antibiotic-Antimycotic (Gibco), and 2 mM glutamine at 37 °C in a 5% CO<sub>2</sub>/ 95% air incubator. Two days before use, cells (1.0 × 10<sup>5</sup>) were seeded on Advanced TC glass-bottomed dishes (CELLview™ Cell Culture Dish, Greiner).



## **8. Confocal fluorescence imaging experiments**

Confocal fluorescence images were acquired with a Zeiss LSM 700 laser-scanning microscope system. Experiments were performed with 20× objective lens or 40× oil-immersion objective lens. For all imaging experiments, Hank's Balanced Salt Solution (HBSS, Gibco) containing calcium and magnesium without phenol red was used. For Fe<sup>2+</sup> uptake experiments, cells were treated with 100 μM Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>•6H<sub>2</sub>O (FAS) (from 10 mM stock solution in water) in MEM without FBS at 37 °C for 30 min. For all imaging experiments, after washing the cells with HBSS (×3), a probe was added (1 μM for HMRhoNox-M and HMRhoNox-E, and 5 μM Ac-HMFluNox-M and Ac-HMFluNox-E). After incubation for 30 min at 37 °C, the cells were washed with HBSS (×1), and then imaged. For chelation experiments, 1 mM 2,2'-bipyridyl (Bpy, from 100 mM stock solution in DMSO) was added to the cells simultaneously with probe. For nuclear staining, Hoechst 33342 (1.0 μg/mL, from 0.1 mg/mL stock solution in water) was added to the cells 10 min before imaging. For co-staining experiments, cells were treated with 50 nM Lyso Tracker<sup>®</sup> Green DND-26 (lysosome, 2 h at 37 °C) or 500 nM ER-tracker<sup>™</sup> Green (endoplasmic reticulum, 30 min at 37 °C) in HBSS prior to treatment with probe in HBSS for 30 min at 37 °C. For all the control experiments, the corresponding amounts of vehicles (FAS: water, Bpy: DMSO) were added to the cells instead.

For transferrin uptake experiments, cells were incubated under the conditions as follows.

Control experiments

MEM (containing total 30% PBS) for 30 min at 37 °C

Transferrin uptake experiments:

5 μM Transferrin (holoTf, from human blood, Wako Pure Chemicals, from 100 μM stock solution in PBS) in

MEM (containing total 30% PBS) for 30 min at 37 °C

Competitive inhibition experiments:

5 μM holoTf and 25 μM apo-transferrin (apoTf, from human blood, Nacalai Tesque, from 100 μM stock solution in PBS) in MEM (containing total 30% PBS) for 30 min at 37 °C

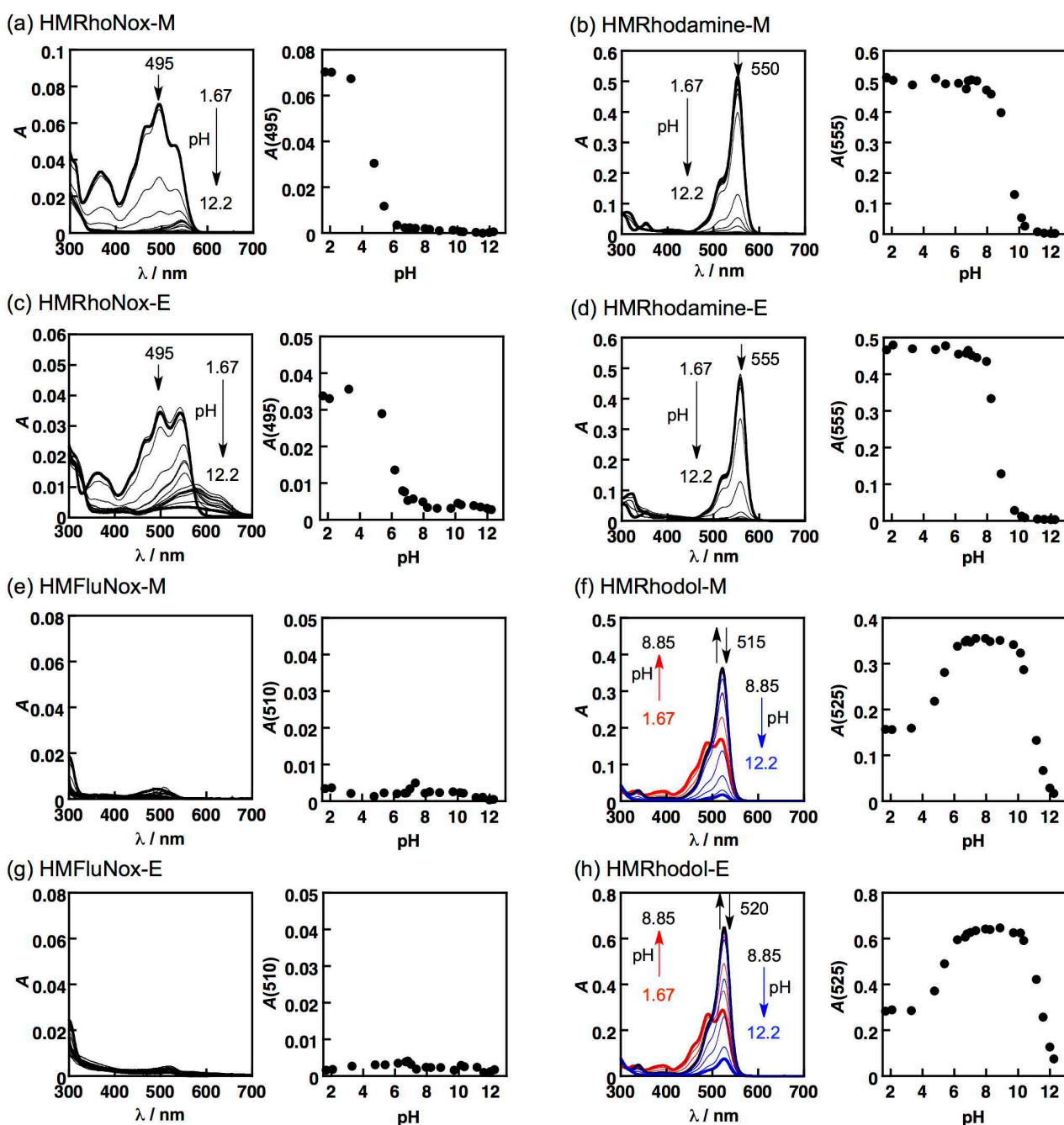
Endocytosis-inhibition experiments:

5 μM holoTf in MEM (containing total 30% PBS) for 30 min at 4 °C

5 μM holoTf and 1 mM NaN<sub>3</sub> (from 100 mM stock solution in PBS) in MEM (containing total 30% PBS) for 30 min at 37 °C.<sup>6</sup>

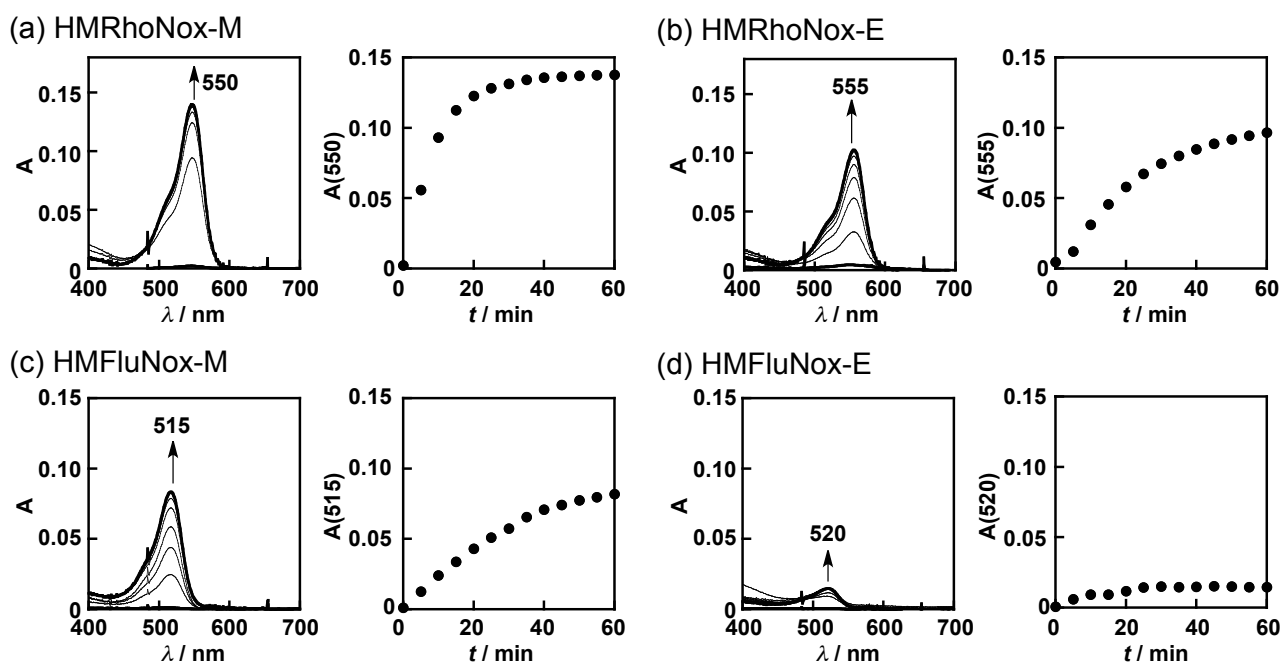
Prior to addition of the probe, the cells were washed with HBSS (×1). After incubation with 1 μM HMRhoNox-M in HBSS for 30 min at 37 °C, the cells were washed with HBSS (×1), and then imaged.

Image analysis was performed with ImageJ (1.45r).

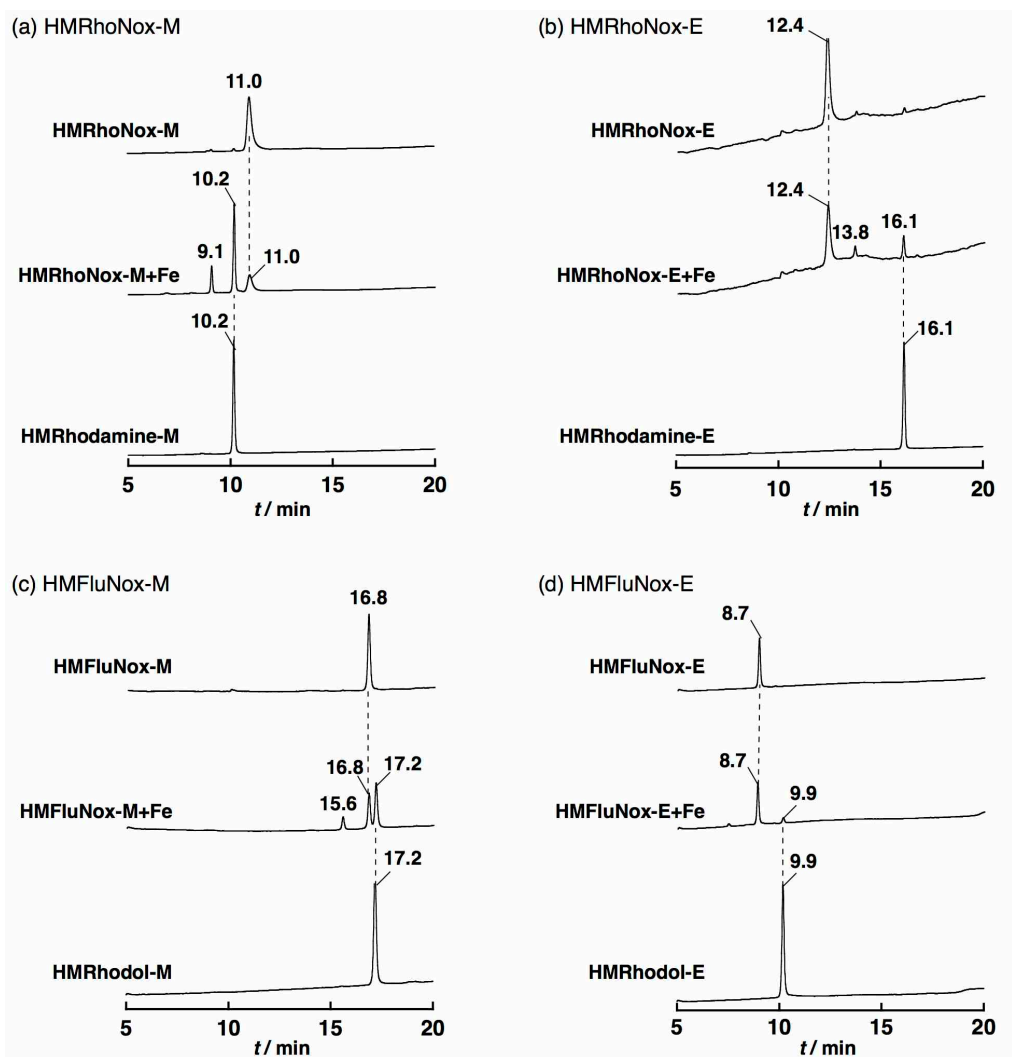


**Figure S1.** Absorbance spectra of (a) HMRhoNox-M, (b) HMRhodamine-M, (c) HMRhoNox-E, (d) HMRhodamine-E, (e) HMFluNox-M, (f) HMRhodol-M, (g) HMFluNox-E, and (h) HMRhodol-E at various pH (1.67, 2.07, 3.30, 4.75, 5.38, 6.18, 6.67, 6.80, 7.00, 7.34, 7.94, 8.24, 8.85, 10.35, 11.14, 11.56, 11.98, 12.24) (left) and plots of the absorbance at each maximal absorption wavelength against pH (right). All the data was collected 5  $\mu$ M probes or dyes and 0.5% DMF as a co-solvent.

The extinction coefficients of HMRhodamine-M, HMRhodamine-E, HMRhodol-M, and HMRhodol-E at neutral pH were calculated to be  $9.8 \times 10^4$  (at 550 nm),  $9.8 \times 10^4$  (at 555 nm),  $7.2 \times 10^4$  (at 515 nm), and  $11.4 \times 10^4$  (at 520 nm), respectively.

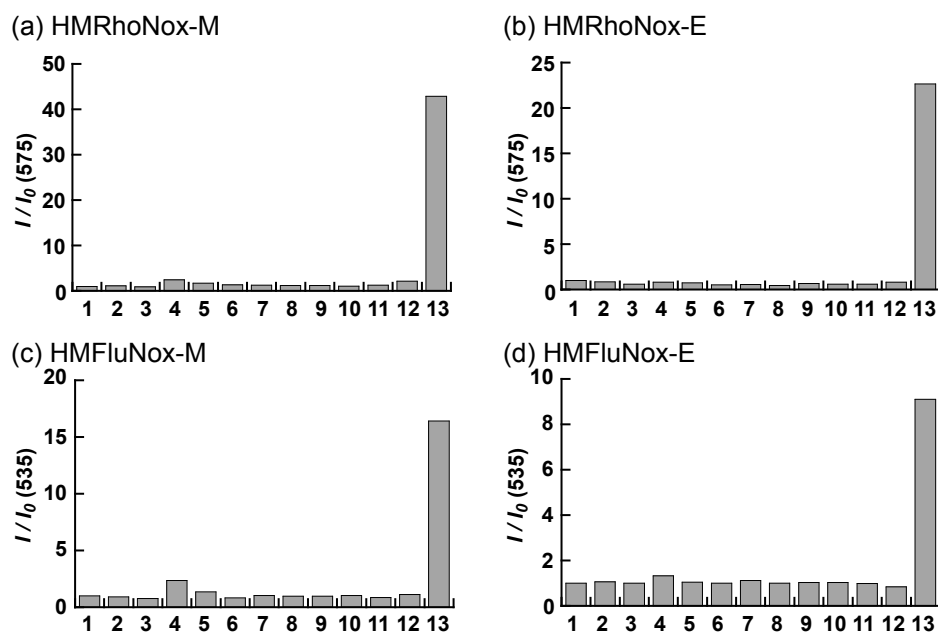


**Figure S2.** Absorbance spectral change of 2  $\mu\text{M}$  (a) HMRhoNox-M (b) HMRhoNox-E (c) HMFluNox-M (d) HMFluNox-E upon addition of 20  $\mu\text{M}$   $\text{Fe}^{2+}$  (left) and plots of absorption at 550 nm (a), 555 nm (b), 515 nm (c) and 520 nm (d) against time (right). The data were acquired in 50 mM HEPES buffer (pH 7.4) containing 0.2% DMF as a co-solvent under Ar atmosphere, and  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  was used as  $\text{Fe}^{2+}$  source.

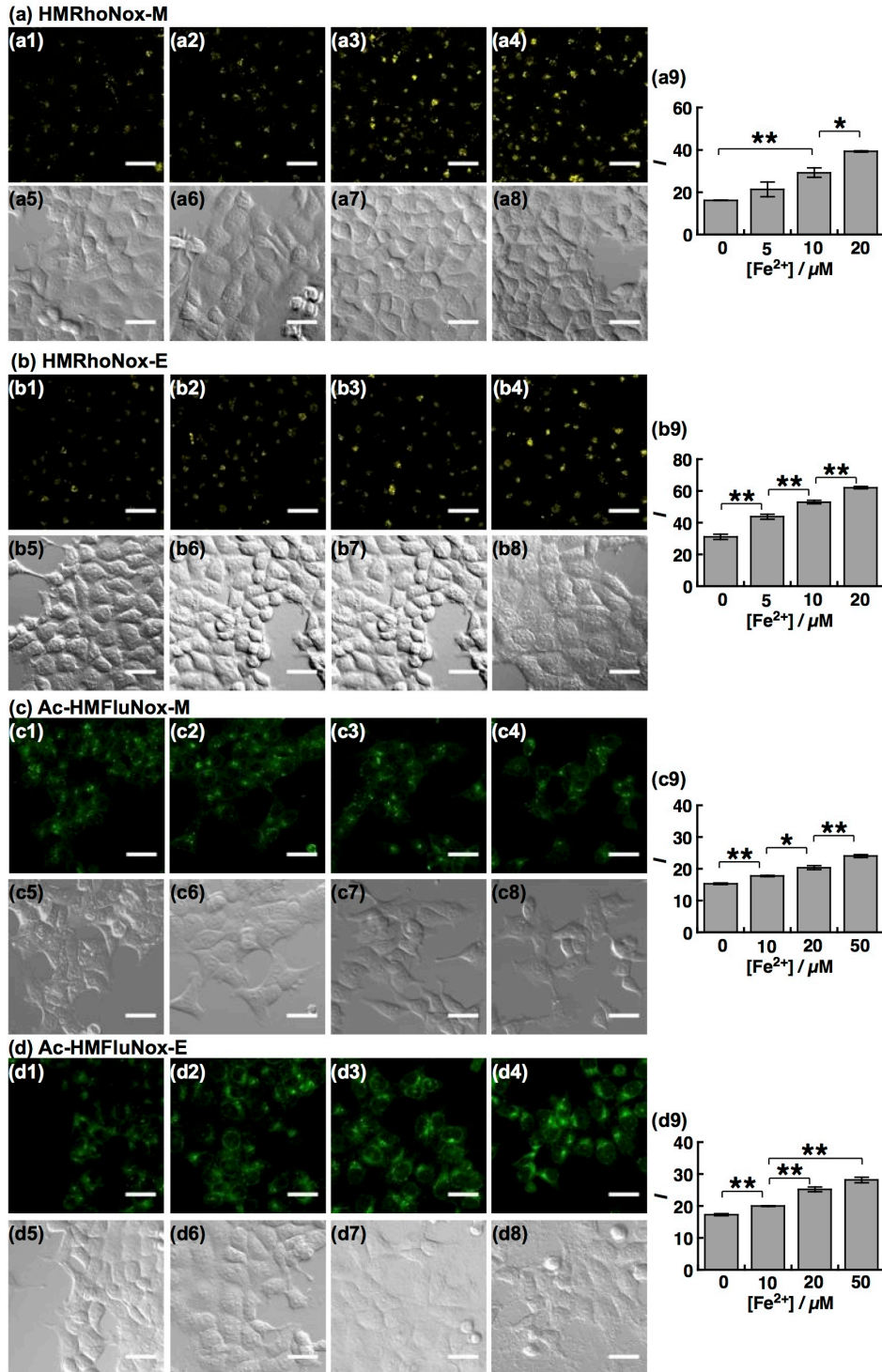


**Figure S3.** LC-MS analysis of the  $\text{Fe}^{2+}$ -triggered deoxygenation reaction of (a) HMRhoNox-M, (b) HMRhoNox-E, (c) HMFluNox-M, and (d) HMFluNox-E. The each probe ( $50 \mu\text{M}$ ) was incubated with  $200 \mu\text{M}$   $\text{Fe}^{2+}$  for 1 h in 50 mM HEPES buffer (pH 7.4).  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  was used as  $\text{Fe}^{2+}$  source. The corresponding deoxygenated dyes, HMRhodamine-M, HMRhodamine-E, HMRhodol-M, and HMRhodol-E were used as the authentic samples in a, b, c, and d, respectively. The reaction mixtures were analyzed by a LC-MS system using a reverse phase column (Waters symmetry C18,  $3.5 \mu\text{m}$ ,  $4.6 \times 75 \text{ mm}$ ) eluted with HPLC gradient as follows;  $\text{H}_2\text{O}/\text{MeCN}$  containing 0.05% formic acid (80:20 to 50:50 over 20 min for a, b, and c, and 90:10 to 70:30 over 20 min for d). The absorbance at 254 nm was monitored. Total ion mass spectra were simultaneously measured to give the mass peaks as follows:

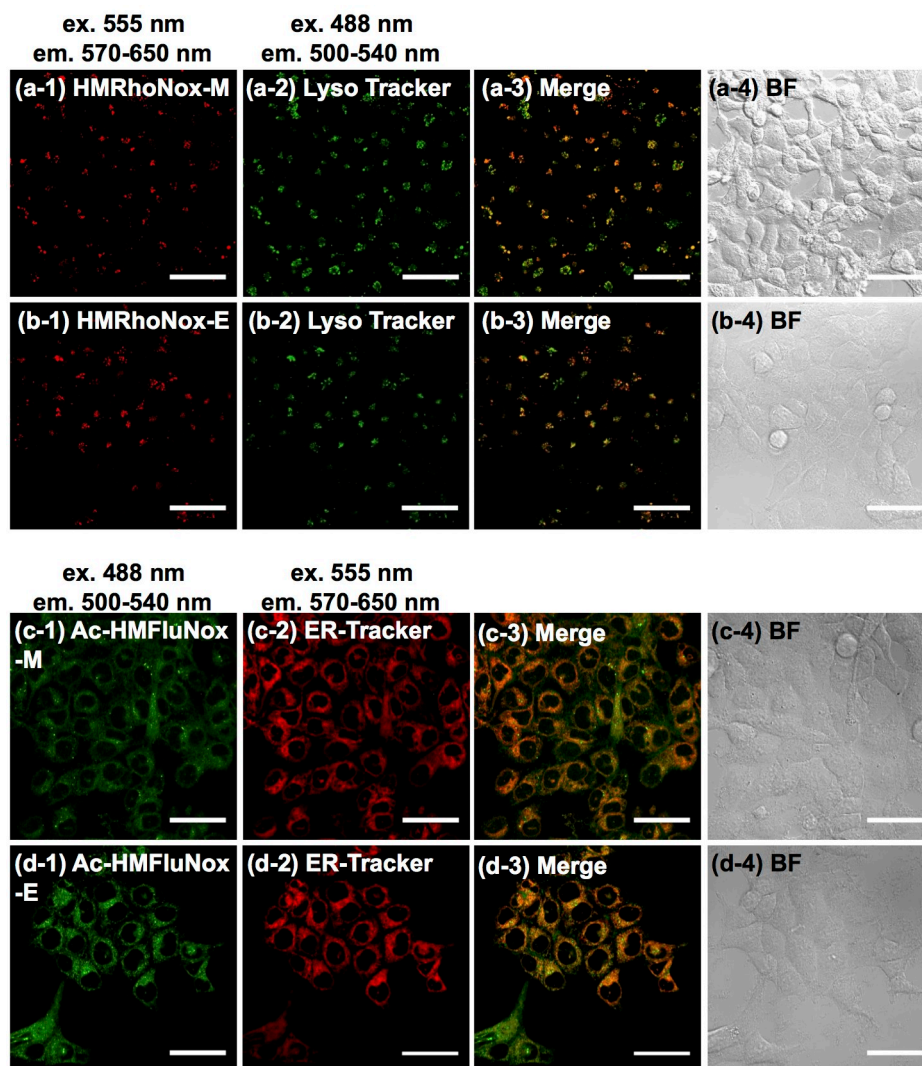
- (a) 389.1 (calcd for  $[\text{HMRhoNox-M}+\text{H}]^+ = 389.2$ ) at 11.0 min, 373.1 (calcd for  $[\text{HMRhodamine-M}+\text{H}]^+ = 373.2$ ) at 10.2 min, and 359.2 (a demethylated product of HMRhodamine-M,  $[\text{M}+\text{H}]^+ = 359.2$ ) at 9.1 min.
- (b) 445.2 (calcd for  $[\text{HMRhoNox-E}+\text{H}]^+ = 445.2$ ) at 12.4 min, 429.2 (calcd for  $[\text{HMRhodamine-E}+\text{H}]^+ = 429.3$ ) at 16.1 min, and 401.2 (M = a deethylated product of HMRhodamine-E,  $[\text{M}+\text{H}]^+ = 401.2$ ) at 13.8 min.
- (c) 362.1 (calcd for  $[\text{HMFluNox-M}+\text{H}]^+ = 362.1$ ) at 16.8 min, 346.1 (calcd for  $[\text{HMRhodol-M}+\text{H}]^+ = 346.1$ ) at 17.2 min, and 332.0 (a demethylated product of HMRhodol-M,  $[\text{M}+\text{H}]^+ = 332.1$ ) at 15.6 min.
- (d) 390.1 (calcd for  $[\text{HMFluNox-E}+\text{H}]^+ = 390.2$ ) at 8.7 min, and 374.2 (calcd for  $[\text{HMRhodol-E}+\text{H}]^+ = 374.2$ ) at 9.9 min.



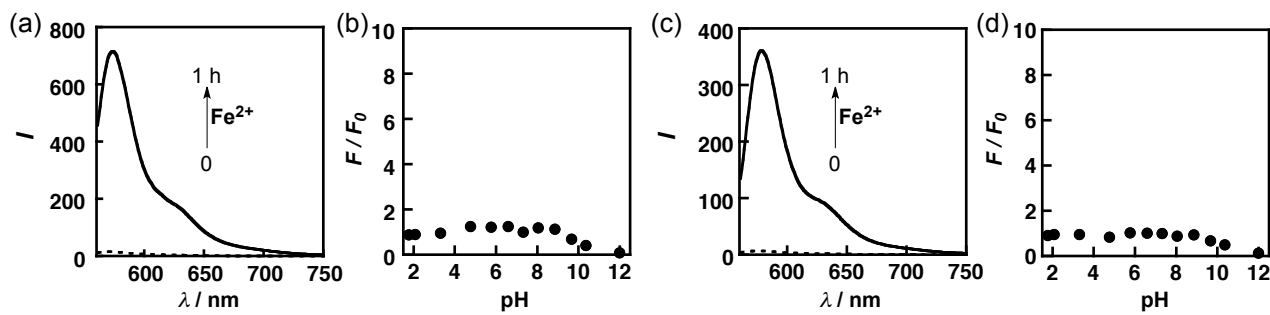
**Figure S4.** Fluorescence response of the probes against various reductants, reactive oxygen species, and chelator. Bars represent relative fluorescence intensities at 575 nm (a, b,  $\lambda_{\text{ex}} = 550$  nm) and 535 nm (c, d,  $\lambda_{\text{ex}} = 515$  nm) 1: apo, 2: 100  $\mu\text{M}$   $\text{Na}_2\text{S}_2\text{O}_3$ , 3: 1 mM sodium ascorbate, 4: 1 mM cysteine, 5: 1 mM glutathione, 6: 100  $\mu\text{M}$   $\text{NaNO}_2$ , 7: 100  $\mu\text{M}$   $\text{O}_2^{\cdot-}$ , 8: 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , 9:  $\cdot\text{OH}$ , 10: 100  $\mu\text{M}$   $\text{NaOCl}$ , 11: 100  $\mu\text{M}$   $\text{NOC-5}$ , 12: 100  $\mu\text{M}$  2,2'-bipyridyl and 20  $\mu\text{M}$   $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ , 13: 20  $\mu\text{M}$   $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ . All data were collected with 2  $\mu\text{M}$  probe after 1 h incubation in 50 mM HEPES buffer (pH 7.4, 0.2% DMF) at room temperature.



**Figure S5.** Confocal fluorescence microscopic images of HepG2 cells supplemented with various concentrations of  $\text{Fe}^{2+}$  and stained with (a) HMRhoNox-M, (b) HMRhoNox-E, (c) Ac-HMFluNox-M or (d) Ac-HMFluNox-E. (a), (b) The cells were treated with (1) 0, (2) 5, (3) 10, or (4) 20  $\mu\text{M}$   $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  for 30 min in MEM. After washing the cells, 1  $\mu\text{M}$  HMRhoNox-M or HMRhoNox-E was added, and the cells were incubated for 30 min. (c), (d) The cells were treated with (1) 0, (2) 10, (3) 20, or (4) 50  $\mu\text{M}$   $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  for 30 min in MEM. After washing the cells, 5  $\mu\text{M}$  Ac-HMFluNox-M or Ac-HMFluNox-E was added, and the cells were incubated for 30 min. Excitation was provided with 555 nm laser (a), (b) or 488 nm laser (c), (d). (5)–(8) Bright field images of the same slices of (1)–(4). (9) Quantification of data in (1)–(4). Statistical analyses were performed with a Student's *t*-test. \* $P < 0.05$ , \*\* $P < 0.01$  ( $n = 3$ ). Error bars show  $\pm$  s.e.m. Scale bars indicate 30  $\mu\text{m}$ .



**Figure S6.** Co-staining experiments of (a) HMRhoNox-M (1  $\mu$ M), (b) HMRhoNox-E (1  $\mu$ M), (c) Ac-HMFluNox-M (10  $\mu$ M), or (d) Ac-HMFluNox-E (10  $\mu$ M). In (a) and (b), HepG2 cells were co-stained with the probe and Lyso Tracker<sup>®</sup> Green. (c), (d) HepG2 cells were co-stained with the probe and ER-tracker<sup>™</sup> Red. (1) Images obtained by detecting the signals from the probes. Band path filters of 570–650 nm with a 555 nm excitation and 500–540 nm with a 488 nm excitation were used for HMRhoNox series (a, c) and HMFluNox series (c, d), respectively. (2) Images obtained by detecting the signals from the corresponding organelle targeting dyes (Lyso Tracker<sup>®</sup> Green for (a) and (b), and ER-tracker<sup>™</sup> Red for (c) and (d)). Band path filters of 500–540 nm with a 488 nm excitation and 570–650 nm with a 555 nm excitation were used for Lyso Tracker<sup>®</sup> Green (a, c) and ER-tracker<sup>™</sup> Red (c, d), respectively. (3) Merged images of (1) and (2). (4) Bright field images of the same slices as (1)–(3). Scale bars indicate 30  $\mu$ m.



**Figure S7.** Fluorescence spectral change of (a) HMRhoNox-M (2 μM) and (c) HMRhoNox-E (2 μM) upon addition of 20 μM Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>•6H<sub>2</sub>O at pH 5.0. pH profiles of relative fluorescence intensity of (b) HMRhoNox-M (2 μM) and (d) HMRhoNox-E (2 μM).

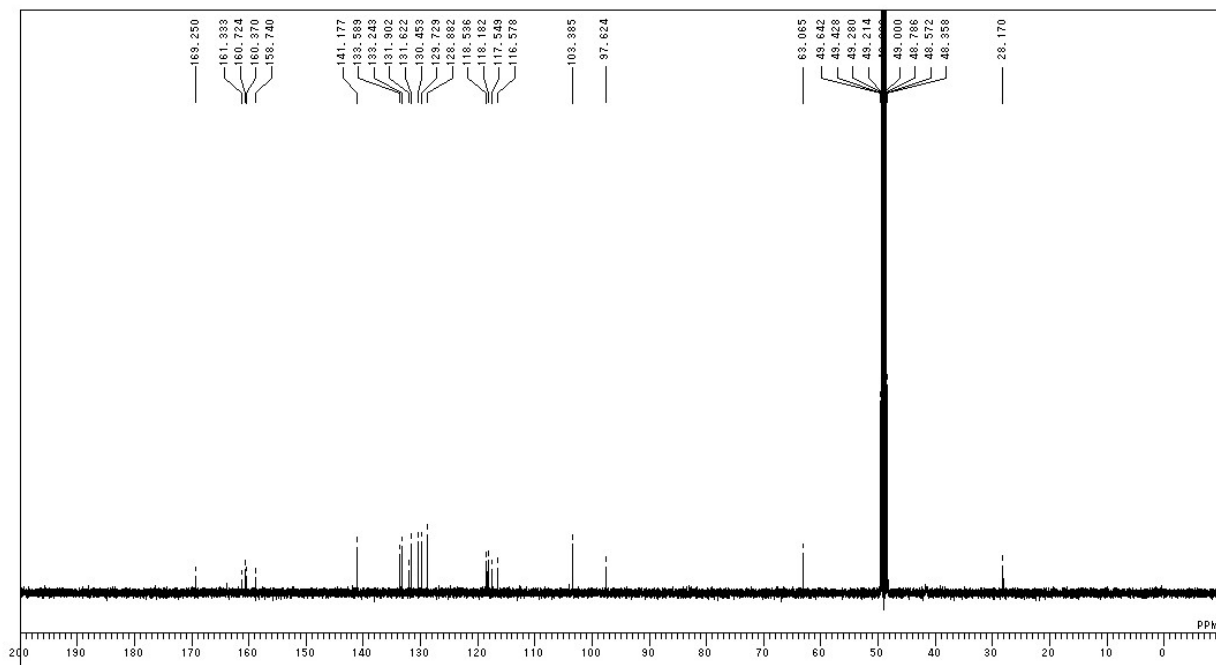
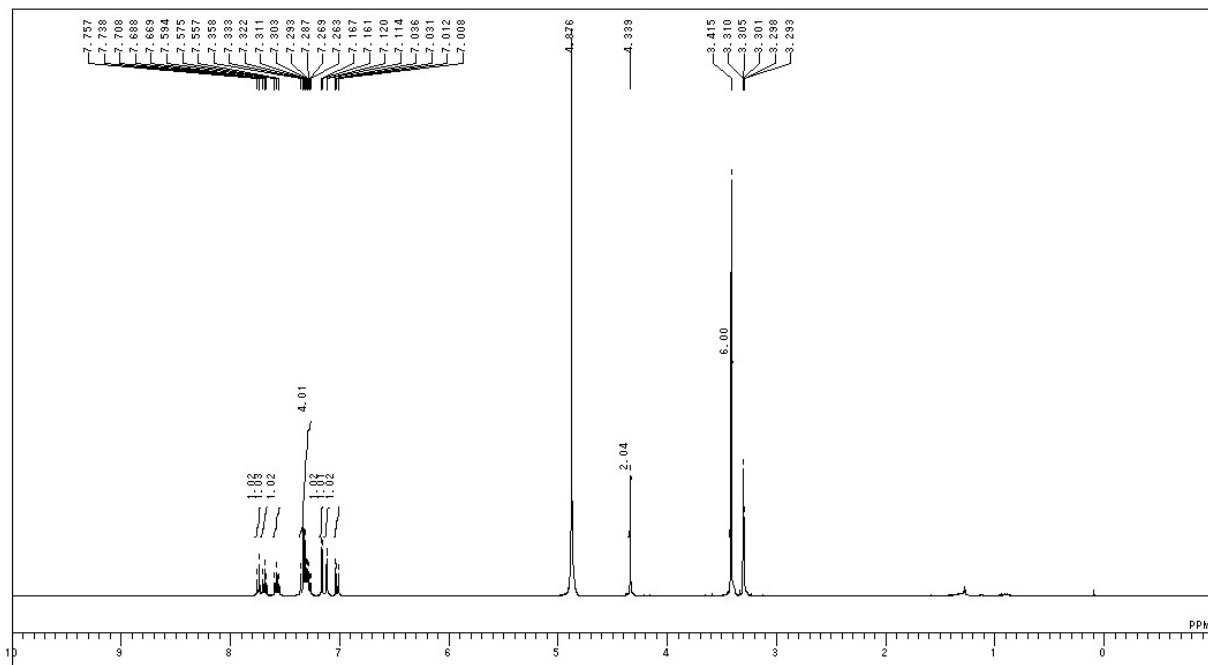


## References

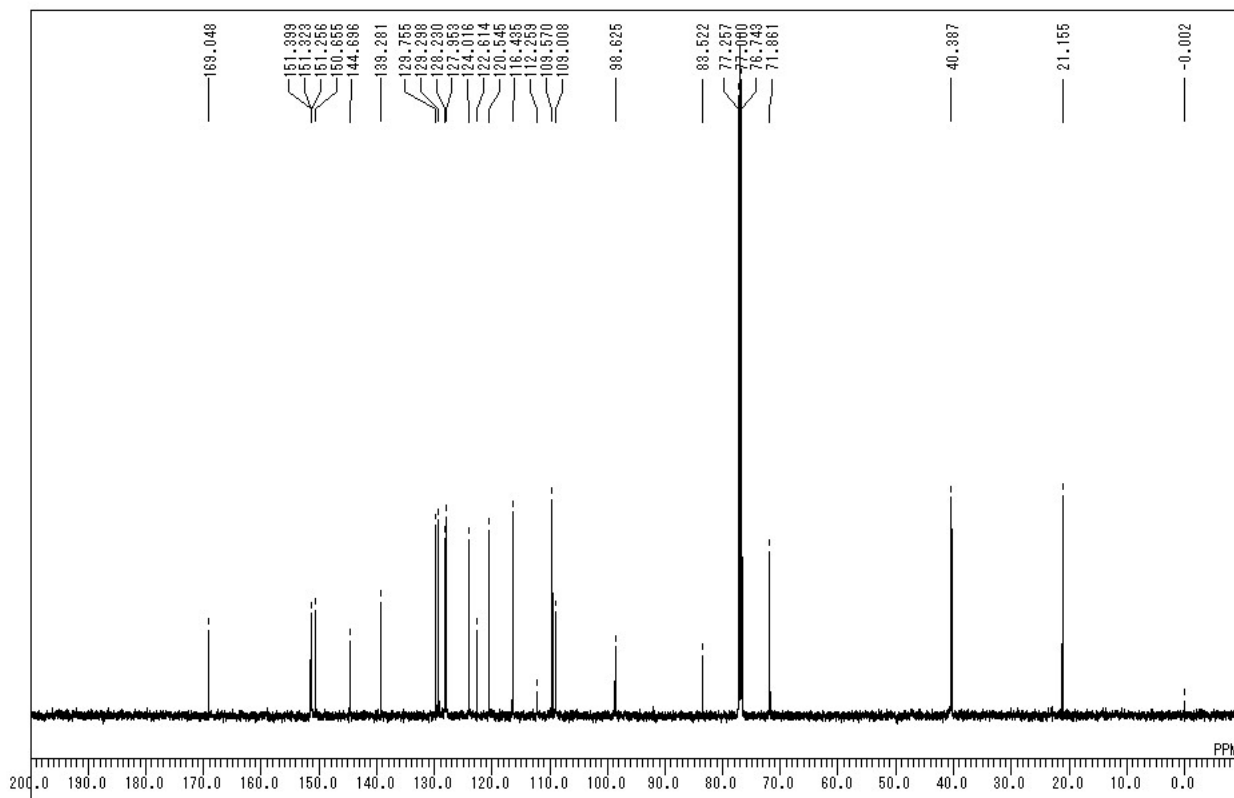
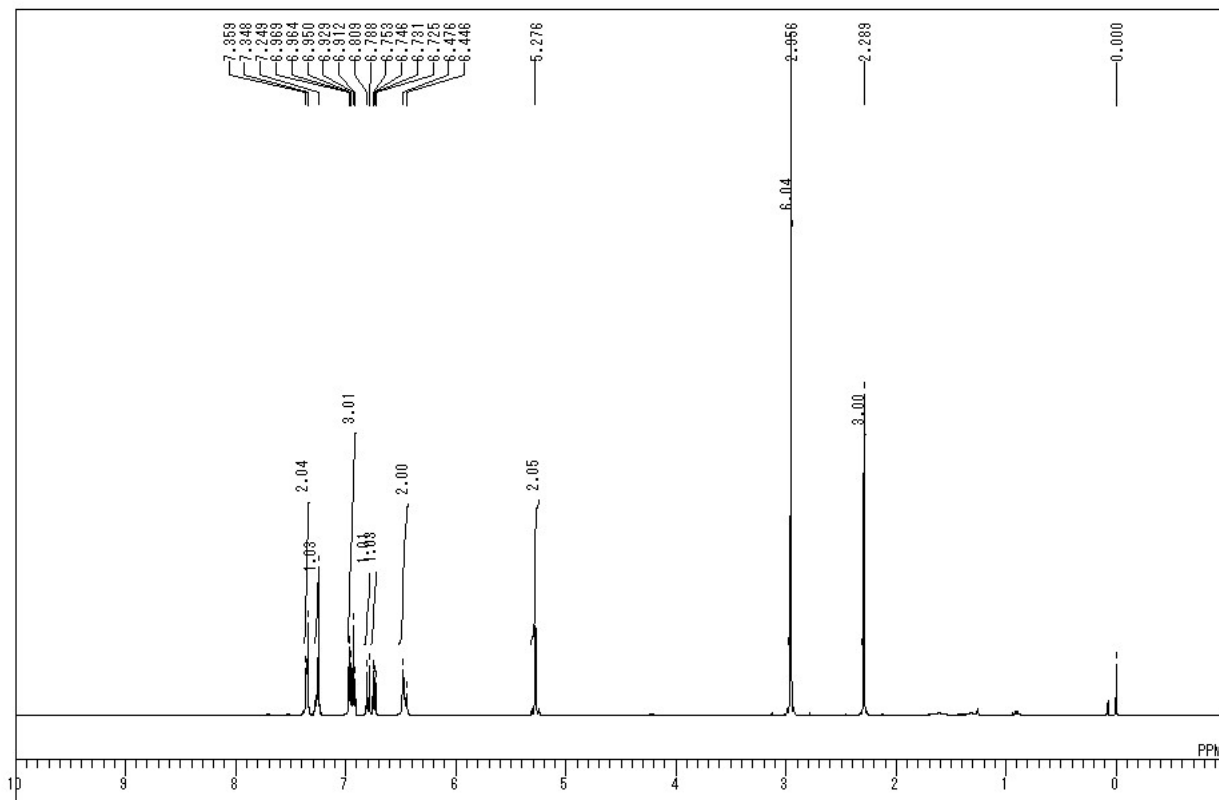
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# <sup>1</sup>H- and <sup>13</sup>C-NMR spectra of the newly synthesized compounds

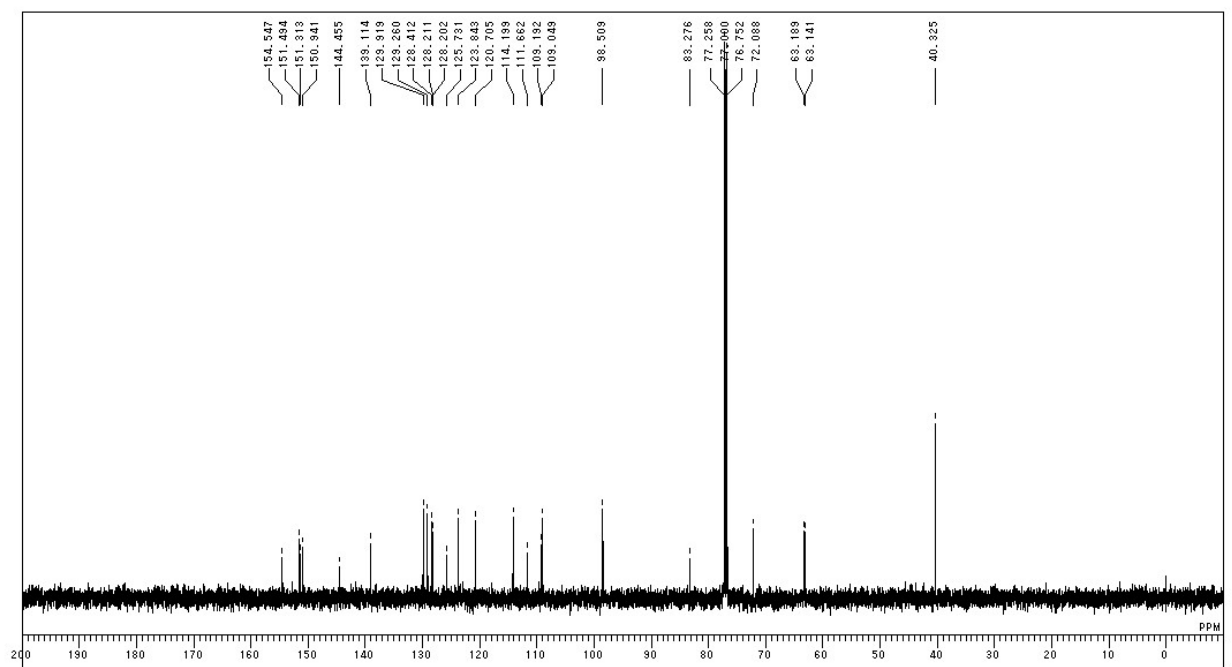
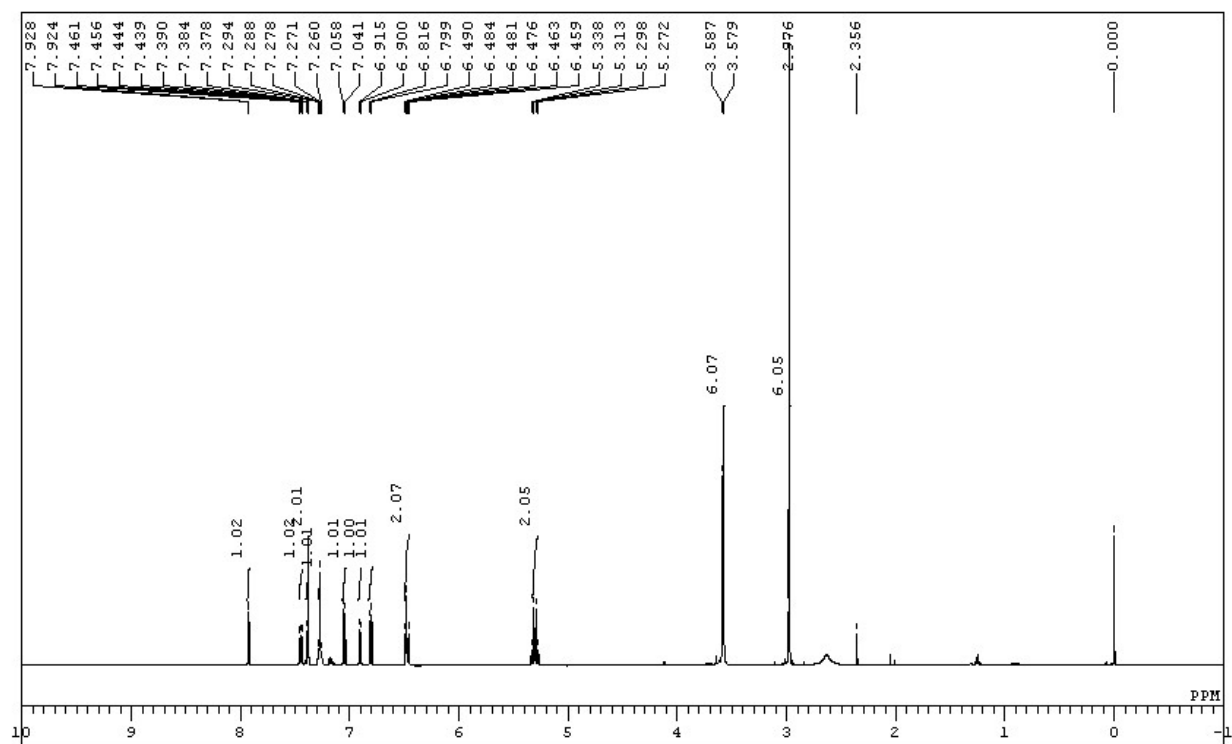
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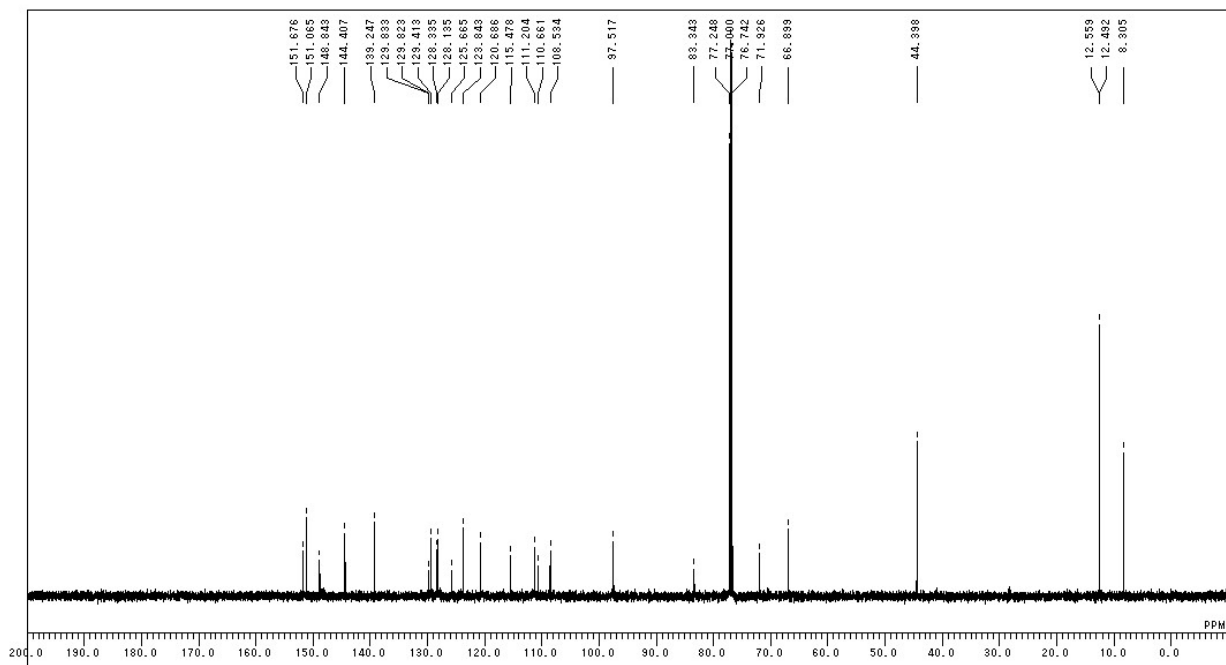
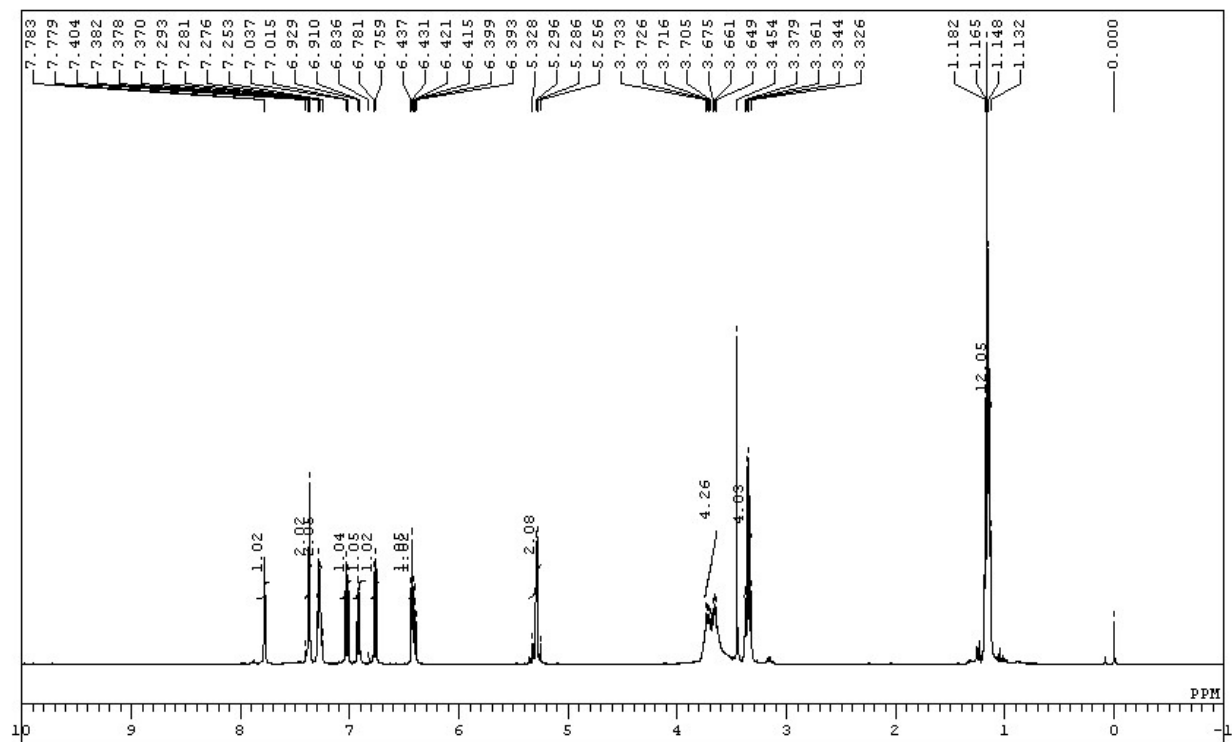
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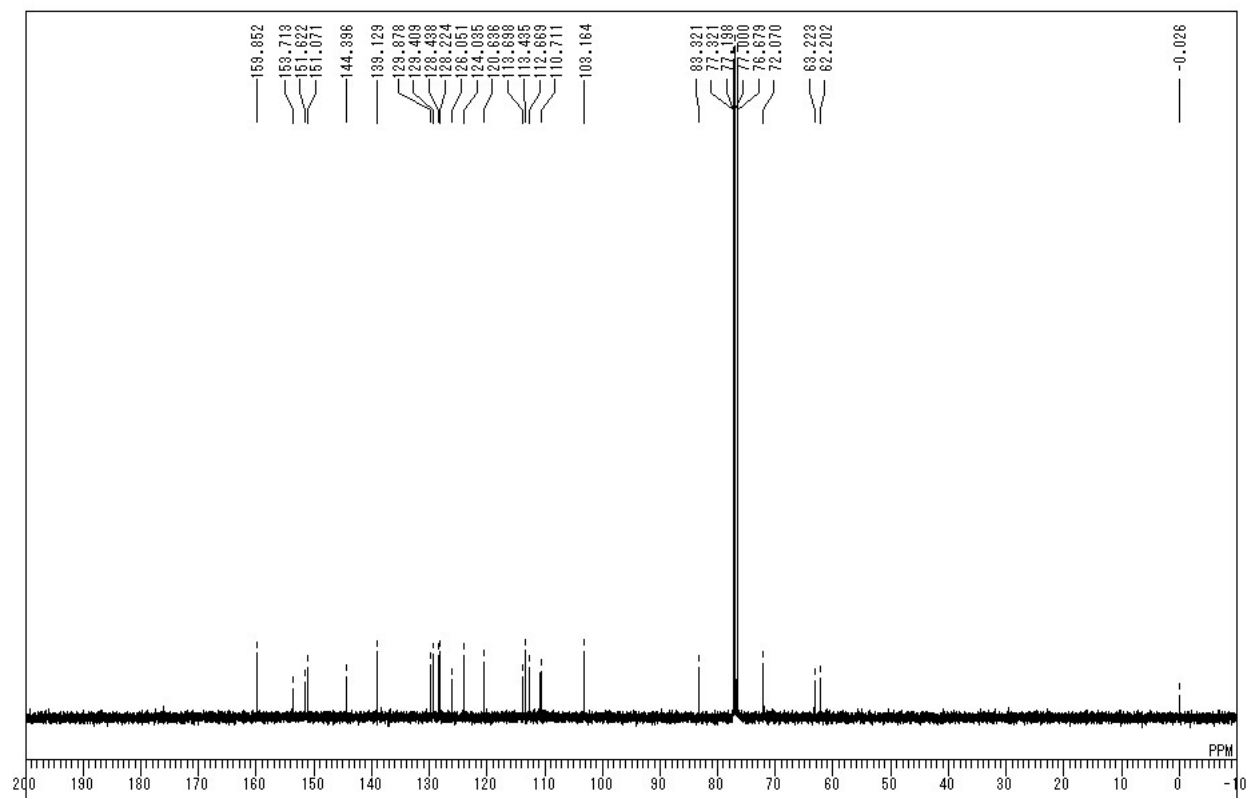
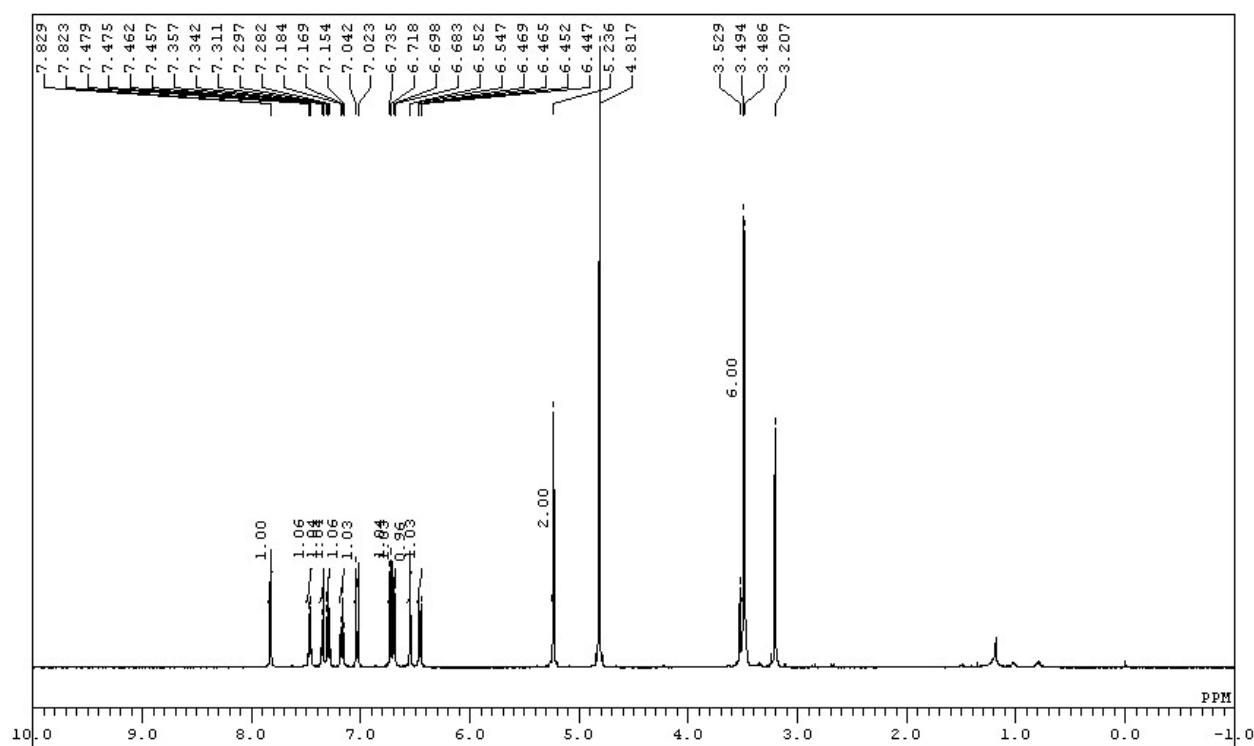
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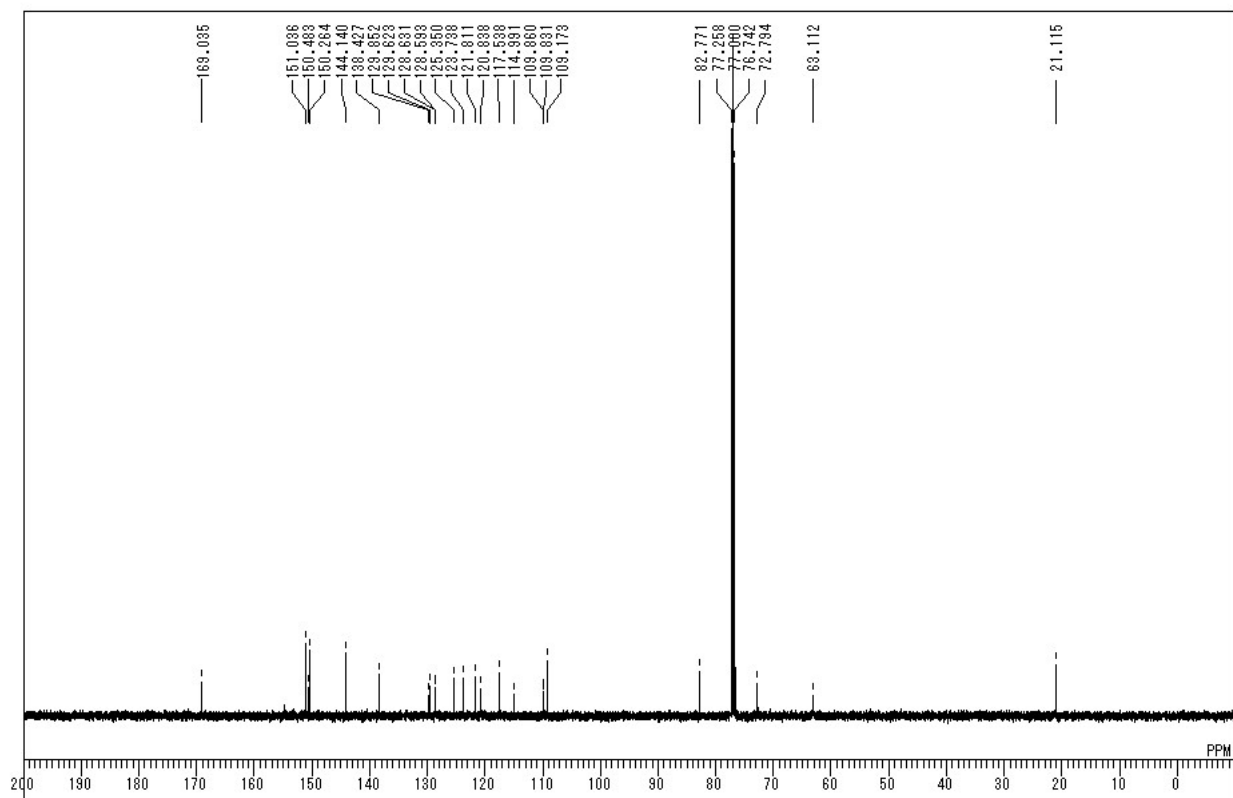
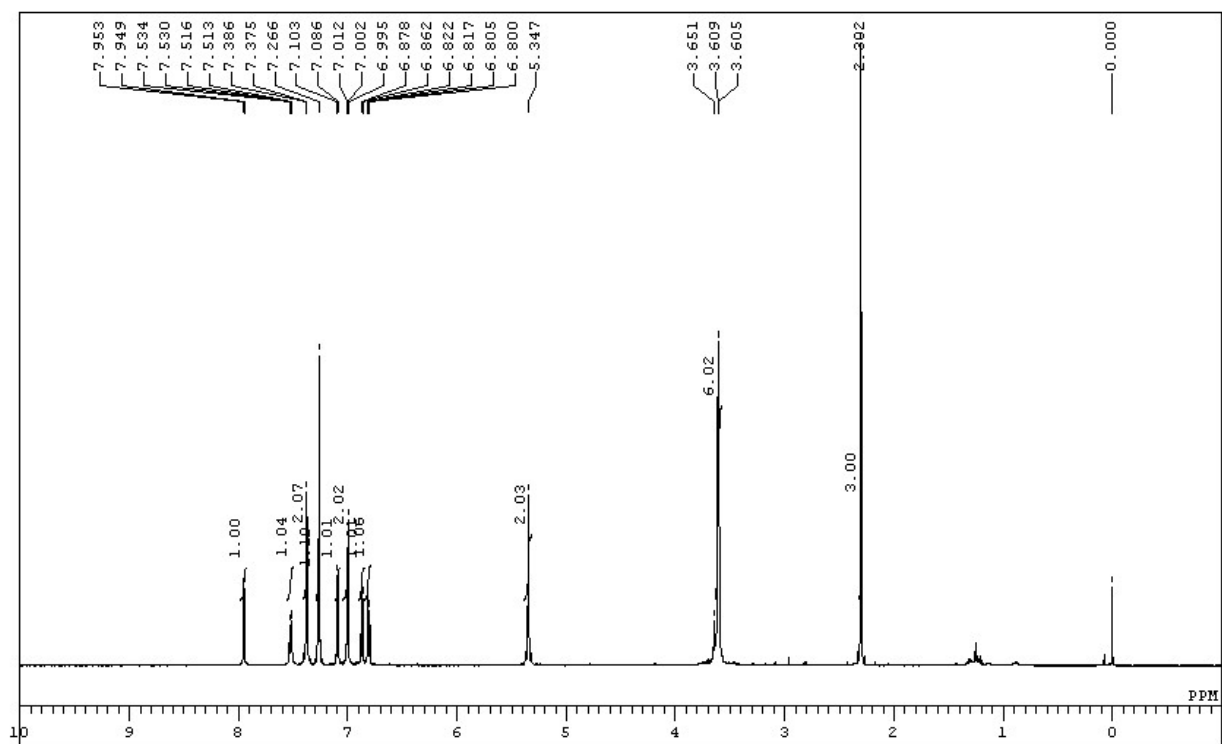
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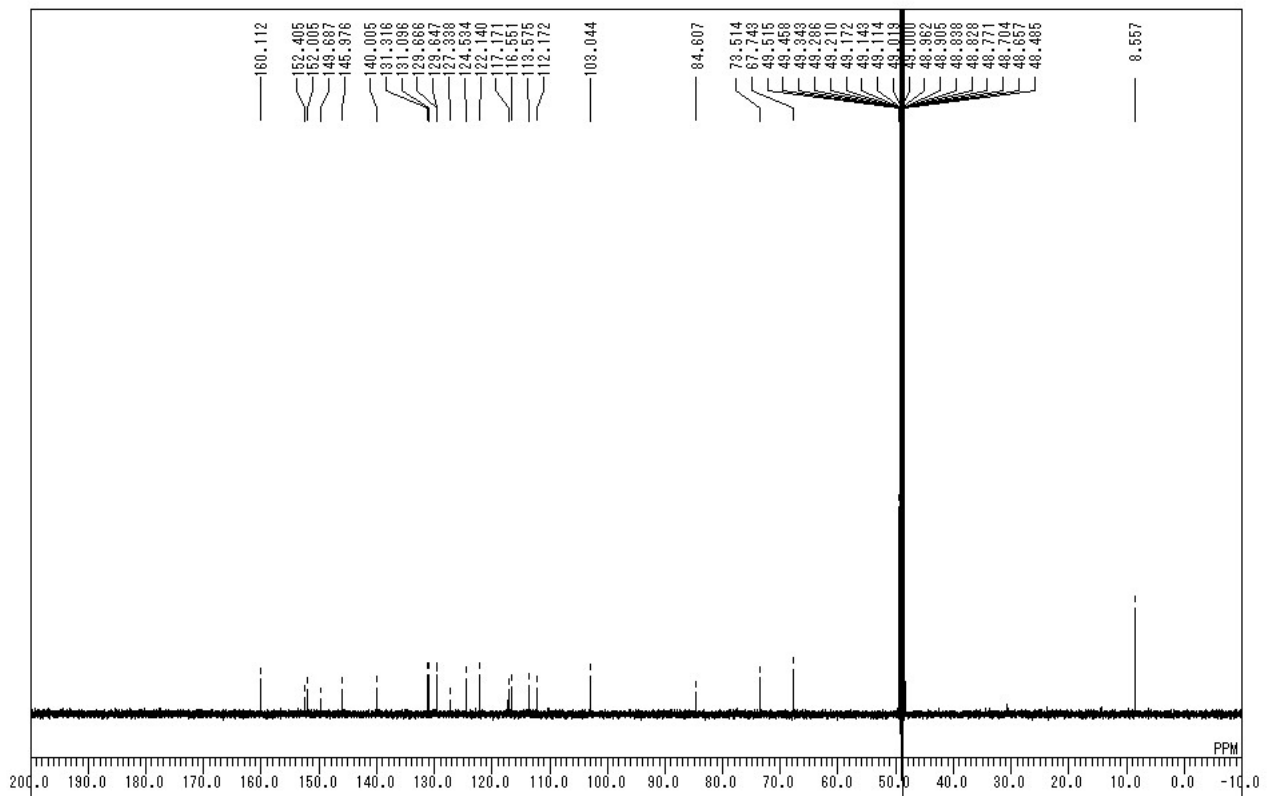
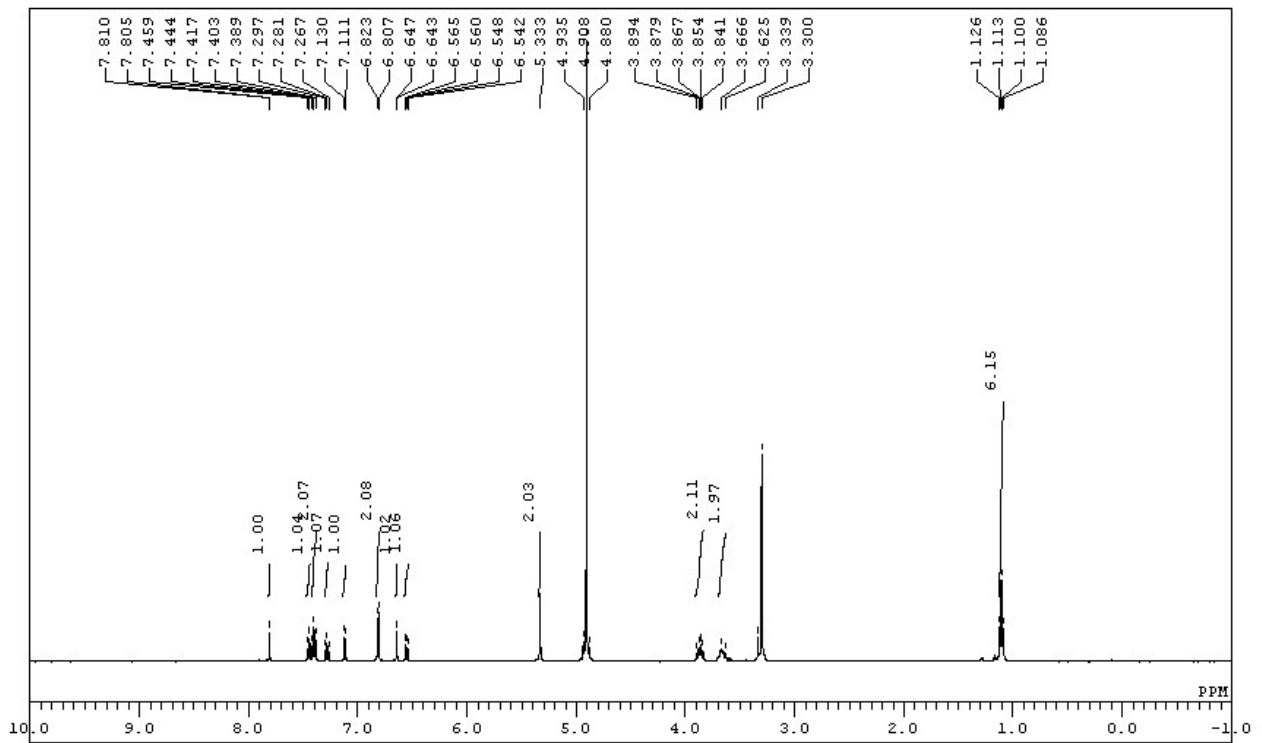
HMFluNox-M (CD<sub>3</sub>OD for <sup>1</sup>H-NMR, CDCl<sub>3</sub> for <sup>13</sup>C-NMR)



Ac-HMFluNox-M (CDCl<sub>3</sub>)



HMFluNox-E (CD<sub>3</sub>OD)





Ac-HMFluNox-E (CDCl<sub>3</sub>)

