Electronic Supplementary Information (ESI)

Reaction-based two-photon probes for *in vitro* analysis and cellular imaging of monoamine oxidase activity

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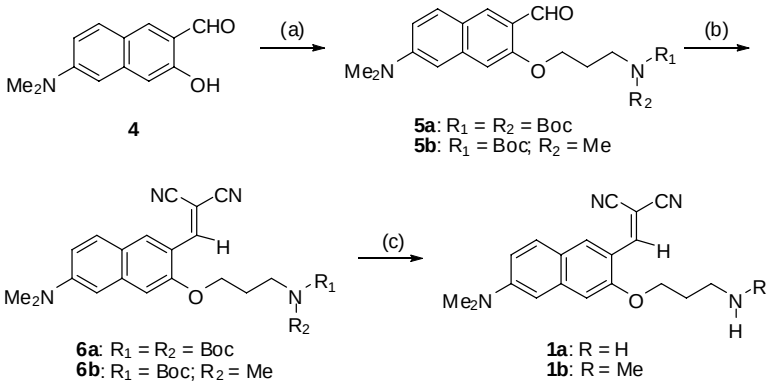
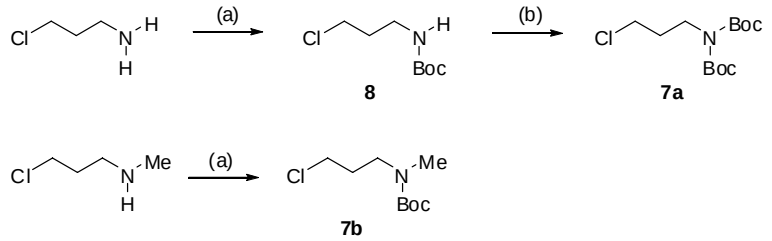
Synthesis

Scheme S1. Synthesis of probes **1a** and **1b**.



Reagents and conditions: (a) compound **7a** or **7b** (see below), K_2CO_3, DMF. (b) malononitrile, piperidine, EtOH. (c) TFA-CH_2Cl_2 (1:1, v/v), TIPS.

Scheme S2. Synthesis of compounds **7a** and **7b**.



Reagents and conditions: (a) $(Boc)_2O$, Et_3N, THF. (b) $(Boc)_2O$, DMAP, CH_3CN.

Experimental Procedures

Materials and methods. The chemical reagents were purchased from Aldrich or TCI. Monoamine oxidase A and B (2.5 mg/vial) were purchased from Sigma Aldrich. Pargyline was purchased from Sigma. Commercially available reagents were used without further purification. Anhydrous solvents for organic synthesis were prepared by passing through a solvent purification tower. Thin-layer chromatography (TLC) was performed on precoated silica gel 60F-254 glass plates. ^{1}H and ^{13}C NMR spectra were measured with a Bruker DPX-300 and DPX-500. Coupling constants (J value) are reported in Hertz. Mass spectral analysis was recorded with Jeol JMS 700 and was reported in units of mass to charge (*m*/*z*). HRMS was performed at the Korea Basic Science Center, Kyungpook National University.

***N*-*tert*-Butyloxycarbonyl-3-(chloropropyl)carbamic acid *tert*-butyl ester (7a).** To a solution of 3-chloropropylamine hydrochloride (1.30 g, 10 mmol) in dichloromethane (4.5 mL) was added triethylamine (1.67 mL, 12 mmol) dropwise, and the resulting solution was stirred for 15 min. Next, a solution of di(*tert*-butyl)dicarbonate (2.18 g, 10 mmol) in dichloromethane (4.5 mL) was added dropwise to the amine solution over a period of 1 h, and the resulting mixture was stirred overnight at room temp. The reaction mixture was diluted with dichloromethane (10 mL), and it was washed sequentially with 1 N HCl (10 mL), water (2 × 5 mL), saturated aqueous $NaHCO_3$ (1 × 10 mL), and brine (1 × 10 mL). The organic layer was separated, dried over Na_2SO_4, and concentrated by rotary evaporation to give compound **12** as a colorless oil (1.5 g, 82% yield), which was used for the next step without further purification: ^{1}H NMR ($CDCl_3$, 300 MHz, 293K): δ 4.71 (s, 1H), 3.59 (t, 2H), 3.29 (m, 2H), 1.96 (m, 2H), 1.52 (s, 9H); ^{13}C NMR ($CDCl_3$, 75 MHz, 293K): δ 156.00, 79.22, 42.31, 37.87, 32.60, 28.33. Compound **12** was dissolved in acetonitrile (20 mL) and treated

with 4-(dimethylamino)pyridine (559 mg, 4.56 mmol) at room temp, and the resulting mixture was stirred for 10 min. Di-*tert*-butyl-dicarbonate (1M in THF, 15.0 mL, 15.25 mmol) was added to the reaction mixture, and the resulting mixture was stirred over night at room temp. The reaction was quenched by the addition of water, and the aqueous phase was extracted with EtOAc (3 × 40 mL). The combined organic extracts were washed with 1 M aqueous HCl (50 mL) and brine (4 × 50 mL), and concentrated in vacuo. The residue was purified by silica gel column chromatography (eluent: 10% EtOAc in hexane) to afford compound **7a** as a colorless liquid (538 mg, 60%). ^{1}H NMR ($CDCl_3$, 300 MHz, 293 K): δ 3.73 (m, 2H), 3.58–3.53 (t, 2H), 2.05 (m, 2H), 1.51 (s, 18H). ^{13}C NMR ($CDCl_3$, 75 MHz, 293 K): δ 152.80, 82.86, 44.49, 42.75, 32.43, 28.42. HRMS (*m*/*z*): calcd $[M+H]^+$ for $C_{13}H_{24}ClNO_4$ 294.1472; found, 294.1468.

3-Chloropropyl-*N*-(methyl)carbamic acid *tert*-butyl ester (7b). To a solution of 3-chloropropyl-*N*-methylamine (590.6 mg, 3.05 mmol) in acetonitrile (20 mL) were added 4-(dimethylamino)pyridine (559 mg, 4.56 mmol) followed by di-*tert*-butyl-dicarbonate (1 M in THF, 15.0 mL, 15.25 mmol), and the reaction mixture was stirred over night at room temp. After an extractive work-up and column chromatography (eluent: 10% EtOAc in hexane), compound **7b** was obtained as a colorless liquid (388 mg, 55%). ^{1}H NMR ($CDCl_3$, 300 MHz, 293 K): δ 3.55 (t, 2H), 3.34 (t, 2H), 2.87 (s, 3H), 1.99 (m, 2H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$, 75 MHz, 293 K): δ 156.01, 147.04, 85.39, 79.77, 46.61, 28.70, 27.68.

6-Dimethylamino-3-{3-[*N*,*N*-bis(*tert*-butyloxycarbonyl)amino]propyloxy}naphthalene-2-carbaldehyde (5a). Under a nitrogen atmosphere, 6-dimethylamino-3-hydroxy-naphthalene-2-carbaldehyde[1] (**4**, 176.7 mg, 0.82 mmol), compound **7a** (242 mg, 0.82 mmol), and potassium carbonate (227 mg, 1.64 mmol) were combined in anhydrous DMF (5 mL). The resulting mixture was heated at 50°C for 27 h, and then it was cooled to room temperature, and diluted with EtOAc (100 mL). The mixture was washed with water (3 × 100 mL) and brine (1 × 100 mL), and the organic layer was separated, dried over Na_2SO_4, and concentrated. The residue was purified by silica gel column chromatography (eluent: 20% EtOAc in hexane) to afford compound **5a** as a yellow solid (271 mg, 71%). ^{1}H NMR (CDCl3, 500 MHz, 293K): δ 10.51 (s, 1H), 8.24 (s, 1H), 7.73–7.71 (d, 1H), 7.02–7.00 (dd, 1H), 6.93 (s, 1H), 6.73–6.72 (d, 1H), 4.22–4.19 (t, 2H), 3.90–3.87 (t, 2H), 3.12 (s, 6H), 2.22–2.20 (t, 2H), 1.51 (s, 18H). ^{13}C NMR (CDCl3, 75 MHz, 293K): δ 189.70, 157.83, 152.63, 150.68, 139.88, 131.28, 130.38, 122.02, 120.66, 114.27, 104.47, 103.99, 82.47, 66.01, 43.94, 40.34, 28.84, 28.07. HRMS (*m*/z): calcd $[M+H]^+$ for $C_{26}H_{36}N_2O_6$ 472.2573; found, 472.2571.

6-Dimethylamino-3-{3-[*N*-methyl-*N*-(*tert*-butyloxycarbonyl)-amino]propyloxy}naphthalene-2-carbaldehyde (5b). Compound **5b**, prepared similarly as **5a** using compound **4** and compound **7b**, was obtained as a yellow solid (70%). ^{1}H NMR (CDCl3, 300 MHz, 293K): δ 10.48 (s, 1H), 8.21 (s, 1H), 7.71–7.69 (d, 1H), 7.00–6.98 (dd, 1H), 6.92 (s, 1H), 6.71–6.70 (d, 1H), 4.17–4.15 (t, 2H), 3.49–3.46 (t, 2H), 3.10 (s, 6H), 2.90 (s, 3H), 2.12 (s, 2H), 1.43 (s, 9H). ^{13}C NMR (CDCl3, 75 MHz, 293K): δ 189.53, 157.73, 155.86, 150.75, 139.90, 131.28, 130.79, 121.99, 120.68, 114.31, 104.54, 103.98, 46.03, 40.33, 29.71, 29.26, 28.44, 14.13. HRMS (*m*/z): calcd $[M+H]^+$ for $C_{22}H_{30}N_2O_4$ 386.2206; found, 386.2208.

2-{3-[*N*,*N*-bis(*tert*-butyloxycarbonyl)amino]propyloxy}-3-(2,2-dicyano)vinyl-7-(dimethylamino)naphthalene (6a). To a stirred solution of compound **5a** (152 mg, 0.32 mmol) and malononitrile (43 mg, 0.64 mmol) in ethanol (3 mL) at room temp under argon was added piperidine (316 μL, 3.2 mmol). The reaction mixture was allowed to stir at room temp for 3 h. The solvent was evaporated under reduced pressure, and the resulting residue was purified by silica gel column chromatography (eluent: 30% EtOAc in hexane) to afford compound **6a** as a red solid (151 mg, 90%). ^{1}H NMR (CDCl3, 500 MHz, 293K): δ 8.71 (s, 1H), 8.37 (s, 1H), 7.72–7.70 (d, 1H), 7.01–7.00 (d, 1H), 6.87 (s, 1H), 6.68–6.67 (s, 1H), 4.16–4.14 (t, 2H), 3.87–3.84 (t, 2H), 3.16 (s, 6H), 2.20 (t, 2H), 1.52 (s, 18H). ^{13}C NMR (CDCl3, 75 MHz, 293K): δ 155.00, 153.69, 152.62, 151.40, 140.12, 131.43, 131.15, 120.74, 117.18, 115.44, 114.47, 104.27, 103.76, 82.58, 66.23, 43.52, 40.28, 28.79, 28.09. HRMS (*m*/z): calcd $[M+H]^+$ for $C_{29}H_{36}N_4O_5$ 520.2686; found, 520.2689.

2-{3-[*N*-methyl-*N*-(*tert*-butyloxycarbonyl)amino]propyloxy}-3-(2,2-dicyano)vinyl-7-(dimethylamino)naphthalene (6b). Compound **6b**, prepared similarly as **6a**, was obtained as a red solid

(90%). ^{1}H NMR (CDCl3, 300 MHz, 293K): δ 8.36 (s, 1H), 7.98 (s, 1H), 7.45–7.42 (d, 1H), 6.83–6.79 (dd, 1H), 6.67 (s, 1H), 6.51 (s, 1H), 3.99–3.95 (t, 2H), 3.36–3.32 (t, 2H), 3.01 (s, 6H), 2.81 (s, 3H), 2.01–1.97 (t, 2H), 1.33 (s, 9H). ^{13}C NMR (CDCl3, 75 MHz, 293K): δ 155.74, 154.76, 152.94, 151.37, 140.15, 131.27, 130.83, 120.53, 116.77, 115.61, 114.51, 114.37, 104.11, 103.73, 79.47, 75.67, 66.00, 46.04, 40.17, 34.44, 28.40, 27.51. HRMS (*m*/z): calcd $[M+H]^+$ for $C_{25}H_{30}N_4O_3$ 434.2318; found, 434.2319.

2-{[3-(3-Aminopropoxy)-6-(dimethylamino)naphthalen-2-yl]methylene}malononitrile (1a). Compound **6a** (51.1 mg, 0.1 mmol) was added to a solution of trifluoroacetic acid (3 mL) and triisopropylsilane (3 μL) in dichloromethane (3 mL), and the resulting mixture was stirred at 0 °C for 1 h. The reaction was followed by TLC. The solvent was concentrated under reduced pressure to give **1a** as a dark red solid, which was pure enough for sensing purpose. ^{1}H NMR (DMSO, 300 MHz, 293K): δ 8.50 (s, 1H), 8.43 (s, 1H), 7.94 (bs, 2H), 7.74–7.71 (d, 1H), 7.14–7.10 (m, 2H), 6.84–6.83 (d, 1H), 4.24–4.22 (t, 2H), 3.11 (s, 6H), 3.10 (m, 2H), 2.15–2.09 (t, 2H). ^{13}C NMR (CDCl3, 75 MHz, 293K): δ 158.33, 157.96, 151.19, 139.74, 130.86, 130.53, 119.95, 116.52, 115.37, 114.81, 114.44, 104.64, 103.54, 76.16, 65.34, 36.43, 26.52.

2-({6-Dimethylamino-3-[(3-methylamino)propoxy]-naphthalen-2-yl}methylene)malononitrile (1b). Compound **1b**, prepared similarly as **1a**, was obtained as a dark red solid, which was pure enough for sensing purpose. ^{1}H NMR (DMSO, 300 MHz, 293K): δ 8.54 (bs, 1H), 8.45 (s, 1H), 8.42 (s, 1H), 7.74–7.71 (d, 1H), 7.14 (s, 1H), 7.11–7.10 (d, 1H), 6.83 (d, 1H), 4.23–4.19 (t, 2H), 3.20 (m, 2H), 3.11 (s, 6H), 2.64–2.63 (t, 3H), 2.18–2.13 (m, 2H). ^{13}C NMR (CDCl3, 75 MHz, 293K): δ 154.55, 154.25, 151.23, 139.75, 130.90, 130.61, 119.99, 116.51, 115.41, 114.87, 114.47, 104.68, 103.57, 76.27, 65.39, 45.84, 32.63, 25.18.

Moclobemide [4-chloro-(2-morpholinoethyl)benzamide]. To a solution of 4-chlorobenzoyl chloride (0.977 mL, 7.62 mmol) in THF (35 mL) were added 4-(2-aminoethyl)morpholine (1.0 mL, 7.62 mmol) and triethylamine (1.06 mL, 7.62 mmol) at 0 °C over 1 h. During addition, the temperature of the reaction mixture was kept below 5 °C. The resulting mixture was stirred at room temp for 12 h, and then was treated with EtOAc (100 mL) and water (200 mL). The organic layer was separated and evaporated under reduced pressure; the resulting white solid was recrystallized from EtOAc and hexane to give pure Moclobemide (1.95 g, 95%). ^{1}H NMR (DMSO, 300 MHz, 293K): δ 8.51 (t, 1H), 7.89–7.86 (dd, 2H), 7.56–7.49 (dd, 2H), 3.59 (m, 4H), 3.37 (m, 2H), 2.44 (m, 6H). ^{13}C NMR (DMSO, 75 MHz, 293K): δ 165.05, 135.87, 133.23, 129.02, 128.28, 66.14, 57.24, 53.23, 36.57.

Spectroscopic analysis

One- and two-photon spectroscopic analysis of IminoPOS. UV/Vis absorption spectra were obtained using a HP 8453 UV/Vis spectrophotometer. Fluorescence spectra were recorded on a Photon Technical International Fluorescence System (PTI, Photon Technology International) with a 1–cm standard quartz cell. The concentration of IminoPOS and acedan, coumarin 153 dyes were 10 μM. The fluorescence quantum yield was determined by using rhodamine 6G and coumarin 153 as the references. The measurement of two photon emission spectra was performed by using home-built cavity-dumped Ti:sapphire oscillator pumped by a 5.0 W output of a frequency-doubled Nd:YVO4 laser (Verdi, Coherent), which allows us to use excitation wavelengths at 780, 800, and 820 nm. The energy of the output pulses was 40 nJ at the repetition rate of 380 kHz. A 15–cm focal length singlet lens was used to focus the excitation beam to the sample in a 1–mm quartz cell, and the fluorescence was collected in a backscattering geometry using a parabolic mirror. Two–photon cross section (GM) was checked by TPACS (Two-photon induced fluorescence) method and 1 units (GM) value is 10^{-50} cm^4 s $photon^{-1}$ $molecule^{-1}$.[2,3]

Table S1. Absorption and emission properties of acedan, coumarin 153, and imino-POS.

	λ_{abs} (nm)	ε (L mol^{-1} cm^{-1})[b]	λ_{fl} (nm)[b]
IminoPOS	446	12,436	585
Acedan	365	14,690	501
Coumarin 153	425	19,280	548

[a] All data were obtained for a 10 μM solution of the fluorophore dissolved in EtOH. [b] Measured at each absorption λ_{max}.

Table S2. Quantum yield and GM value of acedan, coumarin 153, and imino-POS.

Solvent	Fluorophore	Quantum yield (Φ_F) [b]			Two photon absorption cross-section (GM) [c]		
		390 nm	400 nm	410 nm	780 nm	800 nm	820 nm
EtOH	IminoPOS	0.602	0.628	0.634	131.31	157.02	179.89
	Acedan	0.547	0.530	0.516	145.64	151.43	34.22
	Coumarin 153	0.509	0.470	0.501	113.18	141.45	99.45
Water	IminoPOS	0.148	0.166	0.175	32.77	19.69	19.60
	Acedan	0.249	0.175	0.209	53.88	48.27	18.75
	Coumarin 153[d]	-	-	-	-	-	-

[a] All data were obtained for a 10 μM solution of the fluorophore dissolved in each solvents. [b] Fluorescence quantum yields were determined using rhodamine 6G (Φ_F = 0.6) as a standard.[2] [c] σ (GM) values were determined using rhodamine 6G (GM = 700) as a standard.[3] [d] Not determined due to low solubility in pure water.

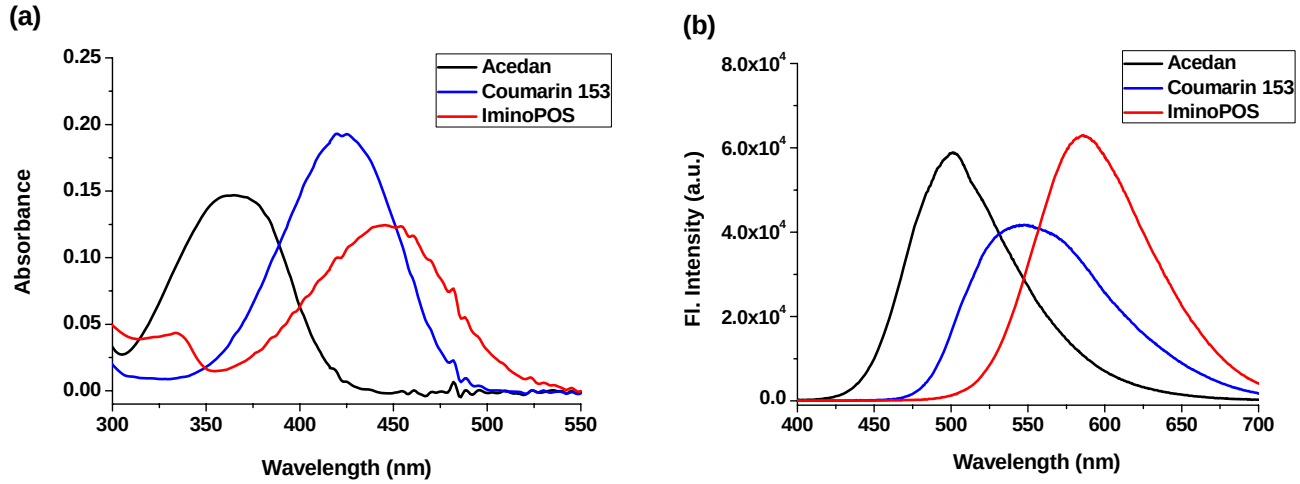


Fig. S1. Absorption and emission spectra of acedan, coumarin 153, and IminoPOS in EtOH, at 10 μM concentration for each. (a) Absorbance spectra. (b) Fluorescence spectra obtained by excitation at each of the maximum absorbance wavelengths.

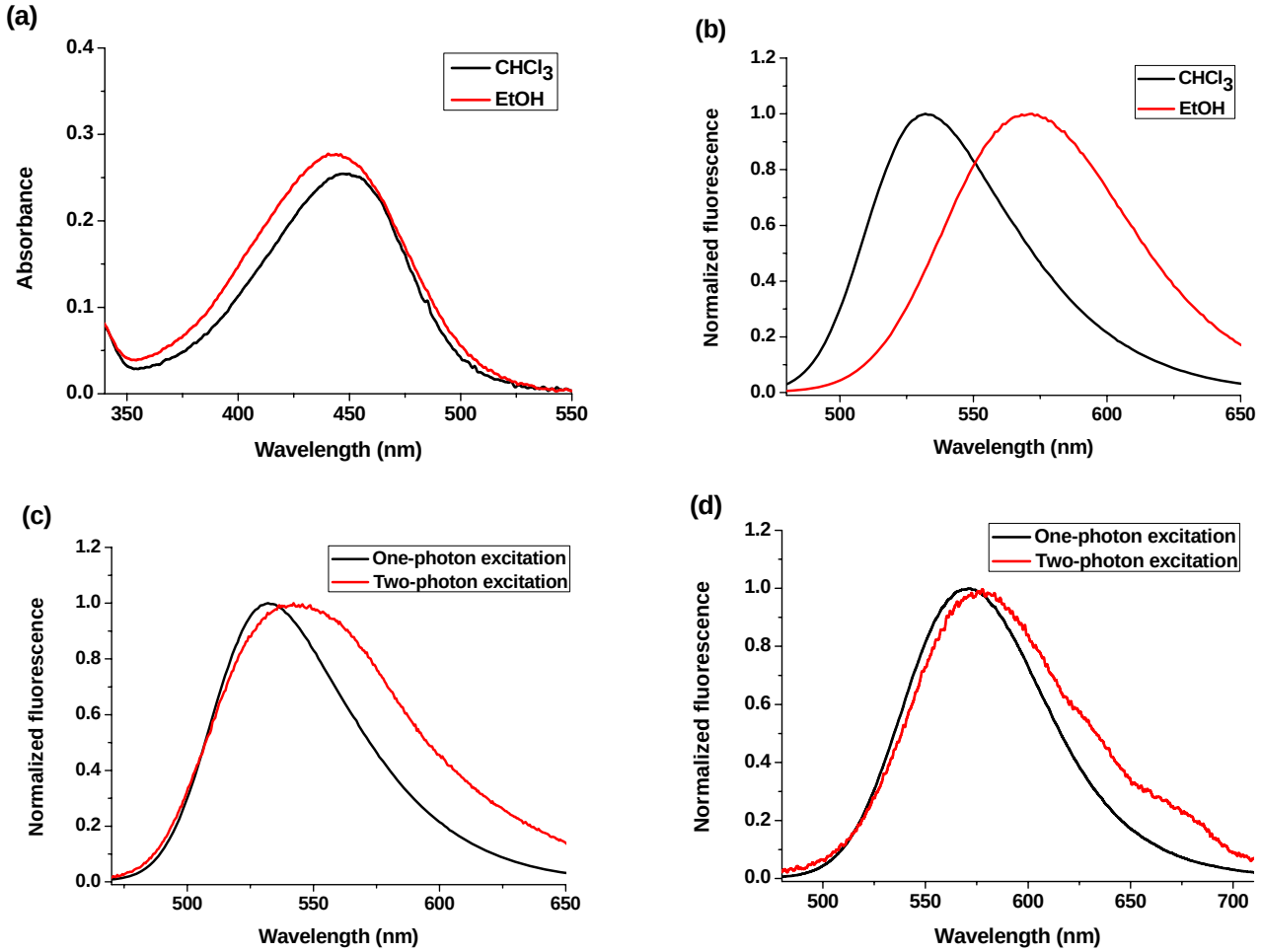


Fig. S2. One- and two-photon absorption and emission spectra of IminoPOS (10 μM) in $CHCl_3$ and EtOH, respectively. One- and two-photon excitation experiments were carried out by exciting at 400 nm and 800 nm. (a) Absorbance spectra obtained in $CHCl_3$ and EtOH respectively. (b) Normalized fluorescence spectra measured in $CHCl_3$ and EtOH respectively. (c) Normalized fluorescence spectra obtained by one- and two-photon excitation in $CHCl_3$. (d) Normalized fluorescence spectra by one- and two-photon excitation in EtOH.

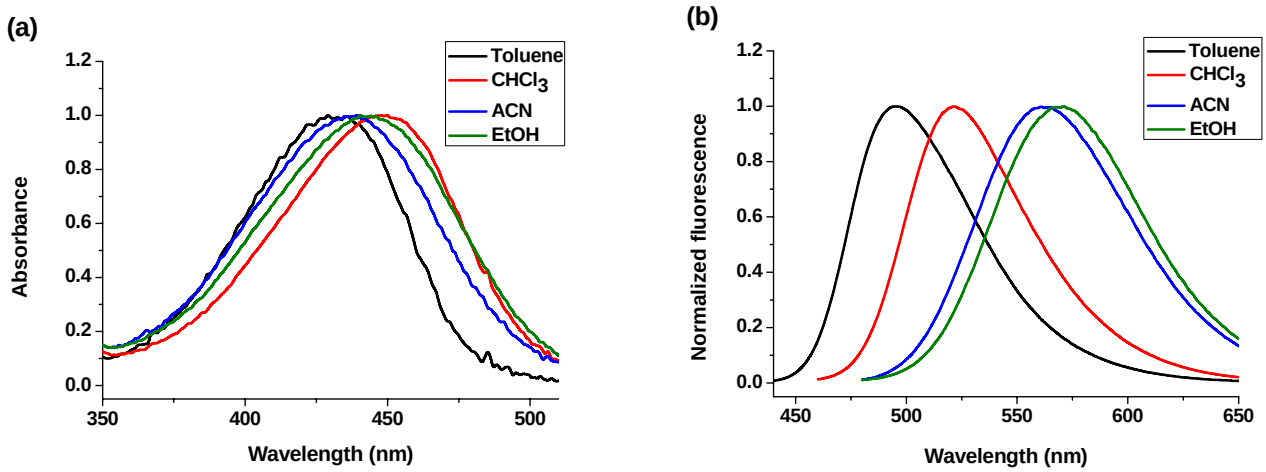


Fig. S3. Absorption and emission spectra for IminoPOS dissolved in various solvents (10 μM). (a) Normalized absorption spectra, (b) Normalized emission spectra at each maximum absorbance wavelength.

Absorption and Emission Properties of Probes 1, IminoPOS, and a Mixture of Probe 1a and MAO A. UV absorption spectra were obtained using a HP 8453 UV/Vis spectrophotometer. Fluorescence spectra were recorded on a Photon Technical International Fluorescence system with a 1-cm standard quartz cell. The concentrations of probe **1** and IminoPOS are 10 μM in buffer (100 mM HEPES, pH 7.4 with 5% glycerol and 1% DMSO) respectively. The emission spectra of probes **1** and IminoPOS were obtained with excitation wavelength at 448 nm (Figure S4 a, b). A reaction mixture of probe **1a** (70 μM) and MAO A (50 μg/mL) gives fluorescence when excited at 448 nm (Figure S4 c). In the normalized emission spectra, the fluorescence spectra of IminoPOS and that of reaction mixture of probe **1a** and MAO A were matched but not completely, possibly owing to a different nature of the "MAO-bound" IminoPOS (Figure S4 d).

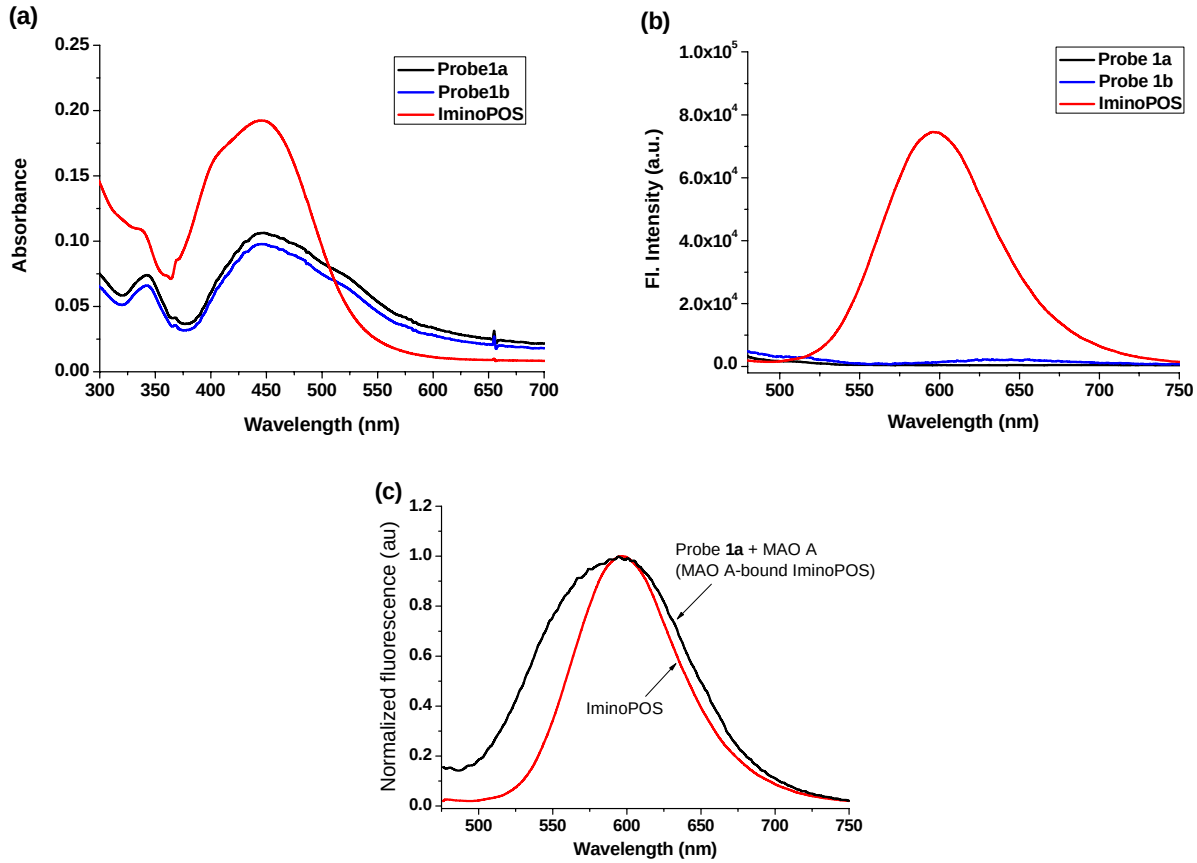


Fig. S4. (a) Absorption spectra of probe **1** and IminoPOS (10 μM in the buffer) respectively. (b) Emission spectra of probe **1** and IminoPOS, measured at each maximum absorbance wavelength, respectively. (c) Normalized emission spectra of IminoPOS and a mixture of probe **1a**/MAO A.

Fluorescence response of probes 1a and 1b toward MAO A and MAO B. Typically, the enzyme's concentration is fixed at 50 μg/mL. The probe's concentration is fixed at the K_m value (**1a**/MAO A; 70 μM, **1b**/MAO A; 252 μM, **1a**/MAO B; 75 μM, **1b**/MAO B; 210 μM). The fluorescence responses and time-dependent fluorescence enhancements were monitored by fluorescence spectrometer with excitation wavelength 448 nm. Time-dependent fluorescence enhancements were monitored at 10 min-intervals at the same conditions (Figure S6).

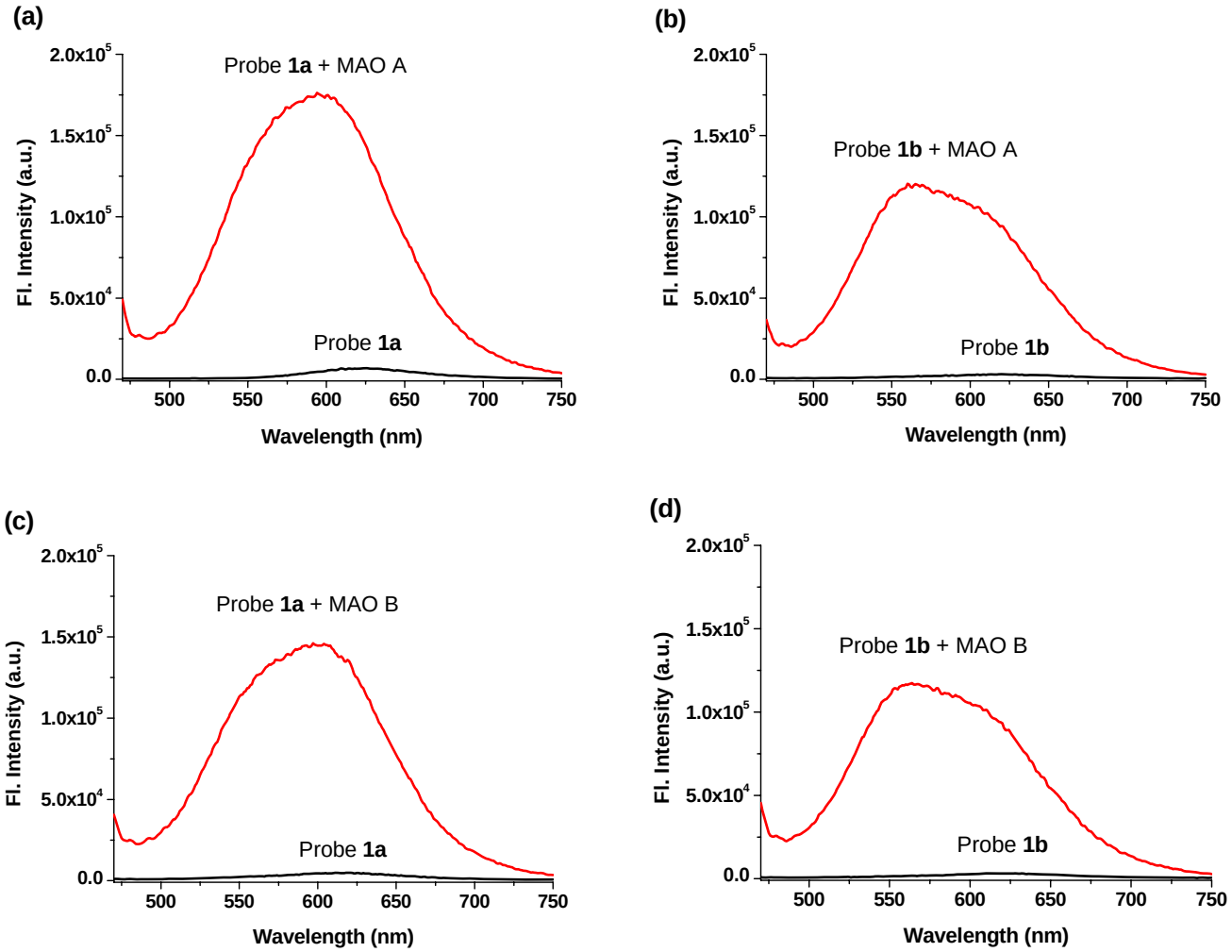


Fig. S5. Fluorescence changes of probes **1** upon treatment with MAOs A and B. Each of the spectra was checked after 90 min (excitation wavelength: 448 nm): (a) probe **1a**/MAO A, (b) probe **1b**/MAO A, (c) probe **1a**/MAO B, and (d) probe **1b**/MAO B.

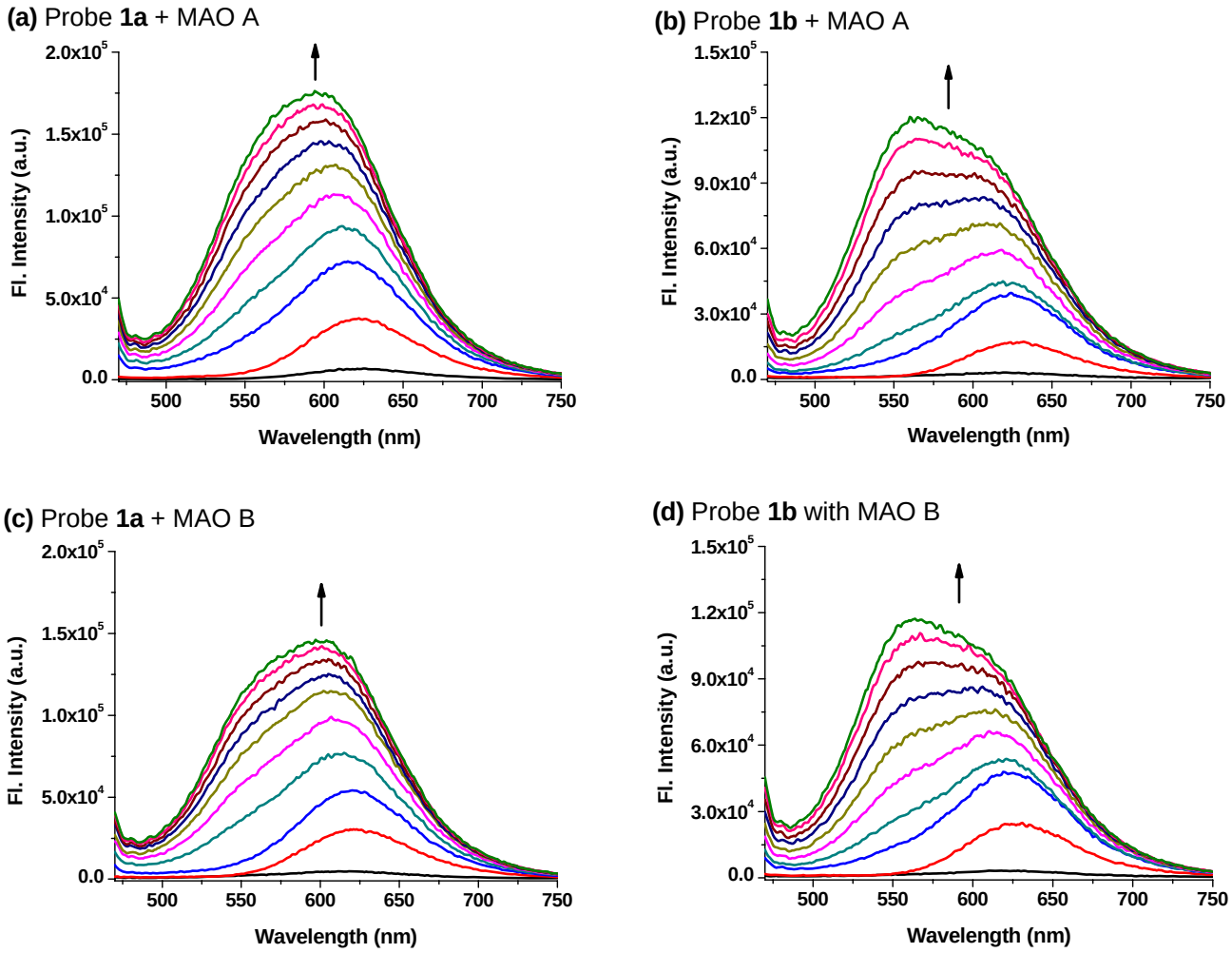


Fig. S6. Time-dependent fluorescence changes of probes **1** upon treatment with MAOs A and B. Each of the data was recorded for a reaction period from 0 min to 90 min at 10-min time intervals (excitation wavelength: 448 nm): (a) probe **1a**/MAO A, (b) probe **1b**/MAO A, (c) probe **1a**/MAO B, and (d) probe **1b**/MAO B.

Enzyme kinetic assays. A 96-well fluorescence assay plate was used for this assay. Changes in the fluorescence intensity for each of the reaction mixtures between probes **1** and MAOs (the final enzyme concentration: 10 μg/mL) were monitored after incubating at 37 °C for 3 h, using a microplate spectrofluorometer (VICTOR 3 V^{TM} multilabel counter, Perkin Elmer–Wellesley, MA, USA). Each of the enzyme reactions was performed at 37 °C in a buffer (100 mM HEPES, pH 7.4 with 5% glycerol and 1% DMSO). The fluorescence enhancement was recorded after the 3 h-incubation for each mixture, using a fluorescence VICTOR multilabel counter (the excitation wavelength: 465 nm; the detection wavelength: 600 nm).

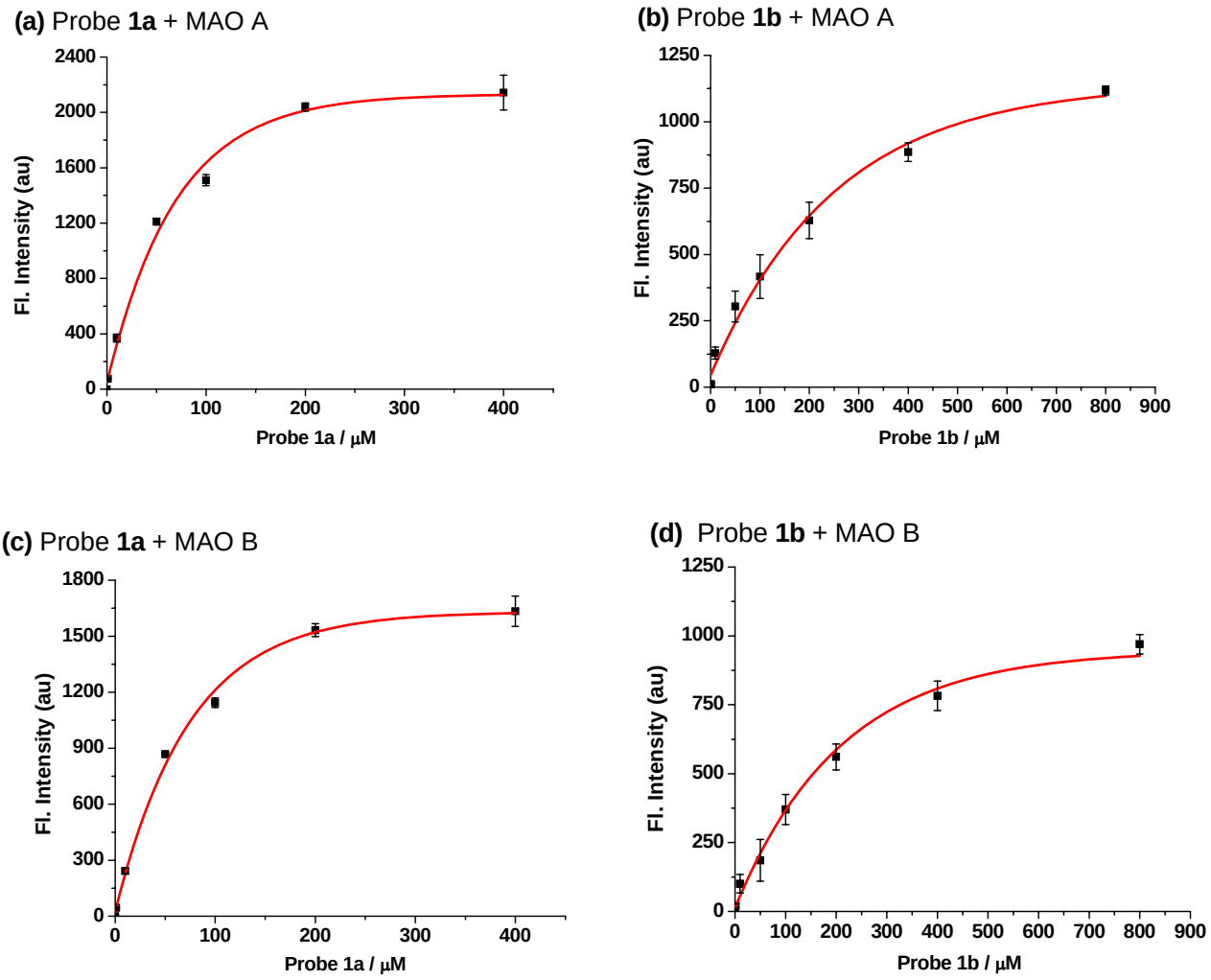


Fig. S7. Plots of the fluorescence intensity versus varying concentrations of probe **1a** or **1b** (the final concentration of the enzyme: 10 μg/mL): (a) probe **1a**/MAO A, (b) probe **1b**/MAO A, (c) probe **1a**/MAO B, and (d) probe **1b**/MAO B.

Table S2. K_m values (μM) of probe **1a** and **1b** for MAOs[a]

	MAO A	MAO B
1a	69 ± 8	74 ± 6
1b	252 ± 36	210 ± 22

[a] Probe **1a** and **1b**: 0–400 μM; MAOs A and B: 10 μg mL^{-1}; after incubation for 3 h at 37 °C.

Influence of MAO inhibitors on the enzyme assay with probes 1a. A 96-well fluorescence assay plate was used for this assay. MAO A or B was incubated in the presence of Moclobemide (70 μM) or Pargyline (70 μM) for 2 h at 37 °C, and then it was treated with probe **1a** (70 μM) and further incubated for 3 h at 37 °C (the final concentration of the enzyme was 10 μg/mL), during which period the time-dependent fluorescence enhancements were measured at 10-min intervals, using a microplate spectrofluorometer (VICTOR 3 V^{TM} multilabel counter, Perkin Elmer–Wellesley, MA, USA) (the excitation wavelength: 465 nm; the detection wavelength: 600 nm).

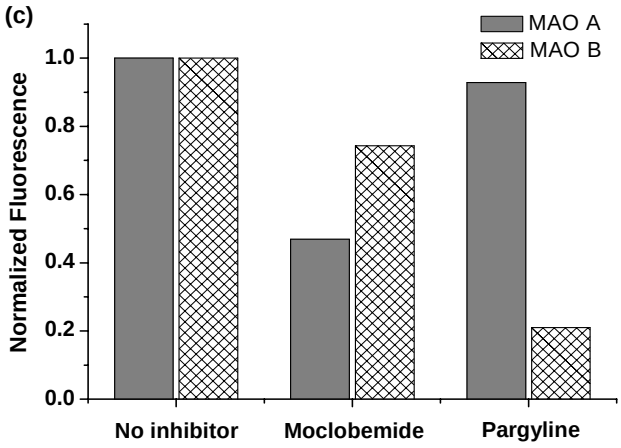


Fig. S8. Enzyme inhibition assays. Fluorescence response of probe **1a** (70 μM) toward MAO A or MAO B (the final concentration: 10 μg mL^{-1}) in the absence and presence of inhibitors, Moclobemide or Pargyline (70 μM for each). Comparison of the normalized fluorescence intensity data at each of the K_m values.

Cell culture and imaging

Preparation of bovine chromaffin cells and cell culturing. Chromaffin cells were isolated from the bovine adrenal gland medulla by two-step collagenase digestion as previously described.[4] Cells were grown at the density of 2×10^6 cells per 60-mm dish. C6 glioma cells and chromaffin cells were maintained in DMEM (HyClone) with 10% fetal bovine serum (Hyclone) and 1% antibiotics (WelGENE). Cells were incubated in a humidified atmosphere of 5% CO_2–95% air at 37 °C.

Chemical treatment and acquiring fluorescent microscope images. For live cell imaging for the MAO activity, primary chromaffin cells were seeded at a density of 2×10^6 in a 60-mm dish and cultured for 24 h. Control C6 glioma cell lines were cultured with the same protocol as chromaffin cells. After 24 h of culture, the live cells were treated with 100 μM of IminoPOS (as control) or the MAO specific probe and incubated for 1 h. The cells were washed with Phosphate Buffered Saline (PBS). An Axiovert 200M fluorescence imaging microscope (Carl Zeiss) was used to capture fluorescent images with an excitation filter (435–455 nm) and a dichromatic emission filter (555–575 nm). In the inhibition investigation, the live chromaffin cells were pretreated separately with 100 μM Moclobemide or pargyline for 1 h. The control probe (IminoPOS) or the MAO specific probe was then added to the cells with a final concentration of 100 μM and incubated for another 1 h at 37 °C. The cells were then washed with PBS. The fluorescence imaging was acquired with the fluorescence imaging microscope.

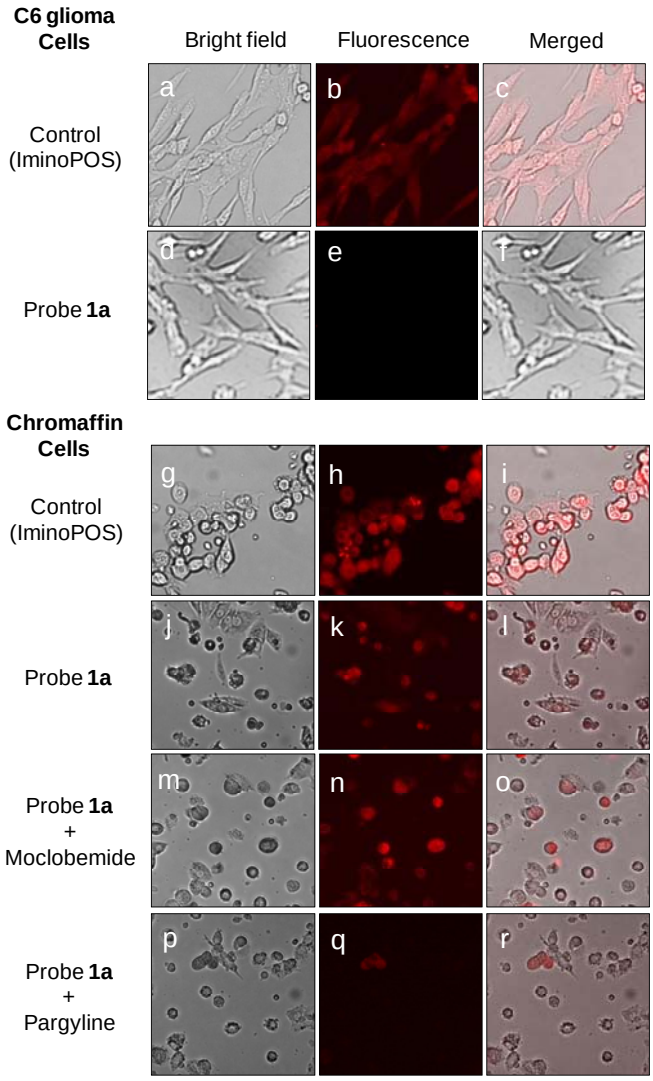


Fig. S9. Representative one-photon fluorescence images of C6 glioma cells (upper, a–f) and chromaffin cells (lower, g–r)

Two-photon fluorescence microscopy. A point scanning two-photon microscopy (TPM) was used to obtain probe-labeled images. System was build using an upright microscope (BX51; Olympus) with a 20× objective lens (XLUMPLFLN-W 20×, NA 1.0 water immersion, Olympus) and the images were obtained by exciting the probes with a mode-locked femtosecond Ti:Sapphire laser (Chameleon Ultra II, Coherent). The laser source could emit near infrared (NIR) wavelength between 680 nm to 1020 nm with a repetition rate of 80 Mhz and a pulse width of 140 fs. In order to scan the specimen, we used two galvanometer mirror (6215H, Cambridge Technoloogy) allowed x-axis and y-axis scanning. We set the wavelength at 790 nm and 900 nm, which corresponded to approximately 17 mW and 10 mW average power in the focal plane, respectively. In the microscope, the excitation light went through a 680 nm short pass dichroic mirror (DM1, 680DCSPXR, Chroma) and another 680 nm long pass dichroic mirror (DM2, 680DCXXR, Chroma). The emitted fluorescence was passed through the objective and reflected to the detection path by the 680 nm long pass dichroic mirror. It was collected by photomultiplier tube (PMT, H5783P, Hamamatsu) and the signals were constructed an 8 bit unsigned 256 × 256 pixels at 50 ps pixel residence rate (20 Khz). Based on this experiment conditions, fluorescence emission spectra was observed by 900 nm excitation by TPM at 10 mW laser power and 0.382 frame/s imaging speed.

Cell viability assessments. Cell viability was assessed by measuring their ability to metabolize 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)[5] in C6 glioma cell line. Cells were seeded onto 96-well plates at a density of about 5×10^3 cells per well in the growth medium and incubated until about 70–80% confluency. Following the treatments as indicated, 25 μL of MTT solution (5 mg/mL) was added to each well, and cells were maintained for 2 h at 37 °C. 100 μl of a solubilizing solution (50% dimethylformamide and 20% SDS, pH 7.4) was then added. After an overnight incubation at 37 °C, absorbance at 570 nm was measured.

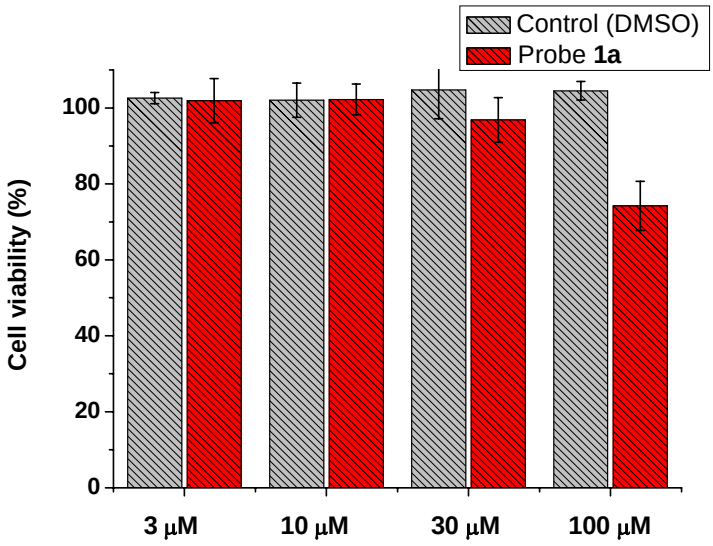


Fig. S10. Cell viability of substrate probe **1a** in C6 glioma cell line. Cell culture with DMSO solution as a control.

Reference

1) The synthesis of compound **4** will be reported in a full account.
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