Supporting Information for

Visualization of Tyrosinase Activity in Melanoma Cells by a BODIPY-Based Fluorescent Probe

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Experimentals

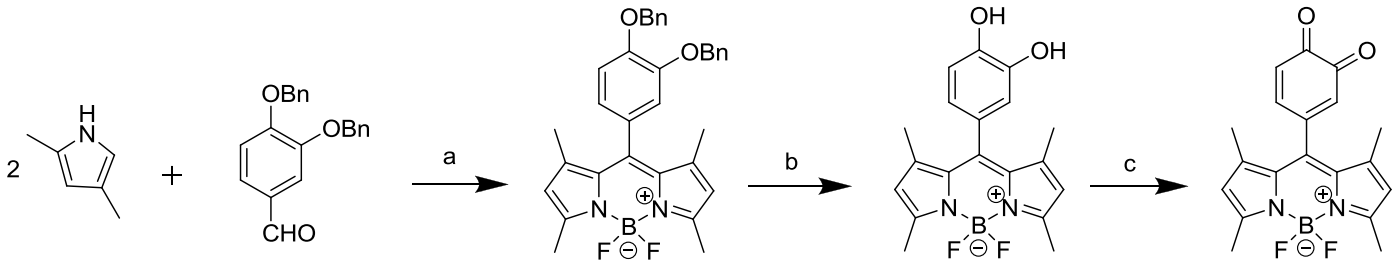
Materials

All reagents were of the highest commercial quality and used as received without further purification. All solvents were spectral grade unless otherwise noted. Anhydrous CH_2Cl_2 and THF were obtained as a sure-seal bottle from Aldrich Co. Inc. (Milwaukee, WI). Silica gel (40 μm) was obtained from Merck Inc. Aqueous solutions were freshly prepared with deionized water from a water purification system (Human Corp. Korea). 2,4-Dimethylpyrrole (**2**), 3,4-bisbenzyloxybenzaldehyde (**3**), L-DOPA were purchased from Aldrich Co. Inc. (Milwaukee, WI). Tyrosinase (EC 1.14.18.1, 4276 U/mg) from mushroom and miconazole were purchased from Sigma. Kojic acid and anisaldehyde for inhibition assay were purchased from TCI Co. Inc. (Tokyo), and benzaldehyde and benzoic acid were purchased from Aldrich Co. Inc. (Milwaukee, WI), respectively.

General methods, instrumentation and measurements

Synthetic manipulations that required an inert atmosphere (where noted) were carried out under argon using standard Schlenk techniques. NMR (^{1}H, ^{13}C) spectra were recorded on Bruker 400 MHz or 500 MHz spectrometers. The chemical shift data for each signal are given in units of δ (ppm) relative to tetramethylsilane (TMS) where δ (TMS) = 0, and referenced to the residual solvent resonances. Splitting patterns are denoted as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). High-resolution FAB-MS or ESI-MS were obtained at national center for inter-university research facilities. Absorption spectra were obtained on a Shimadzu UV-2501 spectrophotometer. Fluorescence measurements were recorded on a Hitachi F-7000 fluorescence spectrophotometer using 10 mm quartz cuvettes with a path length of 1 cm. Fluorescence quantum yields were determined by standard methods, using fluorescein (Φ_F = 0.95 in 0.1 N NaOH) as a standard. The reaction with tyrosinase was measured by monitoring changes in fluorescence intensity using a Synergy Mx Microplate Reader (BioTek, USA).

1. Synthesis of compounds



Scheme S1. Synthetic scheme of probe **1** and **1-Q**, Reagents and conditions a) i) TFA, THF, 6 h, r.t., ii) DDQ, CH_2Cl_2, 5 h, r.t., iii) TEA, $BF_3{\cdot}OEt_2$, overnight, r.t., 49%; b) H_2, Pd/C, MeOH/$CHCl_3$, overnight, r.t., 78%; c) $FeCl_3$, MeOH, r.t., 45%.

8-[3,4-Bis(benzyloxy)phenyl]-1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (4)[1]

To a stirred mixture of 2,4-dimethylpyrrole (410 µL, 4.0 mmol) and 3,4-bisbenzyloxybenzaldehyde (640 mg, 2.0 mmol) in dry THF (250 mL) was added 2 drops of TFA under argon atmosphere. The reaction was stirred at room temperature until TLC showed complete consumption of the 3,4-bisbenzyloxybenzaldehyde. To the reaction solution was added DDQ (500 mg, 2.2 mmol) that was dissolved in dry CH_2Cl_2 (150 mL). After the solution was stirred at room temperature for 6 h, triethylamine (4 mL, 23 mmol) and $BF_3{\cdot}OEt_2$ (3 mL, 24 mmol) were added sequentially to the reaction mixture via syringe, and the resulting solution was stirred for overnight. After the reaction was completed, the reaction solution was evaporated under vacuum and extracted with CH_2Cl_2. The separated organic fractions were dried with $MgSO_4$, filtered, and evaporated under vacuum to yield a black crude compound. The crude product was purified by column chromatography on silica gel using progressively more polar 25:1 to 10:1 hexanes : ethyl acetate as the mobile phase to afford **4** as a red solid (525 mg, 49%).; ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.39-7.16 (m, 10H), 6.92 (d, 1H, J = 8 Hz), 6.72 (s, 1H), 6.65 (d, 1H, J = 8 Hz), 5.85 (s, 2H), 5.14 (s, 2H), 5.08 (s, 2H), 2.45 (s, 6H), 1.22 (s, 6H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 155.1, 149.1, 143.0, 141.1, 136.5, 136.4, 131.4, 128.4, 128.3,

127.8, 127.8, 127.7, 127.3, 127.1, 120.9, 120.8, 115.5, 114.6, 71.2, 70.8, 14.5, 14.2; HR-MS (ESI): calcd. for $C_{33}H_{31}BF_2N_2O_2$. $[M+H]^+$ 537.2517, found 537.2525.

8-(3,4-Dihydroxyphenyl)-1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (1)[1]

A mixture of **4** (165 mg, 0.31 mmol) and 10% Pd/C (183 mg, 56 mol % Pd) in 50 mL of 2:3 $CHCl_3$/MeOH was stirred under a H_2 atmosphere (1 atm) at room temperature for 24 h. The mixture was filtered through Celite, and the filter cake was washed with MeOH and EtOAc. The dark-brown crude solid was purified by column chromatography on silica gel using progressively more polar 3:1 to 1:1 hexanes : ethyl acetate as the mobile phase to afford **1** as a red solid (86 mg, 78%).; ^{1}H-NMR (500 MHz, CD_3OD): δ = 6.93 (d, 1H, J = 4.0 Hz), 6.69 (s, 1H), 6.58 (dd, 1H, J = 2.0, 4.0 Hz), 6.04 (s, 2H), 2.47 (s, 6H), 1.55 (s, 6H); ^{13}C-NMR (125 MHz, CD_3OD): δ = 154.9, 146.4, 143.5, 143.0, 131.8, 126.0, 120.8, 119.3, 116.0, 115.0, 13.5, 13.4; HR-MS (FAB): calcd. for $C_{19}H_{19}BF_2N_2O_2$. $[M]^+$ 356.1507, found 356.1508.

8-(*o*-Quinone)-1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4adiaza-*s*-indacene (1-Q)[1]

A solution of **1** (40 mg, 122 μmol) was mixed with a solution of $FeCl_3$ (911 mg, 5.61 mmol) in 10 mL of MeOH and the resulting mixture was stirred at room temperature for 30 min. The mixture was diluted with H_2O (50 mL) and extracted with $CHCl_3$, The combined organic layer was dried over $MgSO_4$, filtered and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel using progressively more polar 10:1 to 3:1 hexanes : ethyl acetate as the mobile phase to afford **1-Q** as a red-purple solid (18 mg, 45%).; ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 6.91 (d, 1H, J = 10 Hz), 6.57 (d, 1H, J = 10 Hz), 6.45 (s, 1H), 6.03 (s, 2H), 2.49 (s, 6H), 2.13 (s, 6H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 178.7, 178.2, 157.8, 146.8, 141.5, 140.8, 134.2, 131.6, 130.3, 128.3, 122.2, 15.8, 14.8;. HR-MS (ESI): calcd. for $C_{19}H_{17}BF_2N_2O_2$. $[M+Na]^+$ 377.1241, found 377.1247.

2. Studies of photophysical properties

Absorption and emission spectra of compounds

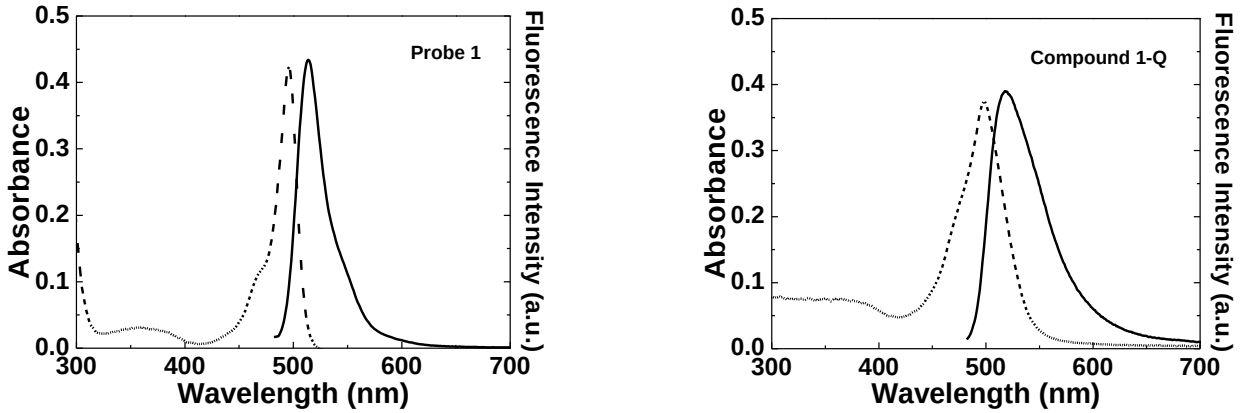


Figure S1. Absorption (dashed lines) and emission spectra (solid lines) of probe **1** (left) and compound **1-Q** (right) in PBS buffer (50 mM, pH 6.3) containing 0.2% DMSO as a cosolvent at 25 ℃. Excited at 465 nm. [**1**] = [**1-Q**] = 5 μM.

Table S1. Photophysical properties of probe **1** and compound **1-Q**[a]

Compound	$\lambda_{abs,\,max}$ [nm]	ε[b] [$M^{-1}cm^{-1}$]	$\lambda_{em,\,max}$[c] [nm]	Φ_{FL}[d]
Probe **1**	496	8.8×10^4	512	0.001
Compound **1-Q**	498	7.5×10^4	515	0.018

[a]Data were obtained in PBS buffer (50 mM, pH 6.3) containing 0.2% DMSO as a cosolvent at 25 ℃. [b]Measured at each absorption maximum. [c]Excited at 465 nm. [d]Quantum yields vs. fluorescein in 0.1 N NaOH (Φ_F = 0.95).[2]

3. Stability studies

Photostability test of probes 1 and compound 1-Q in aqueous solution

We have investigated photostabilities of **1** and **1-Q** in PBS buffer (50 mM, pH 6.3, 0.2% DMSO, 25 ℃) in the presence of oxygen. The photooxidation studies were performed by continuous UV irradiation of each compound using a 150-W steady-state Xe lamp as the irradiation source. The photoinduced degradation was quantified by monitoring the fluorescence intensity of each compound as a function of elapsed photolysis time. Negligible changes in the fluorescence intensity of **1** and **1-Q** were observed after 2.5 hours of irradiation at 465 nm.

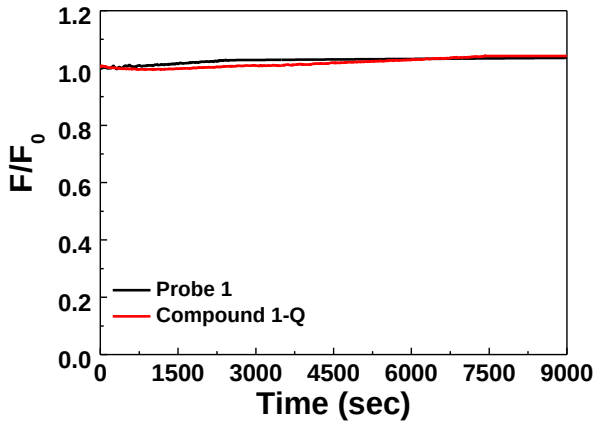


Figure S2. Photostability of probe **1** and compound **1-Q** in PBS buffer (50 mM, pH 6.3, 25 ^{o}C) containing 0.2% DMSO as a cosolvent. Fluorescence intensity was measured at 512 nm for probe **1** and at 515 nm for compound **1-Q**. Excited at 465 nm. [**1**] = [**1-Q**] = 5 μM.

Chemical stability test of probe 1 and compound 1-Q in aqueous solution

The chemical stabilities for probe **1** and compound **1-Q** were examined by monitoring change of fluorescence intensity of each compound in PBS buffer (50 mM, pH 6.3, 0.2% DMSO, 25 ℃) in the presence of oxygen. As a result, negligible changes in the fluorescence intensity of **1** and **1-Q** were observed even after 5 hours. Therefore, background fluorescence due to competing non-enzymatic oxidation would be minimal.

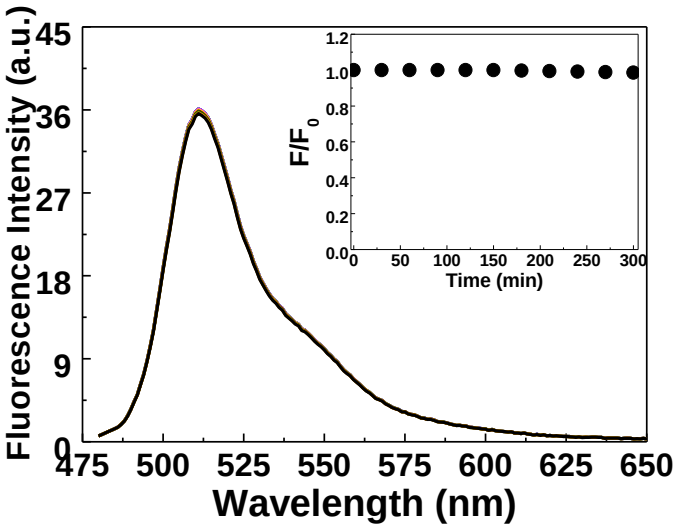


Figure S3. Chemical stability of probe **1** (5 μM) in PBS buffer (50 mM, pH = 6.3, 0.2% DMSO, 25 ^{o}C) in the presence of O_2. The emission spectra were obtained every 0.5 hour (0 – 5 hour). Excited at 465 nm. Inset shows the relative fluorescence intensity at 512 nm versus incubation time.

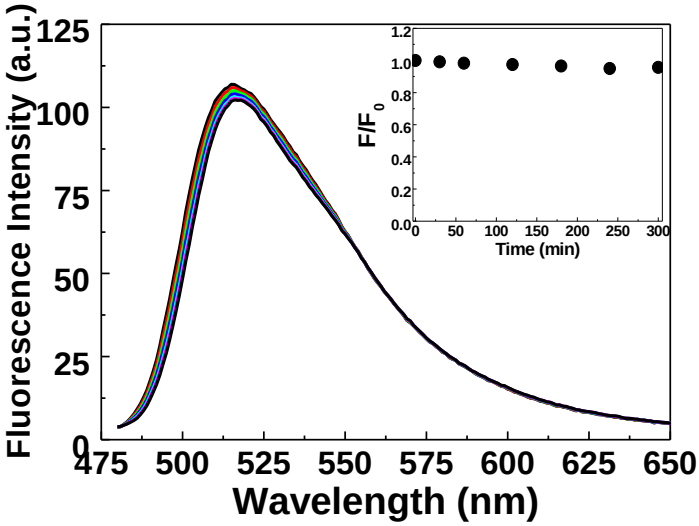


Figure S4. Chemical stability of **1-Q (**5 μM) in PBS buffer (50 mM, pH = 6.3, 0.2% DMSO, 25 °C). The spectra were obtained every 1 hour (0 – 5 hour). Excited at 465 nm. Inset shows the relative fluorescence intensity at 515 nm versus incubation time.

Absorption and emission spectra of probe 1 and compound 1-Q as a function of pH

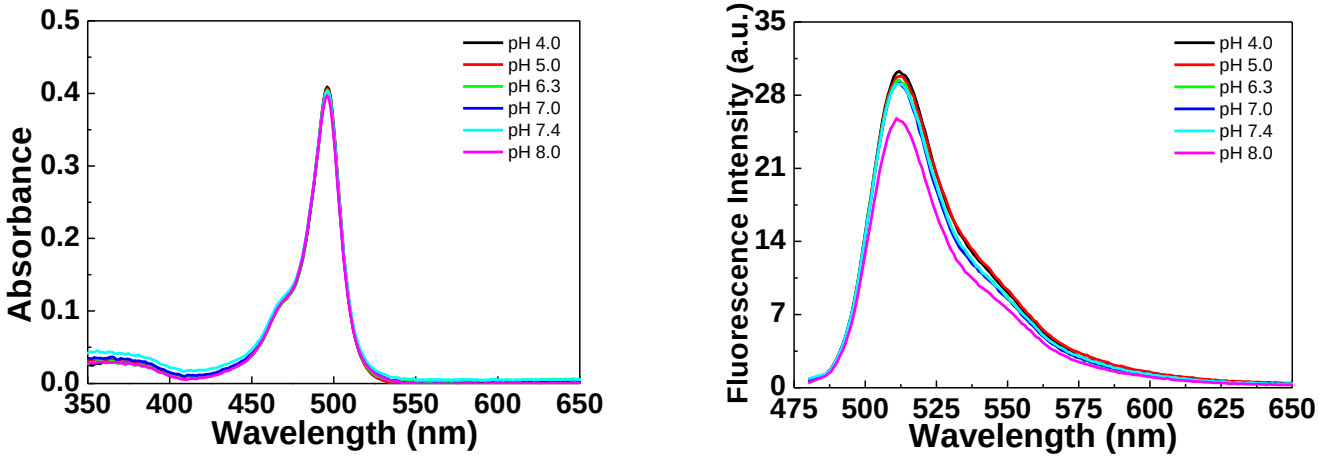


Figure S5. Absorption (left) and emission spectra (right) of probe **1** (5 μM) in different pH buffer systems containing 0.2% DMSO as a cosolvent at 25 °C. Excited at 465 nm.

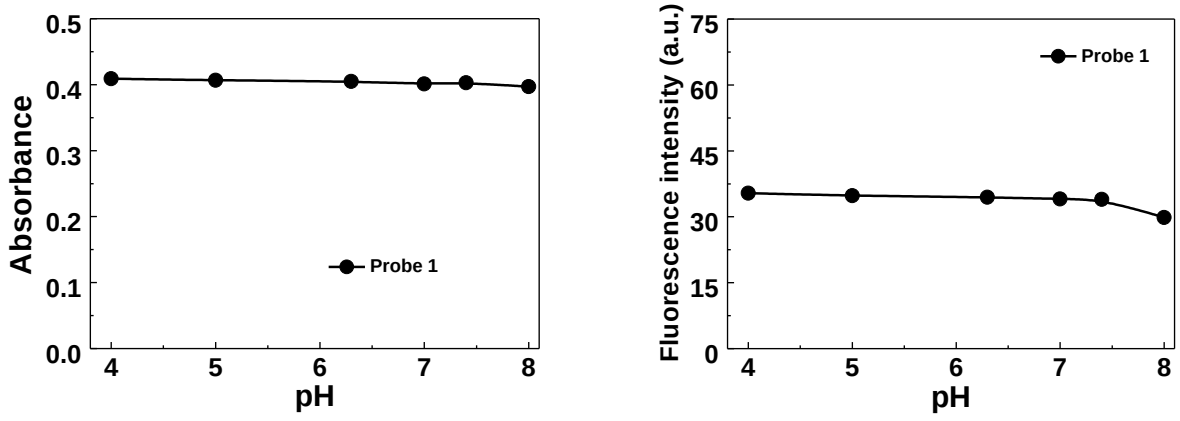


Figure S6. Effect of pH on the absorbance (left) and fluorescence (right) intensities of probe **1** at 25 °C. [**1**] = 5 μM. Absorbance at 496 nm and emission at 512 nm were recorded.

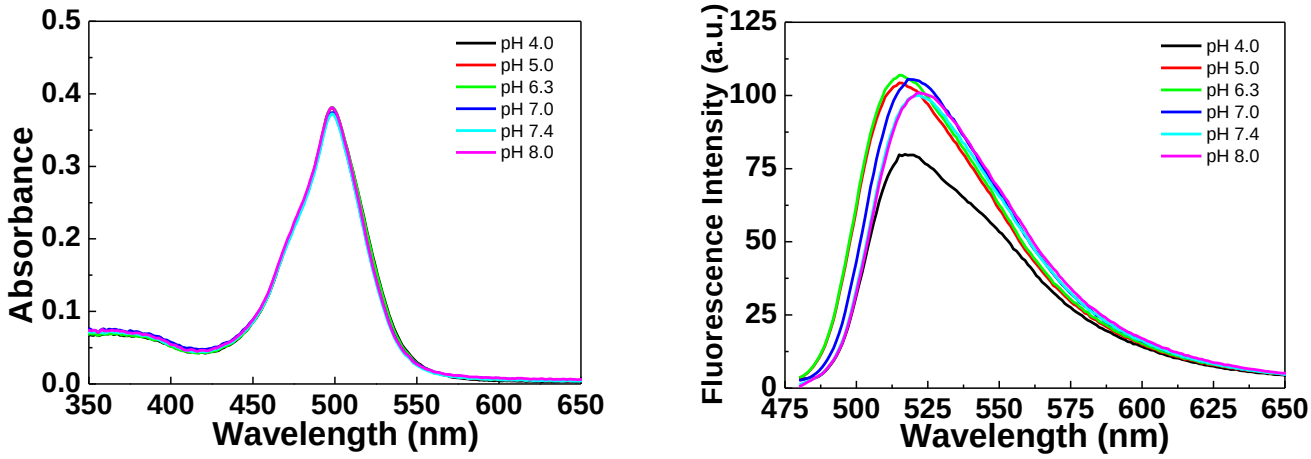


Figure S7. Absorption (left) and emission spectra (right) of probe **1-Q** (5 μM) in different pH buffer systems containing 0.2% DMSO as a cosolvent at 25 ^{o}C. Excited at 465 nm.

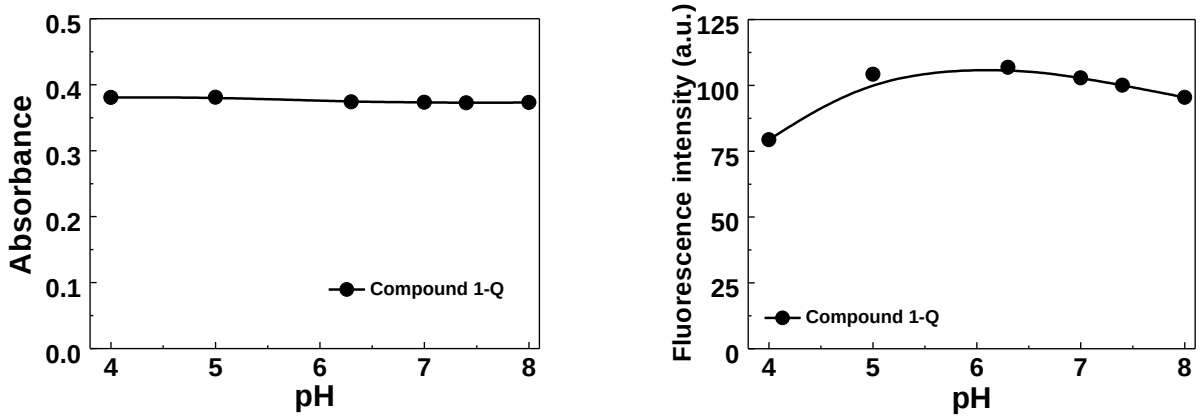


Figure S8. Effect of pH on the absorbance (left) and fluorescence (right) intensities of compound **1-Q** at 25 ^{o}C. [**1-Q**] = 5 μM. Absorbance at 498 nm and emission at 515 nm were recorded.

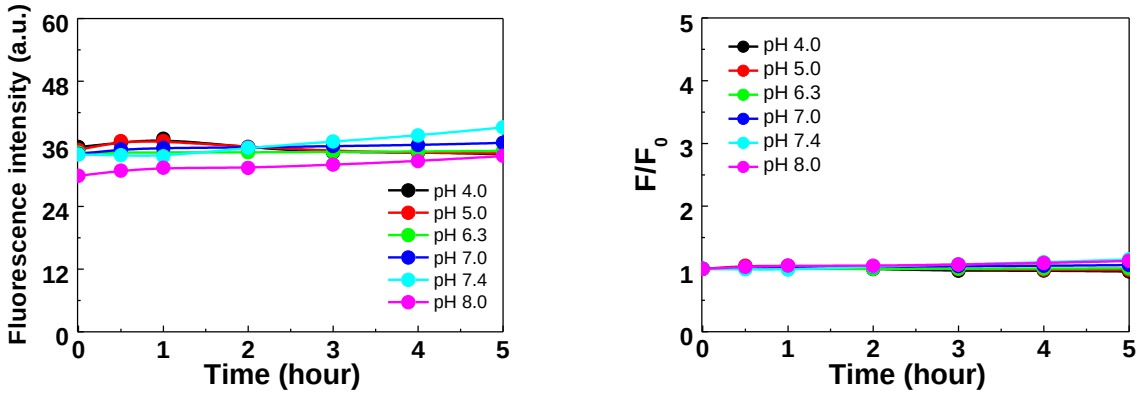


Figure S9. Stability of probe **1** in different pH buffer systems containing 0.2% DMSO as a cosolvent at 25 ^{o}C. The spectra were obtained with excitation at 465 nm every 0.5 hour (0 – 5 hour). Fluorescence intensity was measured at 512 nm. [**1**] = 5 μM.

4. Enzymatic oxidation of probe by tyrosinase

4.1. Optimization conditions for tyrosinase assays

Enzyme assay with probe 1 in various buffer systems

In order to determine the optimal pH condition for the enzyme-catalyzed reaction, tyrosinase-mediated oxidation of probe **1** was investigated in 50 mM phosphate-buffered solution at pH values of 6.3, 7.0, and 7.4. The maximum fluorescence response was observed at pH 6.3 (Figure S10). It was consistent with the optium pH of tyrosinase activity reported in the literature.[3]

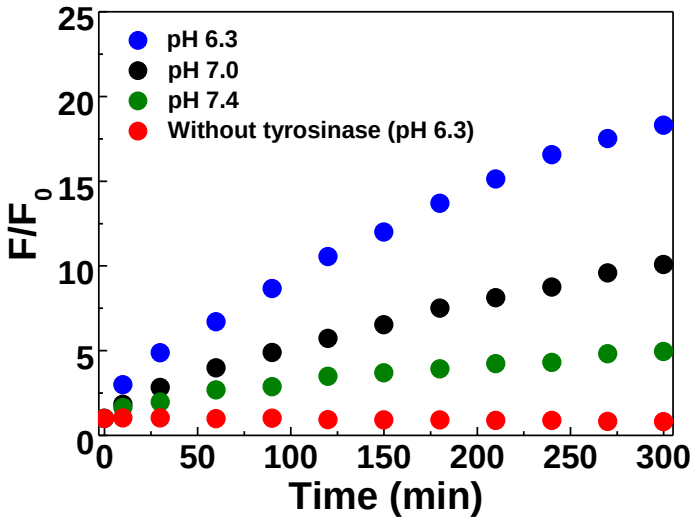


Figure S10. Fluorescence response of probe **1** (1 μM) upon incubation with tyrosinase (90 nM)/O_2 at 37 ^{o}C in PBS buffer solution (50 mM, 0.2% DMSO) at various pH conditions. Excited at 465 nm. The spectra were obtained every 30 min (0 – 300 min). Fluorescence intensity was measured at 515 nm.

Effect of O_2 on the tyrosinase assay

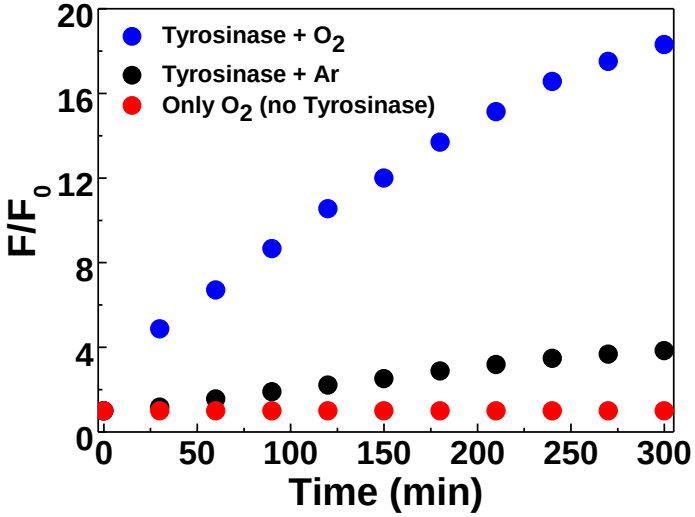


Figure S11. Relative fluorescence response of probe **1** (1 μM) in PBS buffer (50 mM, pH 6.3, 37 ^{o}C) containing 0.2% DMSO as a cosolvent upon incubation with tyrosinase (90 nM) in the presence of oxygen (blue) and in the presence of argon but in the absence of oxygen (black). Each assay solution was prepared by bubbling with oxygen or argon for 30 min, respectively. The spectra were obtained every 30 min (0 – 300 min) with excitation at 465 nm. Fluorescence intensity was measured at 515 nm. The bottom plot (red) shows fluorescence response of probe **1** in the only presence of oxygen but the absence of tyrosinase.

Enzyme assay with probe 1 at various concnetrations

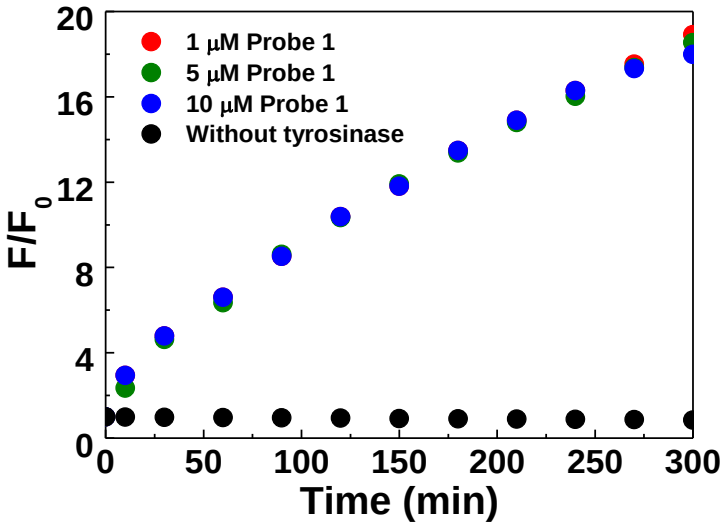


Figure S12. Relative fluorescence intensity (F/F_0) of probe **1** at different concentrations (1, 5, 10 μM) upon incubation with tyrosinase (90 nM)/O_2 in PBS buffer (50 mM, pH 6.3, 37 ^oC) containing 0.2% DMSO as a cosolvent. The spectra were obtained every 30 min (0 – 300 min). Fluorescence intensity was measured at 515 nm with excitation at 465 nm.

Enzyme assay with probe 1 as a function of concentration of tyrosinase

Tyrosinase was dissolved in PBS buffer (50 mM, pH 6.3) containing 0.2% DMSO as a cosolvent and diluted with the buffer solution to make different tyrosinase concentrations. Tyrosinase solution (20 μL) was mixed with the probe **1** (20 μL) solution in buffer solution (50 mM PBS, pH 6.3, 160 μL). Final probe concentration in the solution was 1 μM, and the tyrosinase concentration was 0, 0.1, 0.5, 1.0, 5.0, 10, 22.5, 45, 67.5, 90 nM, respectively. Fluorescence intensity at 515 nm was recorded after incubation for different time periods using a computer-controlled fluorescence plate reader.

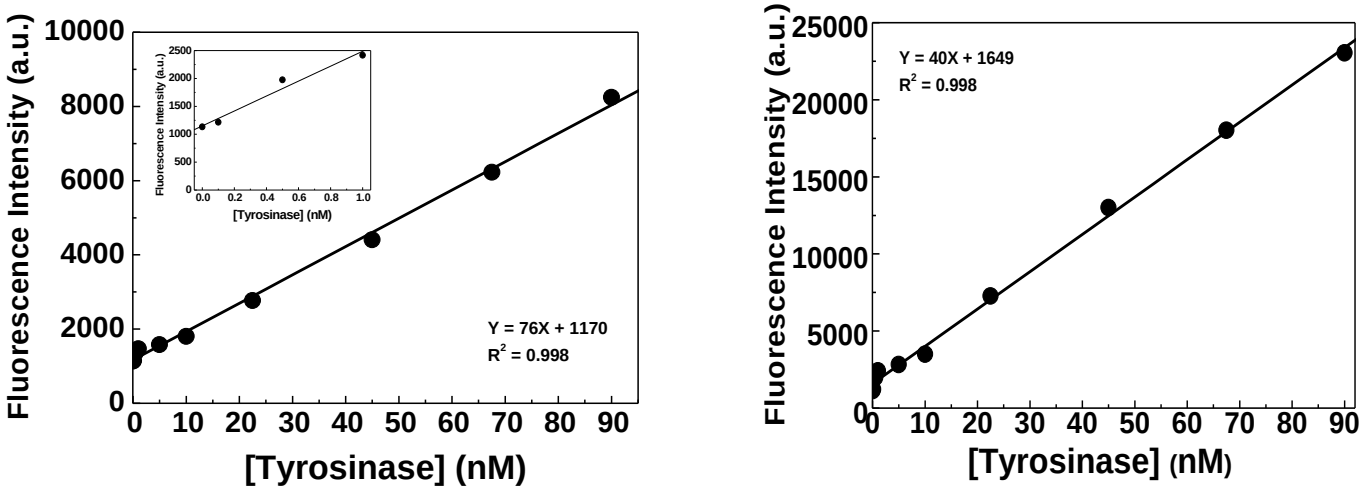


Figure S13. Fluorescence response of 1 μM probe **1** upon (left) 60 min and (right) 300 min incubation with different amounts of tyrosinase (from 0 to 90 nM) in PBS buffer (50 mM, pH 6.3, 37 ^oC) containing 0.2% DMSO as a cosolvent. Excited at 465 nm. Fluorescence intensity was measured at 515 nm. Inset shows fluorescence response of probe **1** upon incubation with low concentrations of tyrosinase (detection limit: 0.5 nM).

Enzyme assay with probe 1 as a function of incubation time

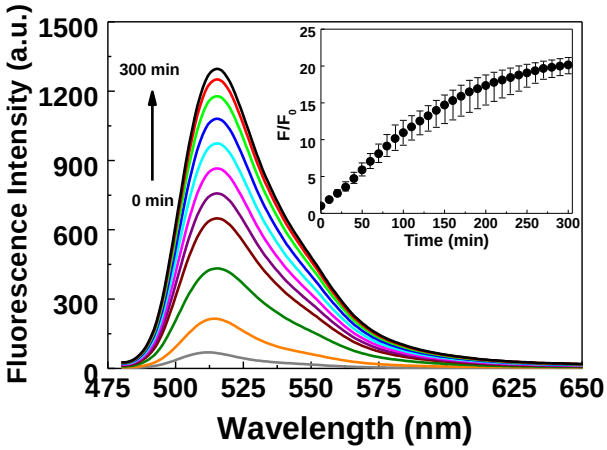


Figure S14. Fluorescence emission spectra of probe **1** (5 μM) upon incubation with tyrosinase (90 nM) for different time periods in PBS buffer (50 mM, pH 6.3, 37 ^{o}C) containing 0.2% DMSO as a cosolvent. Excited at 465 nm. Inset shows the increase of emission peak at 515 nm over 5 hours with error bars corresponding to variations over three trials.

4.2. Kinetic studies of probes

To determine the kinetic constants, probe **1** at a series of the final concentrations (0.1–10 μM) was hydrolyzed by tyrosinase (90 nM) in the presence of O_2. As standards, enzymatic reaction of L-DOPA, commercially available tyrosinase substrate, was also measured under the similar conditions. The reaction was monitored by measuring fluorescence change at 515 nm for probe **1** (excited at 465 nm), and absorbance at 475 nm for L-DOPA at 37 ^{o}C. The initial velocity was calculated from the slope of the each progress curve. The parameters such as K_M and k_{cat} with tyrosinase for probe **1** and L-DOPA were determined by Lineweaver-Burk plot and listed in Table S2.

Determination of kinetic constants for probe 1

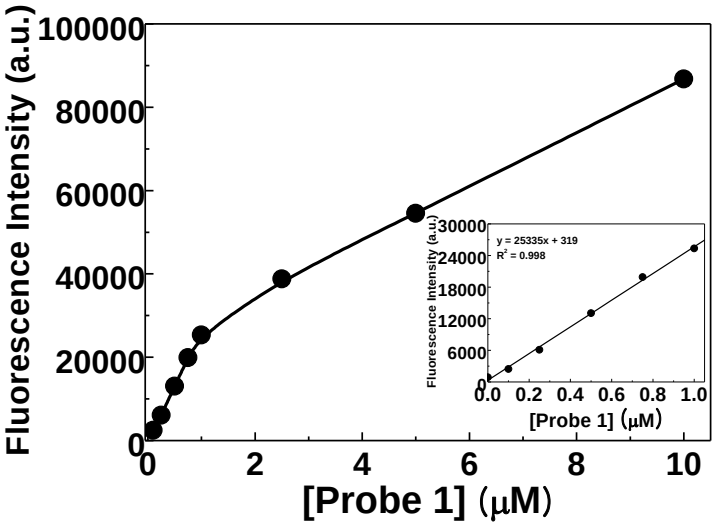


Figure S15. Standard fluorescence curve of probe **1** at different concentrations upon incubation with excess tyrosinase in PBS buffer (50 mM, pH 6.3, 37 ^{o}C) containing 0.2% DMSO as a cosolvent. Fluorescence intensity at 515 nm was measured. [**1**] = 0–10 μM. Inset shows linear increase of fluorescence intensity of probe **1** at low concentrations upon incubation with excess tyrosinase.

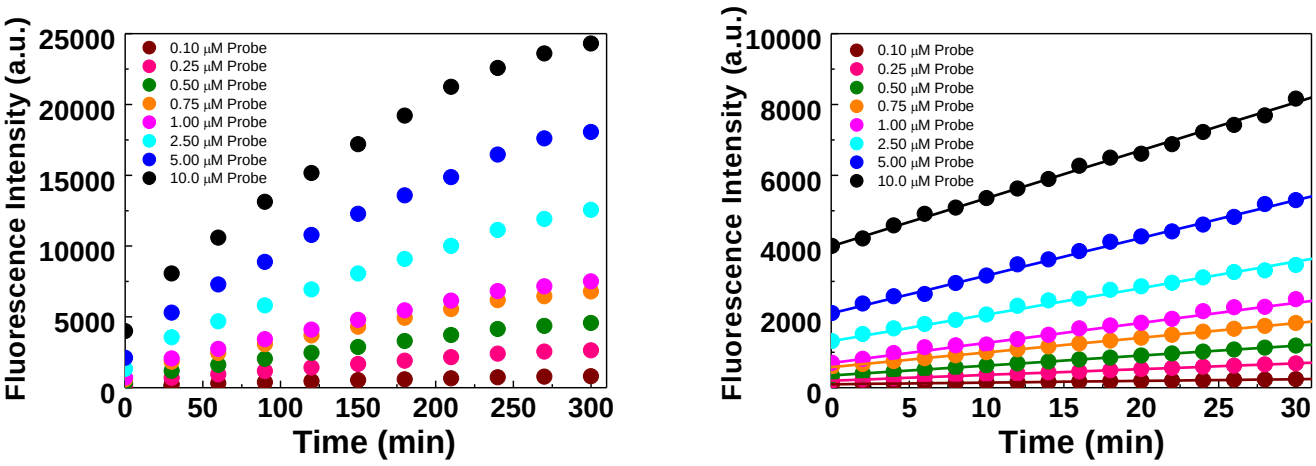


Figure S16. Fluorescence response of probe **1** by tyrosinase. Progress curves of oxidation of probe **1** at various concentrations (0.1–10 μM) upon addition of tyrosinase (25 nM). The spectra were obtained every 30 min upto 300 min (left) and 30 min (right). The enzyme reactions were carried out in 50 mM PBS (pH 6.3) containing 0.2% DMSO as a cosolvent at 37 ^{o}C. Fluorescence intensity was measured at 515 nm with excitation at 465 nm.

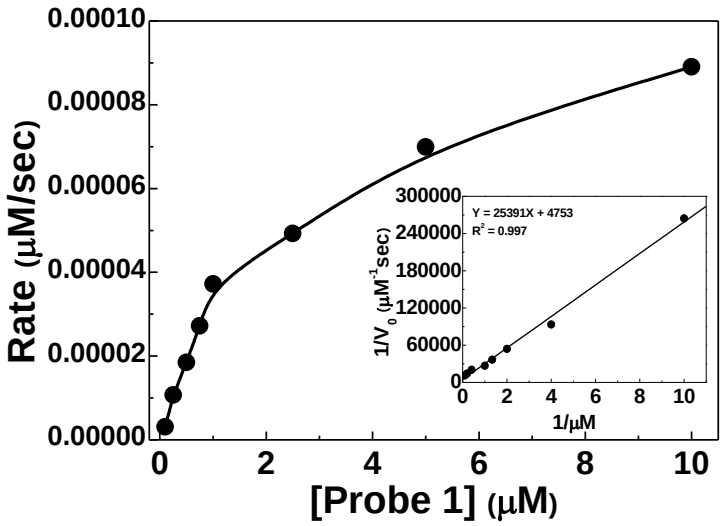


Figure S17. A plot of the initial velocity v_0 of the oxidation of probe **1** by tyrosinase (25 nM) versus concentration of probe **1**. Inset represents a double reciprocal (Lineweaver-Burk) plot.

Determination of kinetic constants for L-DOPA

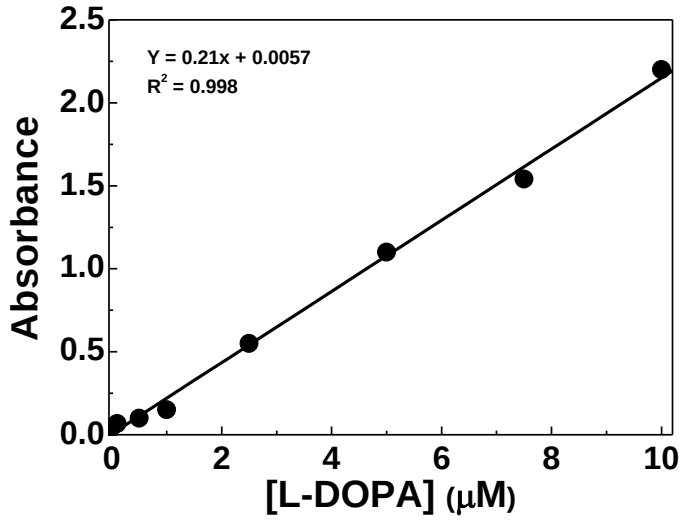


Figure S18. Standard absorbance curve of L-DOPA at different concentrations upon incubation with excess tyrosinase in PBS buffer (50 mM, pH 6.3, 37 ^{o}C) containing 0.2% DMSO as a cosolvent. Absorption intensity at 475 nm was measured. [L-DOPA] = 0–10 μM.

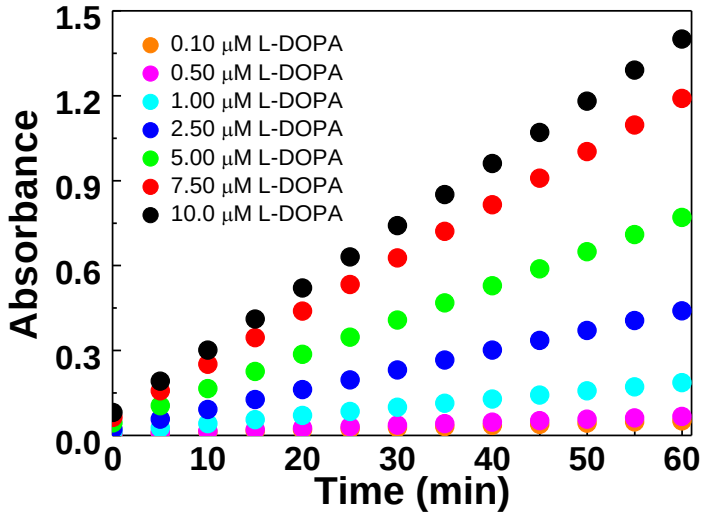


Figure S19. Absorbance response of L-DOPA by tyrosinase. Progress curves of oxidation of L-DOPA at various concentrations (0.1–10 μM) upon addition of tyrosinase (25 nM). The spectra were obtained every 5 min upto 60 min. The enzyme reactions were carried out in 50 mM PBS (pH 6.3) containing 0.2% DMSO as a cosolvent at 37 ^{o}C. Absorption intensity was measured at 475 nm. [Tyrosinase] = 90 nM.

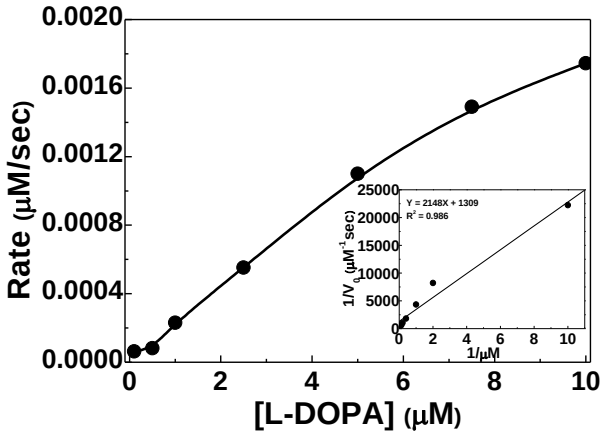


Figure S20. A plot of the initial velocity v_0 of the oxidation of L-DOPA by tyrosinase (90 nM) versus concentration of L-DOPA. Inset represents a double reciprocal (Lineweaver-Burk) plot.

Table S2. Kinetic Data for substrates **probe 1**

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
Probe **1**	5.30	0.01	2.0×10^3
L-DOPA	1.7	0.007	4.0×10^3

4.3. Inhibition assay of tyrosinase activity

For inhibition assay of enzyme activity, the final concentrations of mushroom tyrosinase and probe **1** were fixed to 90 nM and 1 μM, respectively. Mushroom tyrosinase inhibitors, kojic acid, benzaldehyde, anisaldehyde, and benzoic acid were dissolved in PBS buffer (50 mM, pH 6.3, 0.2% DMSO), and then mixed with 20 μL of tyrosinase solution in 140 μL PBS (50 mM, pH 6.3), respectively. Final concentrations of kojic acid in the mixed solution were 0 ~ 3 mM, and final concentrations of benzaldehyde, anisaldehyde, and benzoic acid in the mixed solution were 0 ~ 10 mM. The mixed solutions were incubated at room temperature for 20 min to inhibit enzyme activity. To the inhibitor-treated tyrosinase solution was then added 20 μL of probe **1** solution. Enzymatic reaction was performed by incubating the solution as previously described. Fluorescence at 515 nm was recorded every 30 min for 300 min at 37 ^{o}C using a computer-controlled fluorescence plate reader. IC_{50} value (the inhibitor concentration required to reduce enzyme activity by 50%) was obtained from the plot of relative activity versus inhibitor concentration at 300 min time point.

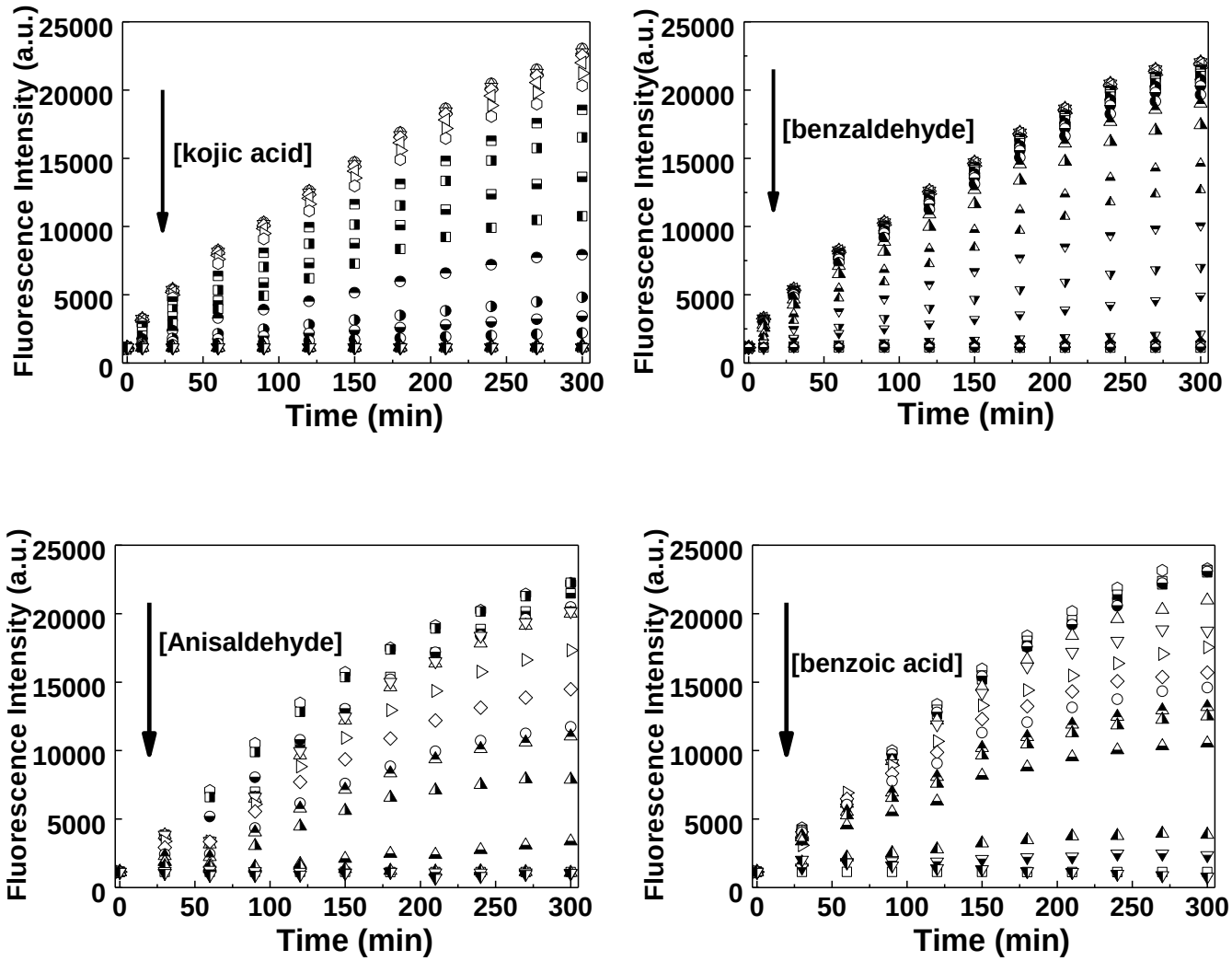


Figure S21. Inhibition assay of tyrosinase activity using probe **1** (1 μM) in PBS buffer (50 mM, pH 6.3, 37 ^{o}C) containing 0.2% DMSO as a cosolvent. Kinetic profiles of probe **1** (1 μM) after addition of the tyrosinase (90 nM) solution, which was pretreated with inhibitor kojic acid (top, left), benzaldehyde (top, right), anisaldehyde (bottom, left), and benzoic acid (bottom, right) at different concentrations, respectively. Excited at 465 nm.

5. Analysis of reaction product by LC-MS

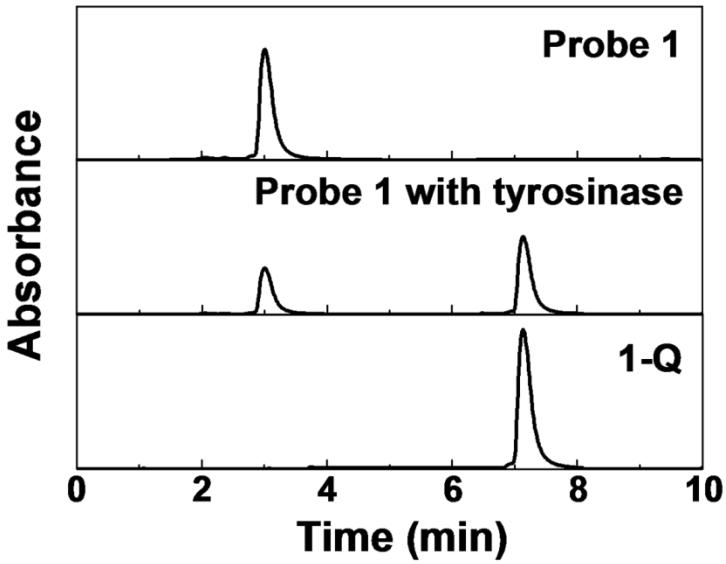


Figure S22. HPLC chromatograms of probe **1** without treatment of tyrosinase (top); with tyrosinase treatment for 3 h at 37 ^{o}C (middle); **1-Q** only (bottom). The samples were analyzed by LC-MS with a linear gradient elution (from 0 to 80% B, A: deionized water, B: methanol, flow rate 0.3 mLmin^{-1}, UV: 340 nm). The MW of the retention time at 3.1 min is 357.1, which corresponds to $[M+H]^+$ for probe **1** and MW of the retention time at 7.2 min is 355.1, which corresponds to $[M+H]^+$ for compound **1-Q**. [**1**] = [**1-Q**] = 10 μM, [tyrosinase] = 90 nM.

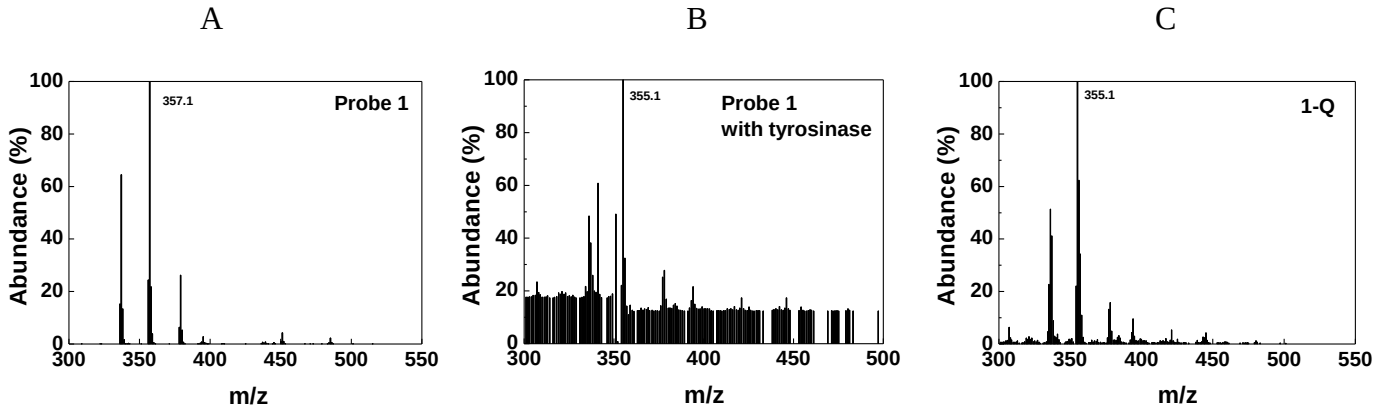


Figure S23. ESI-MS spectra of probe **1** (a) without enzyme treatment, (b) after treating with tyrosinase for 3 h, (c) compound **1-Q**. MW of the retention time at 3.1 min is 357.1, which corresponds to $[M+H]^+$ for probe **1** and MW of the retention time at 7.2 min is 355.1, which corresponds to $[M+H]^+$ for **1-Q**. [**1**] = [**1-Q**] = 10 μM, [tyrosinase] = 90 nM

6. Selectivity test

In order to investigate the selectivity of probe **1** with other oxidized enzyme, we tested fluorescence-turn-on response of probe **1** with alcohol dehydrogenase (ADH), which is known to facilitate the inter-conversion between alcohols and aldehyde or ketone, at various assay conditions in terms of pH of assay solution (pH 6.3 and 8.5) and concentrations of ADH (90 nM and 690 nM).

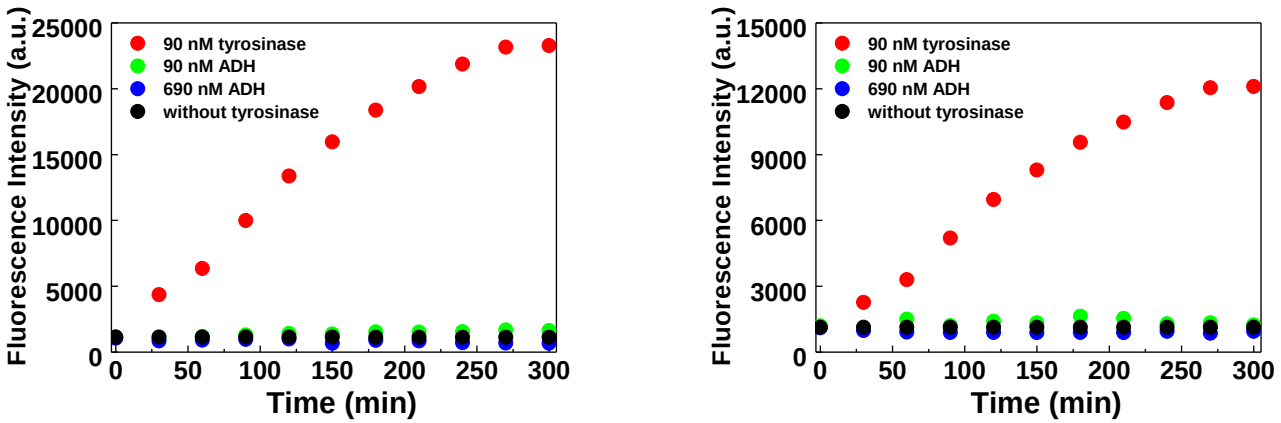


Figure S24. Fluorescence turn-on response of probe **1** upon incubation with tyrosinase (90 nM) and alcohol dehydrogenase (ADH, 90 nM and 690 nM)) in (left) PBS buffer (50 mM, pH 6.3, 37 ^{o}C) containing 0.2% DMSO as a cosolvent and (right) Tris buffer (50 mM, pH 8.5, 37 ^{o}C) containing 0.2% DMSO as a cosolvent, respectively. The spectra were obtained every 30 min (0 – 300 min). Fluorescence intensity was measured at 515 nm with excitation at 465 nm.

7. Cell studies

Cell culture

General Cell Culture

B16F10 cells (mouse melanoma cell line) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% Antibiotic & Antimycotic, in a humidified incubator containing 5% CO_2 at 37 °C.

Cell viability test

B16F10 melanoma cells were seeded in each well of 96-well plates at a density of 20,000 cells/well and incubated for 24 h for cell attachment. The stock solution of probe **1** dissolved in dimethyl sufoxide (2.5 mM) was diluted with a cell culture medium containing 10% FBS to have a final concentration of 0, 0.5, 1, 2.5, and 5 μM. Existing culture medium was replaced with 200 μL of the fresh one containing probe **1**, and the cells were incubated for 24 h. After washing the cells twice, cell viability was measured using a cell counting kit-8 (Dojindo Laboratories). Cell viability was calculated as a percentage compared to untreated control cells. Data are expressed as a mean ± standard deviation. Statistical significance compared to the untreated control cells was calculated by determining *p* values by using the t-test.

Confocal fluorescence imaging of tyrosinase activity in melanoma cells

B16F10 cells were plated at a density of 20,000 cells/well in a LabTek II chambered cover glass (Nalge Nunc International Corp., Rochester, NY) and incubated for 24 h for cell attachment. The stock solution of probe **1** dissolved in dimethyl sufoxide (2.5 mM) was diluted with a cell culture medium to have a final concentration of 2.5 μM. Cells were incubated in the absence or presence of 100 nM α-MSH for 48 h to observe the effect of α-MSH on tyrosinase imaging. To evaluate effect of tyrosinase inhibitors on the enzyme activity, the other sets of the cells were co-incubated with kojic acid (KA) or miconazole (MIC) during α-MSH for 48 h. Then the existing cell culture medium was replaced with fresh medium containing

probe **1**. After the cells were incubated with for 30 min, they were washed three times and a fresh cell culture medium was used. Subsequently, fluorescence images (Ex. 488 nm, Em. 505~550 nm) were acquired using a confocal laser scanning microscope (ZEISS LSM 510 META). For fluorescence imaging, we randomly selected three different areas from each sample. All the data were acquired using identical settings on the microscope in order to ensure reproducibility.

Western Blot analysis

Western blot analysis was performed to compare tyrosinase expression depending on the treatment conditions. B16F10 cells were seeded in 175T flask and incubated for 24 h for cell attachment. Then cells were incubated in the absence or presence of 100 nM α-MSH for 48 h. The other sets of the cells were co-incubated with kojic acid (100 μM, 1 mM, and 5 mM) or miconazole (10 μM and 30 μM) during α-MSH for 48 h. Cell pellets were lysed in phosphate buffer (pH 6.3) containing 1% triton X-100, 2 mM PMSF and protease inhibitor cocktail. The cellular extracts were clarified by centrifugation at 13000 rpm for 10 min at 4 °C. Protein content was measured using the BCA assay kit (Pierce, Rockford, Ill., USA) with BSA as the standard. Equal amounts of protein (40 μg/lane) were separated on bis-tris gel (invitrogen, NuPAGE Novex) and transferred to a nitrocellulose membrane (Amersham, U.K.). To minimize the nonspecific binding, the membrane was incubated at room temperature for 30 min with blocking buffer **(**5% nonfat milk, 0.1% Tween20 containing PBS). Primary anti-β-actin (Santa Cruz biotechnology) was diluted 1:10,000 and anti-tyrosinase (sc-15341, Santa Cruz, U.S.A.) was diluted 1:200. The blot was incubated with each primary antibody until the following day (overnight incubation at 4 °C). The blot was washed three times with PBST buffer for 10 minutes each. And it was incubated with a secondary antibody HRP (horseradish peroxidase)-conjugated mouse immunoglobulin G (Santa Cruz biotechnology) for 1 h at room temperature. The blot was again washed three times with PBST. After final washing, tyrosinase was visualized by enhanced chemiluminescent substrate (supersignal west pico chemiluminescent substrate, Thermo Scientific).

Tyrosinase zymography

Tyrosinase activity was determined as previously described with minor modification.[4] Briefly, cell lysates were prepared using the same procedure as above. Cell supernatant was mixed with 2× protein sample buffer without β-mercaptoethanol. Protein samples were denatured with sodium dodecyl sulfate (SDS). 40 μg of total protein per lane was loaded onto a sodium-dodecyl-sulfate (SDS)-polyacrylamide gel. After electrophoresis, gel was rinsed in 100 mM sodium phosphate buffer (pH 6.5 with oxygen bubbling) for 30 min at room temperature with gentle agitation. After repeating the rinse procedure two more times, the gel was transferred into 200 mL of a staining solution containing the rinse buffer supplemented with 5 mM L-DOPA, and incubated in the dark at 37 °C. Tyrosinase activity was visualized in the gel as a dark melanin containing bend.

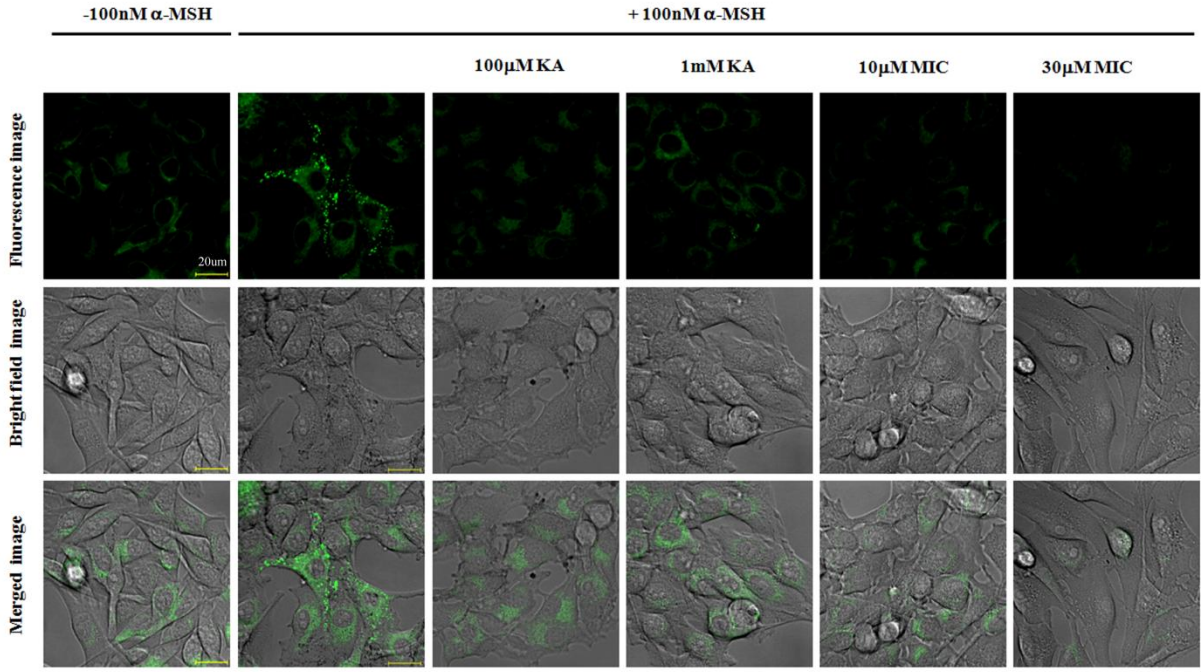


Figure S25. Confocal fluorescence microscope images of B16F10 melanoma cells under different conditions with probe **1**. (a) B16F10 melanoma cells were incubated with 2.5 μM probe **1** for 30 min at 37 °C and then imaged. (b) B16F10 melanoma cells were stimulated with 100 nM α-MSH for 48 h, and incubated with 2.5 μM probe **1** for 30 min at 37 °C and then imaged. Either kojic acid or miconazole at various concentrations was co-incubated during α-MSH stimulation while the other procedures were the same. (top: fluorescence images, middle: brightfield images, bottom: merged images, Ex = 488 nm, Em = 505–550 nm)

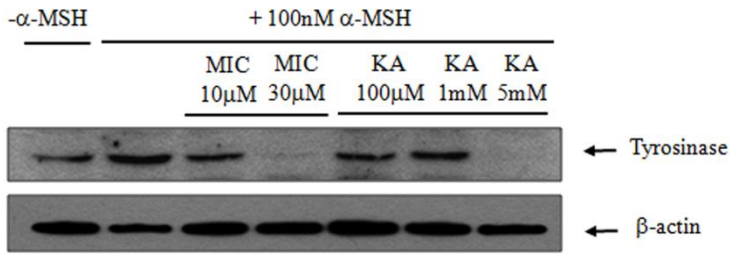


Figure S26. Western blot analysis of tyrosinase protein expression in B16F10 melanoma. Beta-actin was served as a loading control.

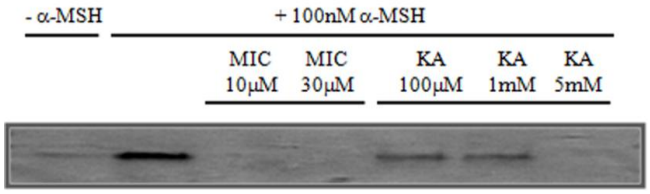


Figure S27. In vitro tyrosinase activity of B16F10. Tyrosinase activity was determined by L-DOPA zymography.

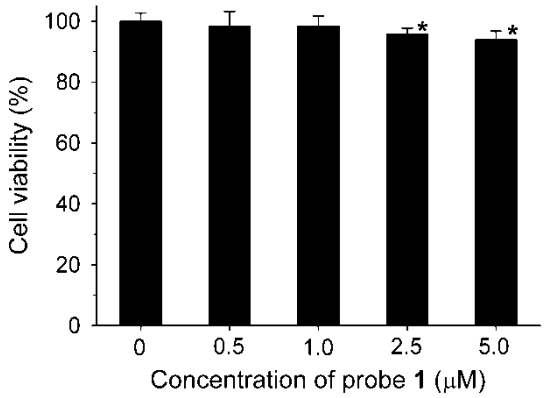
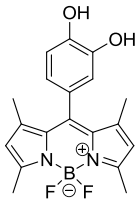
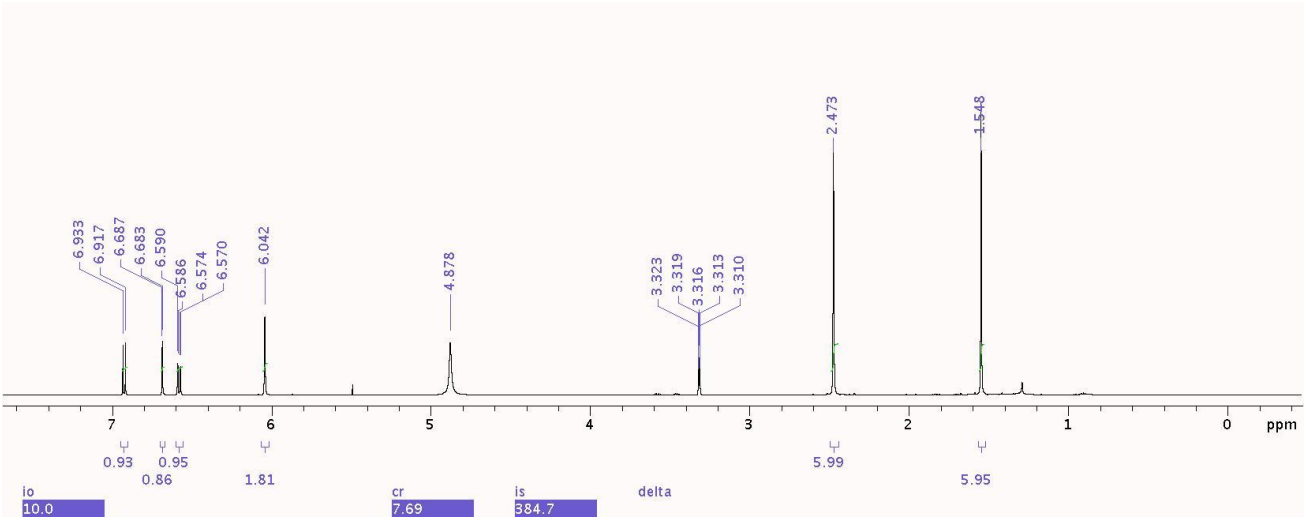


Figure S28. Effect of probe **1** on B16F10 cell viability. The cells were treated with probe **1** at various concentrations for 24 h (n=4). No cell death was observed at the concentration of 1 μM, and 98.6 and 96.5% of the cells were viable when the cells were treated with 2.5 and 5 μM probe **1** for 24 h, respectively. Statistical difference from the control cells: * $p < 0.05$.

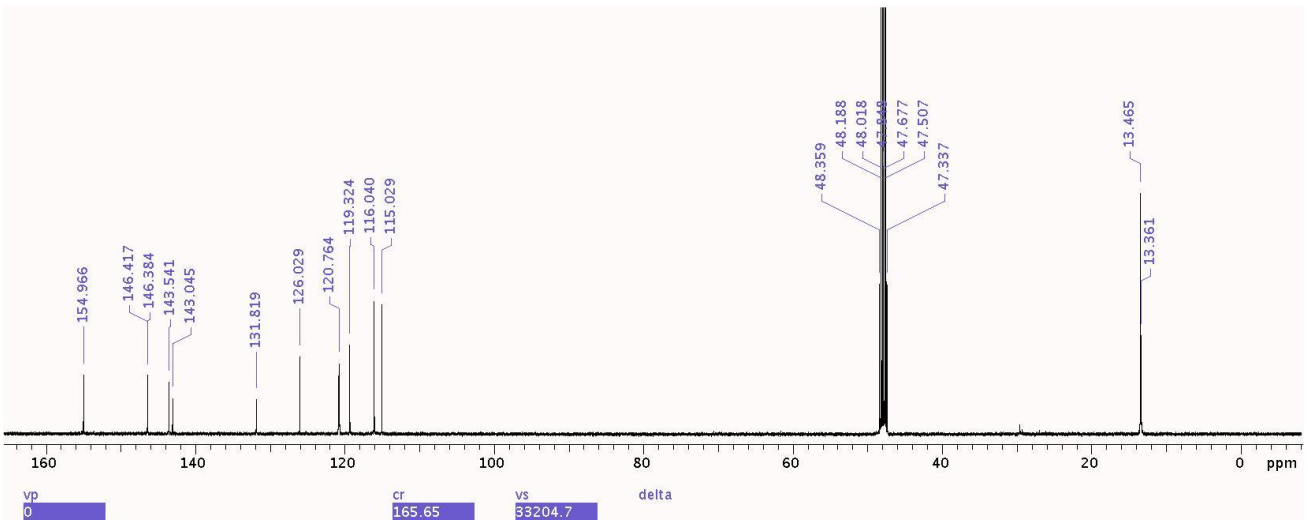
8. ^{1}H-NMR and ^{13}C-NMR Spectra

^{1}H-NMR Spectrum of **1** in $CDCl_3$ (500 MHz):

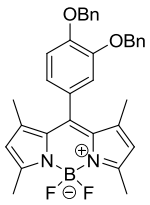


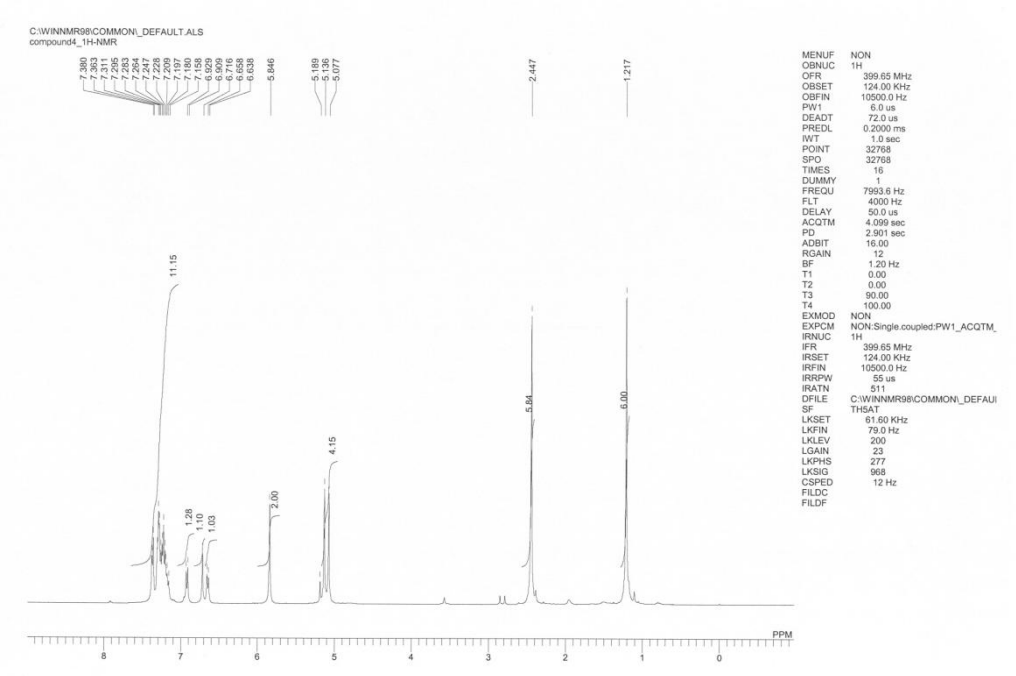


^{13}C-NMR Spectrum of **1** in $CDCl_3$ (125 MHz):

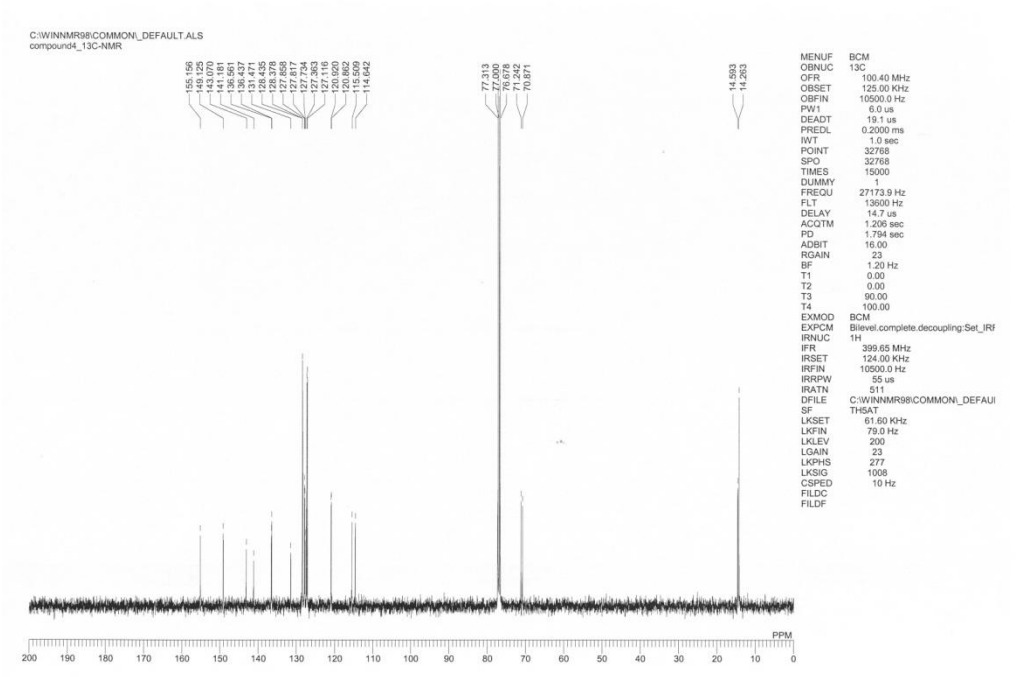


^{1}H-NMR Spectrum of **4** in $CDCl_3$ (400 MHz):

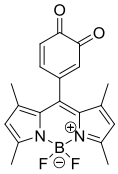


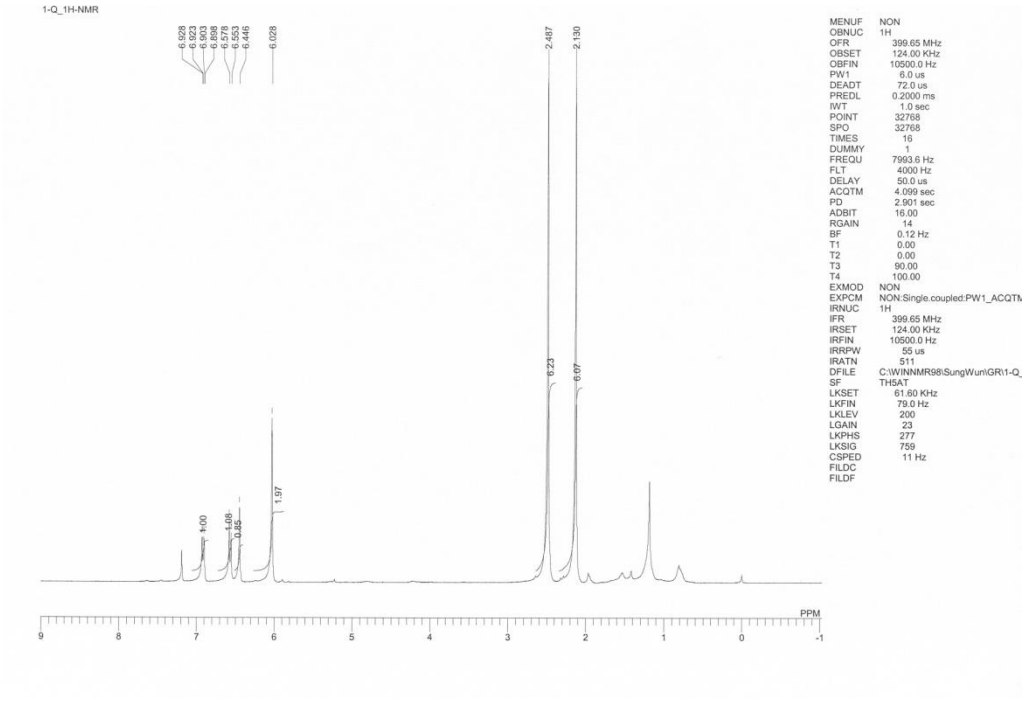


^{13}C-NMR Spectrum of **4** in $CDCl_3$ (100 MHz):

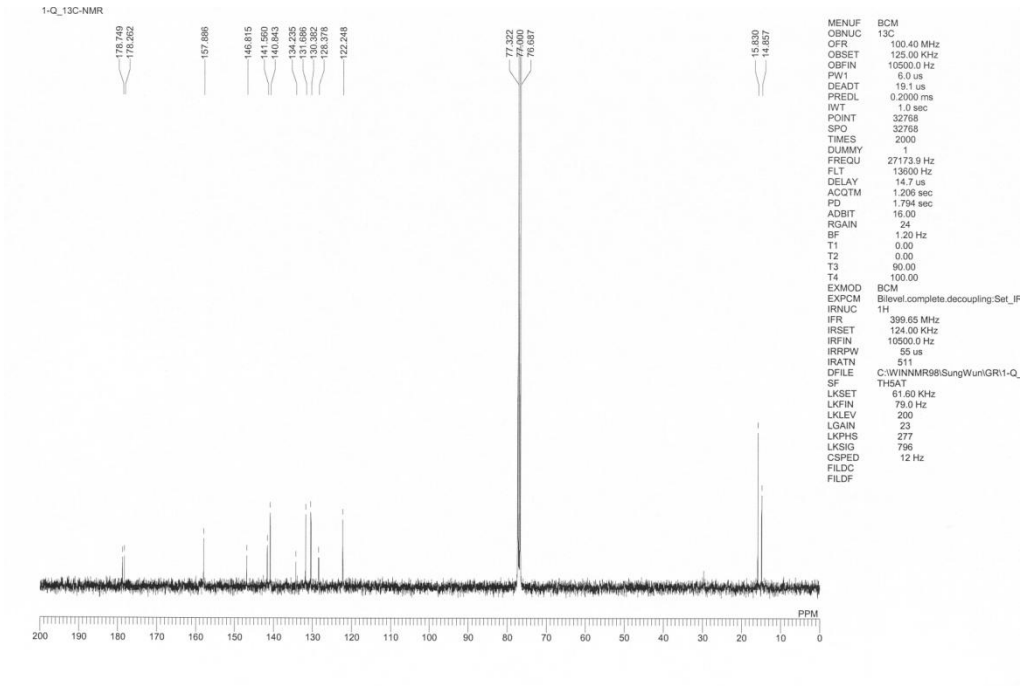


^{1}H-NMR Spectrum of **1-Q** in $CDCl_3$ (400 MHz):





^{13}C-NMR Spectrum of **1-Q** in $CDCl_3$ (100 MHz):



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2 A. T. R. Williams, S. A. Winfield and J. N. Miller, *Analyst*, 1983, **108**, 1067.
3 F. Gauillard, F. Richard-Forget and J. Nicolas, *Anal. Biochem.*, 1993, **215**, 59.
4 Y. J. Shin, C.-S. Han, C. S. Lee, H.-S. Kim, S.-H. Ko, S. K. Hwang, S.-G. Ko, J. W. Shin, S.-K. Ye and M.-H. Chung, *Biol. Pharm. Bull.*, 2010, **33**, 72.