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Supplementary Information

For

BODIPY based Two-Photon Fluorescent Probe Validates Tyrosinase Activity in Live- Cells

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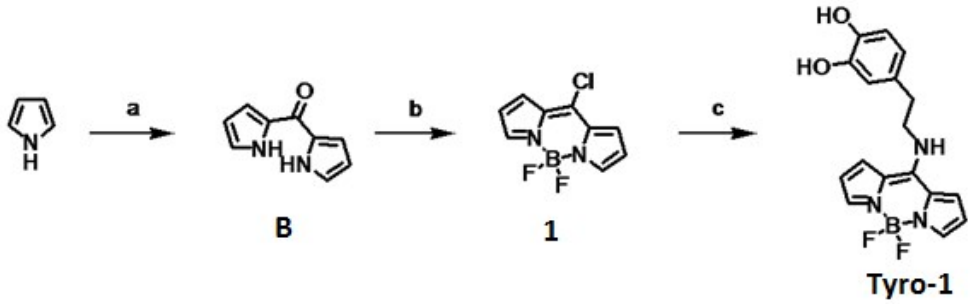
Materials, methods and instrumentations

Pyrrole(Aldrich), triphosgene (TCI), dopamine.HCl(Aldrich), $POCl_3$(Avra), boron trifluoride diethyl etherate(Aldrich), tyrosinase from mushroom (Aldrich), α-MSH (Aldrich), 1, 2-dichloroethane (Avra), diethyl ether (Loba chem., India), pet ether 60-80 (Avra), ethyl acetate (Avra) and DCM (Avra) were purchased commercially and used without further purification. Flash column chromatography was performed using Silica gel (100-200 mesh) and analytical thin layer chromatography was performed using silica gel 60 (pre-coated sheets with 0.25 mm thickness). Mass spectra were recorded on anion SpecHiResESI mass spectrometer. NMR spectra were collected on a 400MHz spectrometer (Bruker, Germany).

Reaction Scheme:



Synthesis of **Tyro-1**, a) (i) Pyrrole, triphosgene, 1,2-dichloroethane, 0°C, 2 h; (ii) Pyrrole, 80°C, 30 min; b) (i) $POCl_3$, 1,2-dichloroethane, 80°C, 2 h. (ii) Et_3N, $BF_3E_{t2}O$; c) Dopamine. HCl, Et_3N, Dichloromethane, 25°C, 6 h.

Synthesis of B and 1

Both **B** and **1** were synthesized according to reported literature.[1]

Synthesis of Tyro-1

Compound **1**(150 mg, 0.664 mmol) was added to a stirred solution of dopamine. HCl (377 mg, 1.99 mmol) and triethylamine (0.28 mL, 1.99 mmol) in DCM (20 mL) at 0°C and stirred for 6 h at room temperature. The reaction was monitored by TLC. After completion of reaction, the reaction mixture was poured into water (50 mL) and extracted with ethyl acetate (3 × 25 mL). The organic layer was washed with water (2 × 50 mL), brine (1 × 50 mL), dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude product was purified by column chromatography on

silica gel (100-200 mesh) using ethyl acetate in pet ether DCM (5:5) as eluent to obtain **1** as pale brown solid (90 mg; 64.47%). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 9.70 (s, 1H); 8.89 (s, 1H); 8.76 (s, 1H); 7.58 (s, 1H); 7.45 (d, *J* = 3.60 Hz, 1H);7.35 (s, 1H); 7.32 (d, *J* = 4.0 Hz, 2H); 6.69 (q, 2H); 6.57 (q, 2H); 6.38 (q, 1H); 3.92 (t, *J* = 7.20 Hz, 2H); 2.95 (q, 2H); ^{13}C-NMR (100 MHz, DMSO-d_6): 149.41, 145.99, 144.01, 134.21, 131.82, 130.21, 124.41, 122.45, 120.01, 117.65, 114.75, 112.89, 47.12, 32.75; ESI- HRMS *m/z* (M+H): calcd. 344. 13, found 344.1375.

General methods for UV-Vis and Fluorescence Spectroscopy

All reagents and solvents used for fluorescence spectroscopy were commercial and used without further purification.

Except under special case, the following methods provide all of measurements for fluorescence spectroscopy. Absorption spectra were recorded on an UV-1800 spectrophotometer (Shimadzu), and fluorescence spectra were recorded using an RF-5301 fluorescence spectrofluorometer (Shimadzu) with a 300 μL volume of 1 cm standard quartz cell. 500 μM Stock solution of the probe was prepared by dissolving probe in DMSO. 100 U/mL Stock solution of the tyrosinase was prepared by dissolving in PBS buffer (pH=7.4). In an eppendorf tube, 3 μL of 500 μM **Tyro-1** and an appropriate volume of tyrosinase or other reagents and PBS buffer solution was mixed. The final 300 μL volume of reaction solution was incubated at 37°C. After then, it was transferred to the quartz cell for measure fluorescence. The fluorescence emission spectra were recorded at excitation wavelength of 400 nm and emission was monitored over wavelength the range of 400-600 nm (λ_{em} = 452 nm) with slit width set at 3/5 nm. The solutions of biologically relevant species were prepared from ascorbic acid, GSH, NaNO2, chloride salts of Ag^{+}, Al^{3+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, K^{+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+} and amino acids (alanine, arginine, asparagine, aspartic acid, cysteine; f) glutamine; g) glutamic acid; h) glycine; i) histidine; j) homocysteine; k) isoleucine; l) leucine; m) lysine; n) methionine; o) phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine) in distilled water. The preparation or generation of ROS were prepared referring to the literature.[2]

Cell culture

Mouse melanoma (B16F10) and human cervical cancer (HeLa) cell lines were purchased from the Korean Cell Line Bank (Seoul, Republic of Korea). B16F10 and HeLa cells were cultured in high glucose Dulbecco's modified Eagle's medium (Welgene, Gyeongsangbuk-do, Republic of Korea) supplemented with 10% fetal bovine serum (VWR International, Radnor, PA, USA) and 1% penicillin-streptomycin from Welgene. All cultured cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Two-photon fluorescence microscopy

1×10^5 cells were seeded on 35-mm glass bottom confocal dishes (SPL Life Science, Gyeonggi-do, Republic of Korea) and allowed to stabilize for one days. When the cells reached 80% confluency, cells were treated with probe **Tyro-1** (10μM in DMSO) at 37°C in 5% CO_2 for 0-3 h. For tyrosinase stimulatory effect evaluated, cells were pretreated with in absence or presence of α-MSH (200 nM) and H_2O_2 (50 μM) for 2 h, and then incubated with probe **Tyro-1** (10 μM) at 37°C in 5% CO_2 for 2 h. For tyrosinase inhibitory effect evaluated, cells were pretreated with in absence or presence of kojic acid (100 μM) for 2 h, and then incubated with probe **Tyro-1** (10 μM) at 37°C in 5% CO_2 for 2 h. Then, cells were washed with PBS. Two-photon fluorescence microscopy images of probe labeled B16F10 cells were obtained with spectral confocal and two-photon microscopes (Leica TCS SP2) with a ×100 (NA = 1.30 OIL) objective lens. The two-photon fluorescence microscopy images were obtained with a DM IRE2 Microscope (Leica) by exciting the probes with a mode-locked titanium-sapphire laser source (Coherent Chameleon, 90 MHz, 200 fs) set at wavelength 740 nm. To obtain images at 420–520 nm range, internal PMTs were used to collect the signals in 8 bit unsigned 512 X 512 pixels at 400 Hz scan speed.

One-photon fluorescence microscopy

Fluorescence images were taken using a confocal laser scanning microscope (Carl-Zeiss LSM 700 Exciter, Oberko- chen, Germany) with same condition. Fluorescence channel was excited at 405 nm with a Si laser and emission was collected by a 420-500 nm band pass filter. Fluorescence images were obtained by using confocal laser scanning microscope (Cal Zeiss).

Cell viability

Approximately 2×10^4 cells were seeded on 96 well microplate (SPL Life Science) and incubated for one days. After incubation, the cells were treated with DMSO and probe **Tyro-1** for 12 h. To analyze cell viability of the probe on cells, we performed cell viability assay using CytoTox96® Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI, USA) followed by manufacturer's instructions. Fluorescence level was analyzed by using SPECTRA MAX GEMINI EM microplate reader (Molecular Devices, Sunnyvale, CA, USA). The wavelength was set at 490 nm. Cell viability was expressed as a percentage of absorbance measured in the treated wells relative to that in the control wells.

Statistical analysis

Statistical significance of the treatment values was analyzed by comparing with control values by using Student's *t*-test. The data represent the mean ± SE, and $*p<0.05$ was considered statistically significant. The *P* values of significant results are shown.

The Linear Range and Detection Limit

The fluorescence titration was used to calculate the detection limit. The fluorescence emission spectrum of probe **Tyro-1** (2.0×10^{-6} M) was measured ten times and the standard deviation of blank measurement was achieved. The fluorescence intensity at 452 nm was plotted as a concentration of tyrosinase. The detection limit was calculated by using following equation.

Detection Limit 3σ/k:

Where σ is the standard deviation of blank measurement, k is the slope between the fluorescence intensity versus tyrosinase concentration.

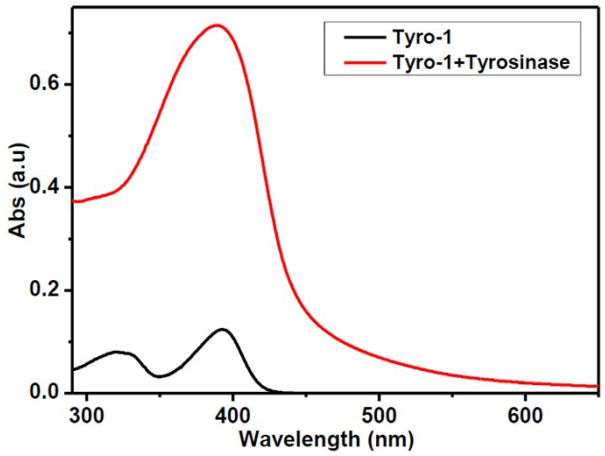


Fig.S1 UV-Vis spectra of **Tyro-1** (5 μM) in the absence (Black line) and presence (Red line) of tyrosinase (200 U/mL) in PBS buffer (pH 7.4; 1% DMSO). Data were recorded after incubation at 25ºC for 30 min.

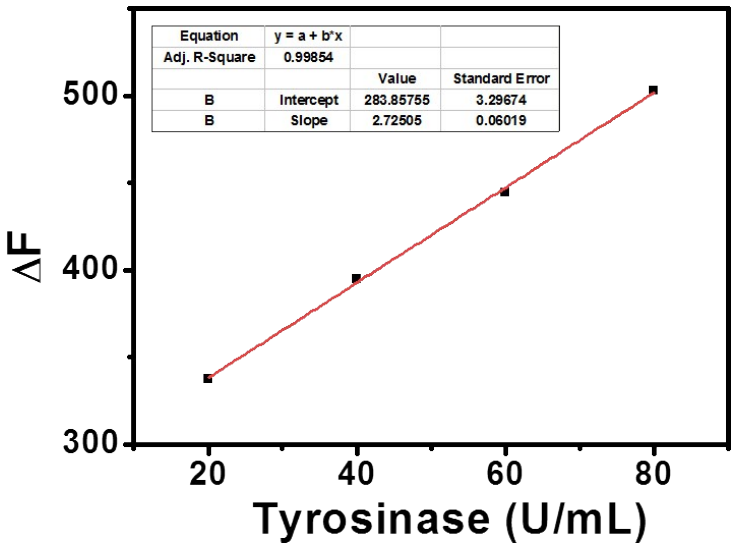


Fig. S2 Linear correlation between ΔF and the concentration of tyrosinase (20-80 U/mL). ΔF means the fluorescence intensity difference of **Tyro-1** (5 μM) before and after incubating with tyrosinase.

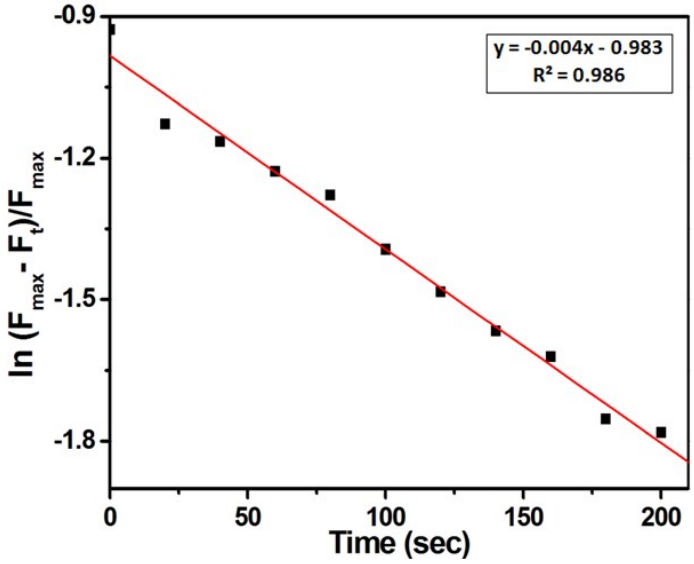


Fig. S3 The estimation of reaction rate constant between **Tyro-1** (5 μM) and tyrosinase (100 U/mL) by applying regression equation; K = 0.004S^{-1}

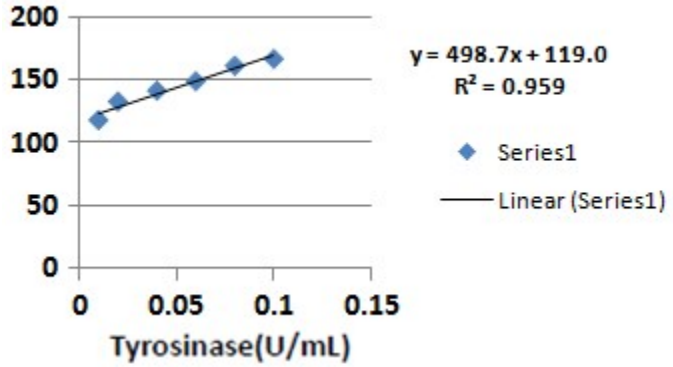


Fig. S4 The changes in the fluorescence intensity of **Tyro-1** (5 μM) at 452 nm against varied concentrations of Tyrosinase (0-0.1 U/mL) in PBS buffer (pH 7.4, 1% DMSO) with the slit width 3/5 nm.

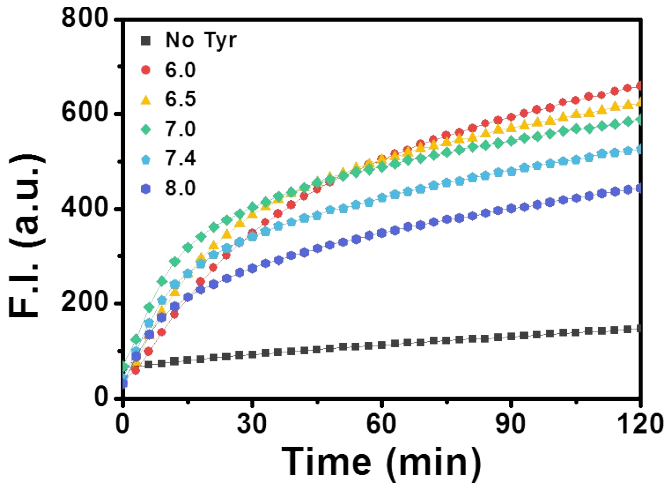


Fig.S5 Fluorescence response of **Tyro-1** (5 μM) upon incubation with tyrosinase (100 U/mL) in different pH buffer solutions (PBS with 1% DMSO) at 37°C, respectively. Fluorescence intensity of each point was recorded at 452 nm, excited at 400 nm with slit set at 3/5 nm.

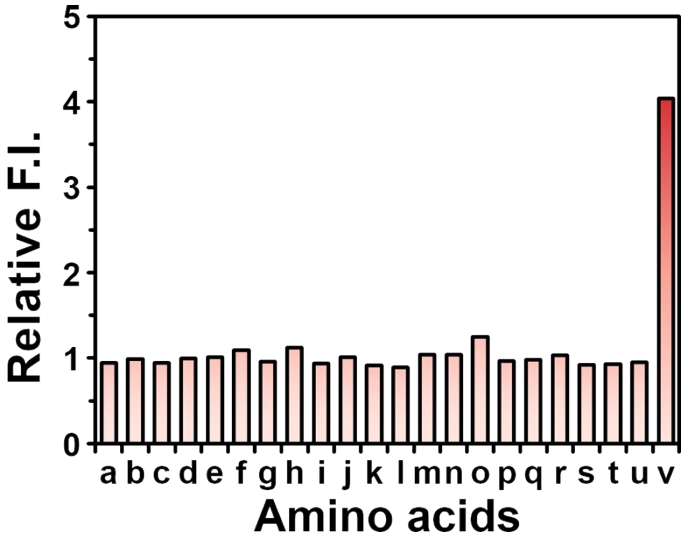


Fig. S6 Fluorescence responses of **Tyro-1** (5 μM) with various amino acids (500 μM): a) alanine; b) arginine; c) asparagine; d) aspartic acid; e) cysteine; f) glutamine; g) glutamic acid; h) glycine; i) histidine; j) homocysteine; k) isoleucine; l) leucine; m) lysine; n) methionine; o) phenylalanine; p) proline; q) serine; r) threonine; s) tryptophan; t) tyrosine; u) valine and v) tyrosinase (100 U/mL) in PBS buffer (pH=7.4; 1% DMSO) at 37°C. All Fluorescence responses were recorded at 452 nm, excited at 400 nm with slit set at 3/5 nm.

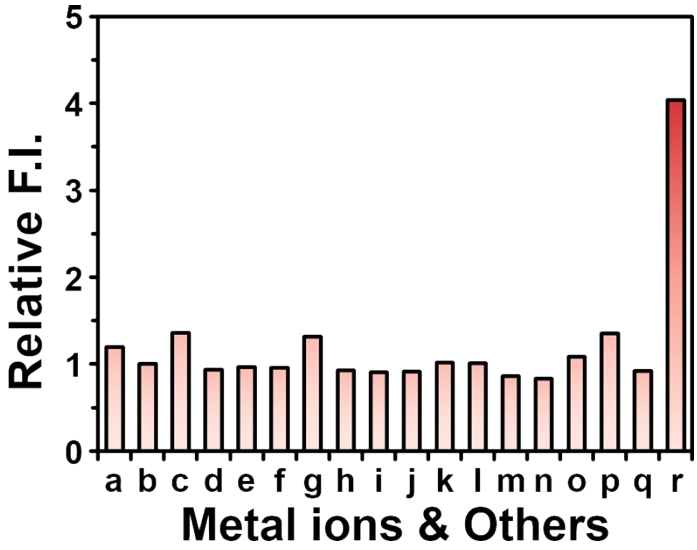
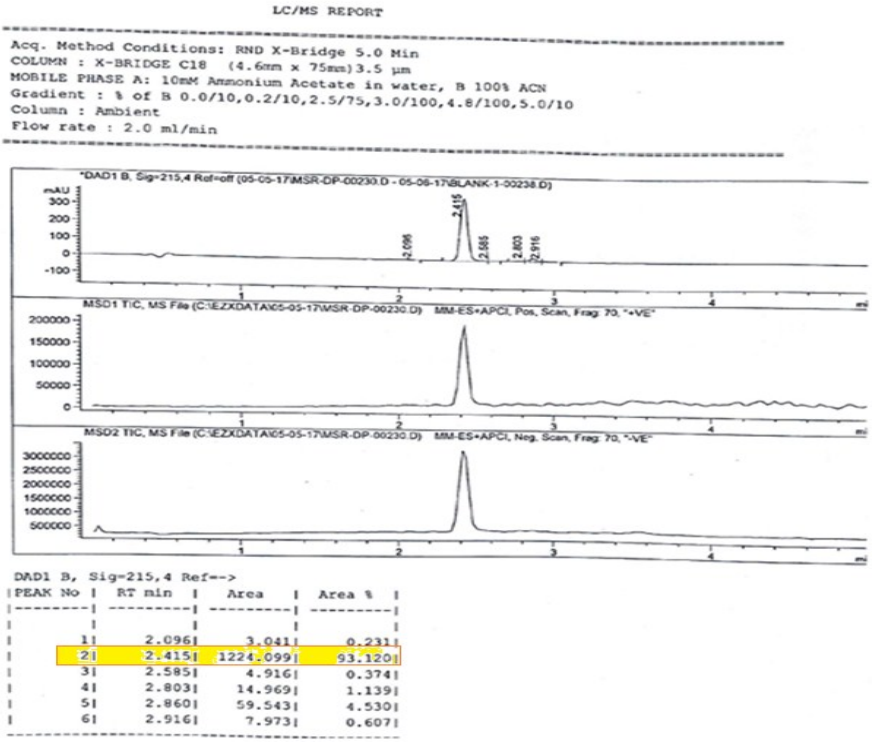


Fig. S7 Fluorescence responses of **Tyro-1** (5 μM) with various metal ions (500 μM): a) Ag^{+}; b) Al^{3+}; c) Ba^{2+}; d) Ca^{2+}; e) Cd^{2+}; f) Co^{2+}; g) Cu^{2+}; h) Fe^{2+}; i) Fe^{3+}; j) K^{+}; k) Mg^{2+}; l) Mn^{2+}; m) Ni^{2+}; n) Pb^{2+}; o) Zn^{2+}; p) ascorbic acid; q) GSH and r) tyrosinase (100 U/mL) in PBS buffer (pH=7.4; 1% DMSO) at 37°C. All Fluorescence responses were recorded at 452 nm, excited at 400 nm with slit set at 3/5 nm.



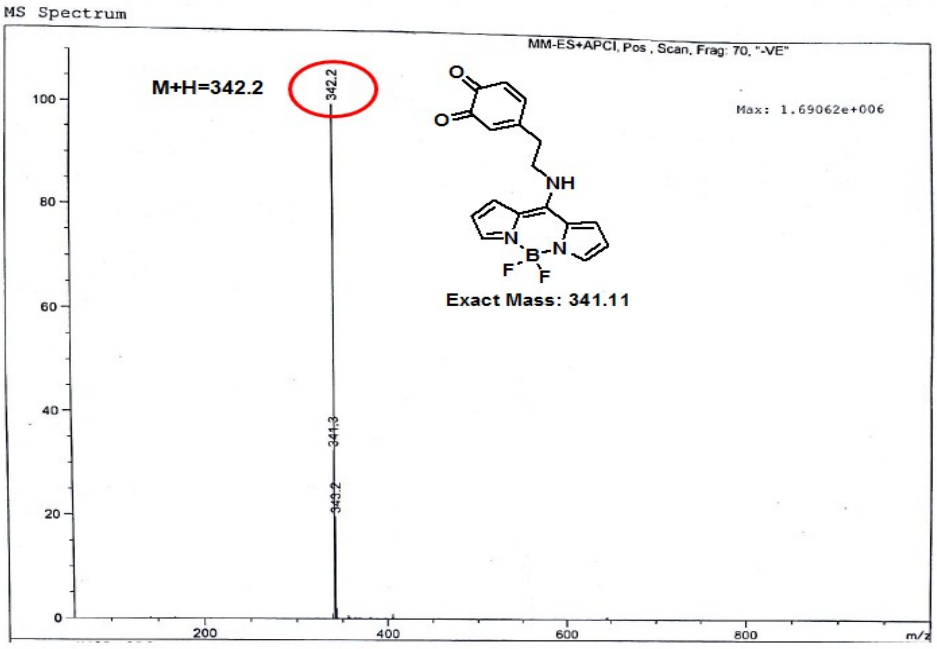


Fig. S8 LC-MS data of tyrosinase treated **Tyro-1**

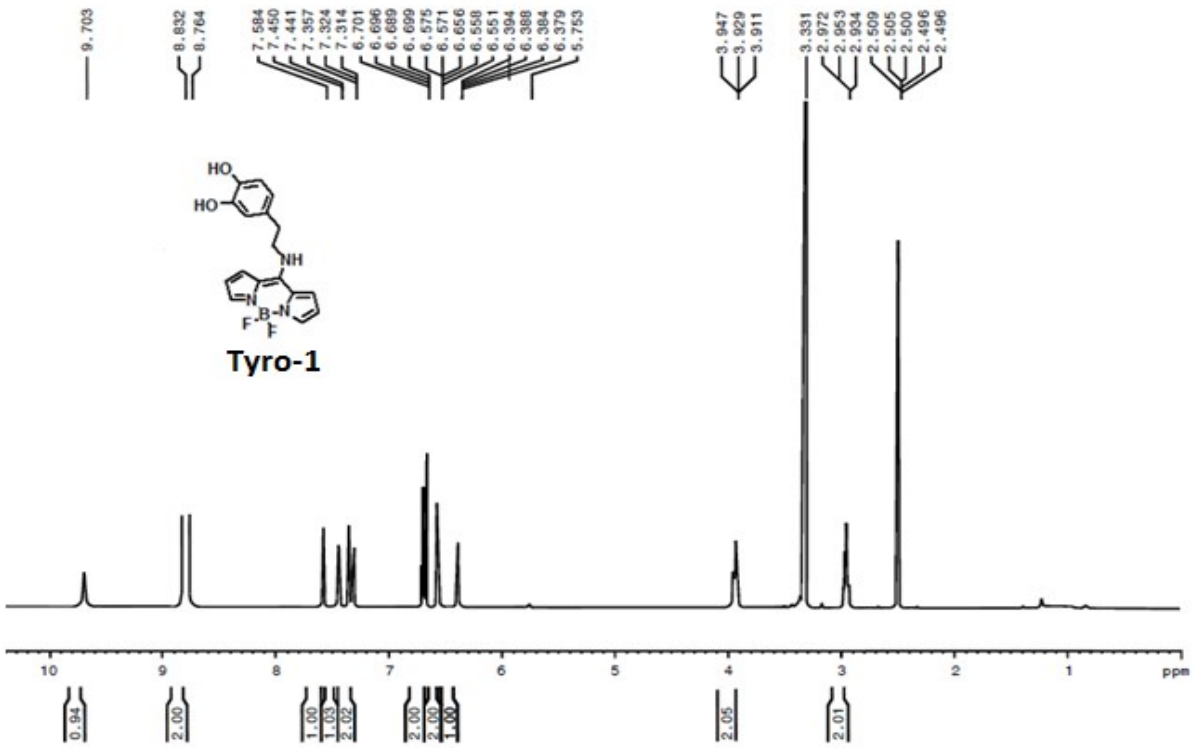


Fig.S9 ^{1}H-NMR of **Tyro-1** in DMSO-d_6

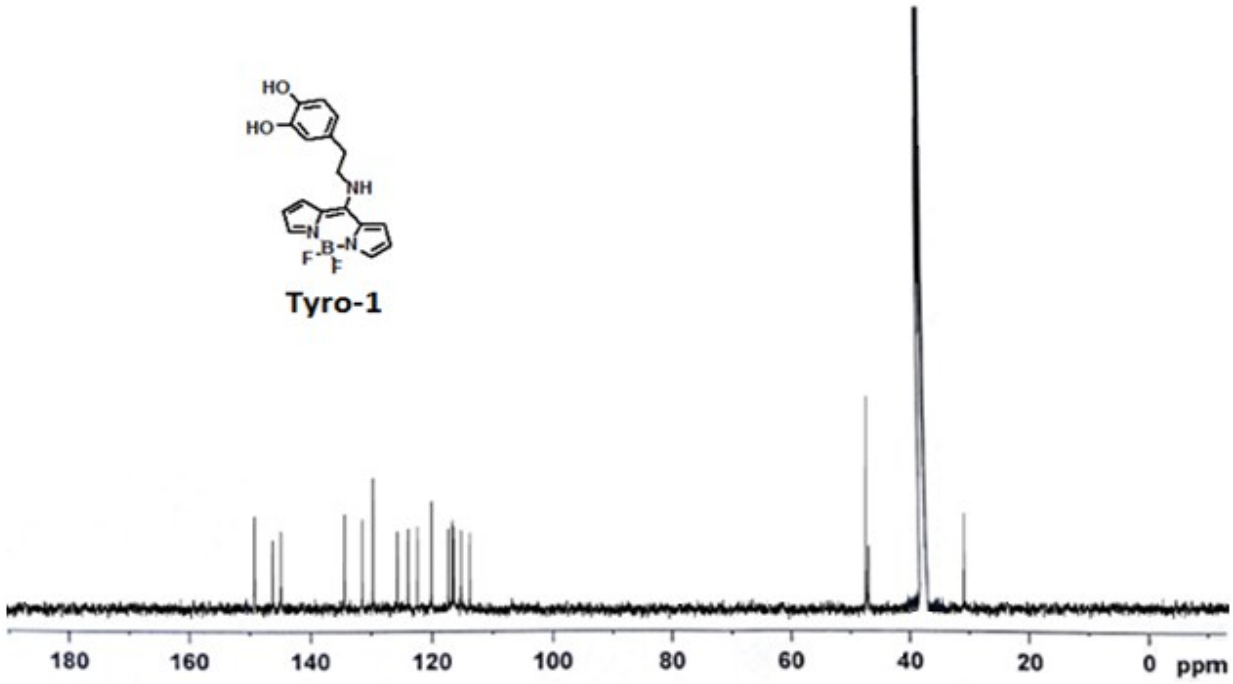


Fig.S10^{13}C-NMR of **Tyro-1** in DMSO-d_6

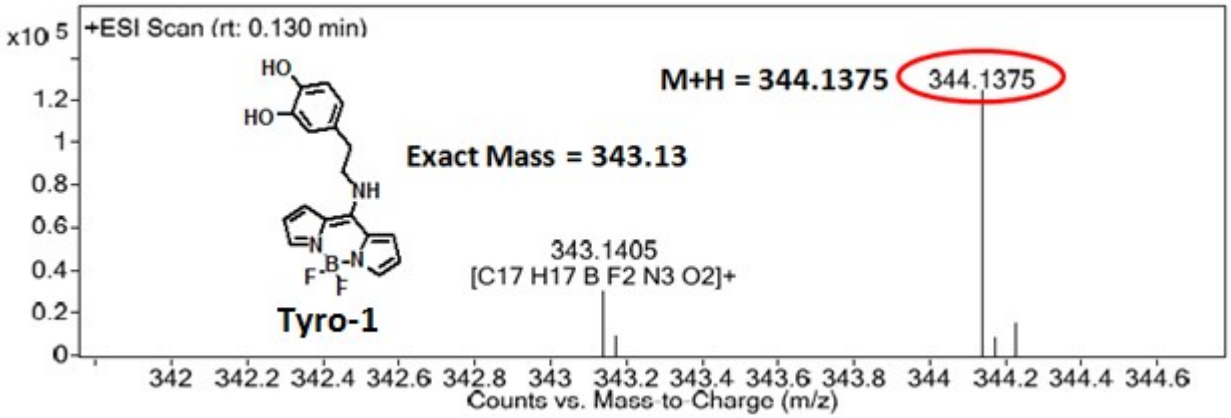


Fig.S11 HRMS of **Tyro-1**

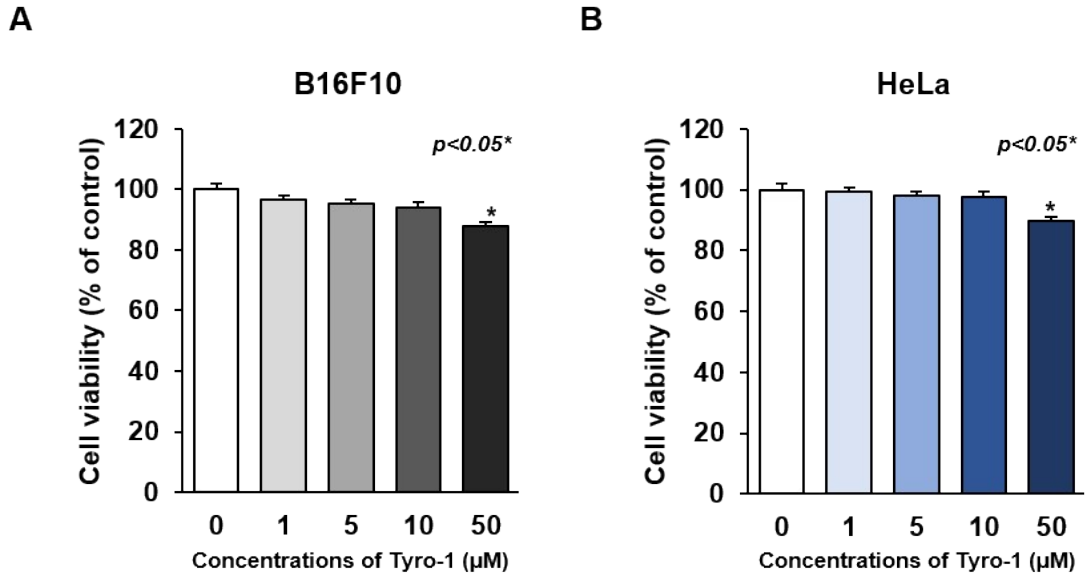


Fig. S12 Cell viability of probe **Tyro-1.** Cells were incubated with 0, 1,5,10 and 50 μM of probe **Tyro-1** for 12 h in B16F10 (A) and HeLa (B) cells. Values represent mean ± SE of three independent experiments performed in triplicate; **p*<0.05.

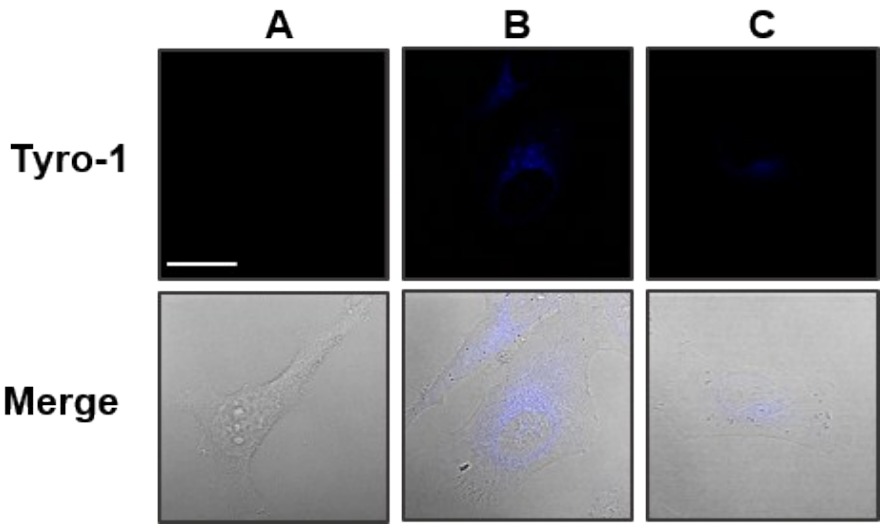


Fig. S13 One-photonimages of the various condition probe **Tyro-1** (10 μM) in HeLa cells. (A) control (cell only); (B) cells incubated with probe **Tyro-1** (10 μM) for 2h; (C) cells pretreated the kojic acid (100 μM) for 2 h and then incubated with probe**Tyro-1** for 2 h. λ_{ex} = 405 nm, λ_{em}= 420-500 nm.Scale bar = 20 μM.

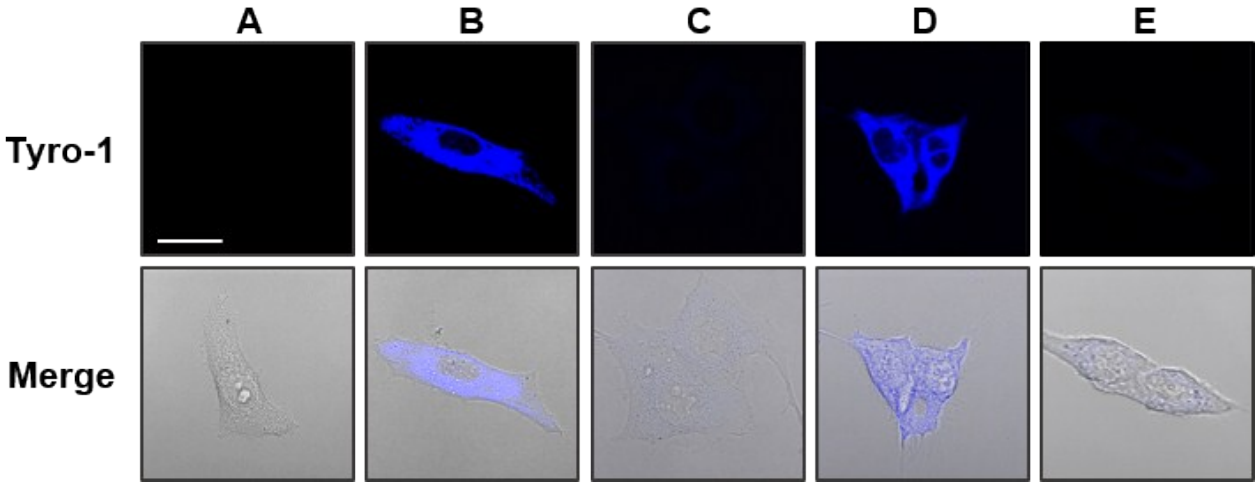


Fig. S14 One-photon laser scanning microscopy images of the various condition probe **Tyro-1** (10 μM) in B16F10 cells (magnification, × 40). (A) Cells only (control); (B) cells incubated with probe **Tyro-1** (10 μM) for 2h; (C) Cells pretreated the kojic acid (100 μM) for 2 h and then incubated with probe **Tyro-1** for 2 h; (D) Cells pretreated the α-MSH (200 nM) for 2 h and then incubated with probe **Tyro-1** for 2 h; (E) Cells pretreated the α-MSH (200 nM) and kojic acid for 2 h and then incubated with probe **Tyro-1** for 2 h. λ_{ex} = 405 nm, λ_{em} = 420-500 nm. Scale bar = 20μM.

References

1. Y. Zhang, X. Shao, Y. Wang, F. Pan, R. Kang, F. Peng, Z. Huang, W. Zhang and W. Zhao *Chem. Commun.,* 2015, **51**, 4245-4548.

2. G. W. Chen, F. L. Song, J. Y. Wang, Z. G. Yang, S. G. Sun, J. L. Fan, X. X. Qiang, X. Wang, B. R. Dou and X. J. Peng, *Chem. Commun.*, 2012, **48**, 2949-2951.