Electronic Supplementary Information for:

Highly selective two-photon fluorescence off-on probe for imaging tyrosinase activity in living cells and tissues

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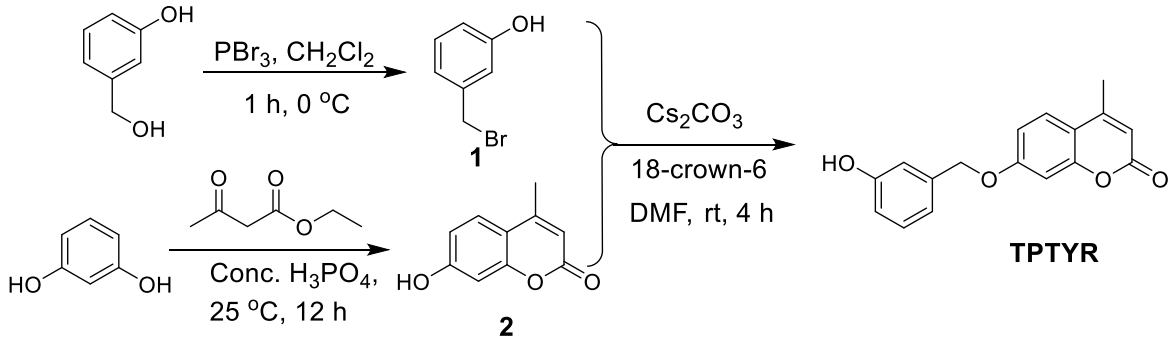
1. Instruments and reagents

UV absorbance spectra were obtained by Thermo Scientific Evolution 201 UV/VIS Spectrometer. Fluorescence emission spectra were obtained by FS-2 spectrophotometer of Scinco. ^{1}H-NMR and ^{13}C-NMR were recorded by Bruker AM 300 MHz spectrometer. Mass spectra were gained from Exactive Plus Orbitrap of Thermo Fisher Scientific. wo-photon fluorescence microscopy images were obtained with multiphoton microscopes (Leica TCS SP8MP) with ×40 oil objectives.

Phosphoric acid (H_3PO_4), dimethyl sulfoxide (DMSO), rhodamine 6G, xanthine oxidase, monoamine oxidase A, monoamine oxidase B, β-glucosidase, trypsin, alkaline phosphatase, NQO1, carboxylesterase, butyrylcholinesterase, acetylcholinesterase, thioredoxin reductase, apyrase, human serum albumin, bovine serum albumin, tyrosinase 3-(hydroxymethyl)phenol, phosphorus tribromide, 18-crown-6, cesium carbonate, 3-hydroxylphenol, and tiazolyl blue tetrazolium bromide (MTT) were bought from Sigma-Aldrich, Korea. MTT assay investigated by Spectramax Microwell plate reader.

2. Synthesis of TPTYR



Compound **1** and **2** was prepared following the reported procedure.[1,2]

Compound **2** (200 mg, 1.14 mmol) was dissolved in acetonitrile (2.5 mL) with 18-crown-6 (0.14 mL), followed by the addition of cesium carbonate (661 mg, 2.03 mmol) and stirred for 30 min at room temperature. Then, 3-(bromomethyl)phenol (630 mg, 3.4 mmol) in 2.5 mL acetonitrile, was slowly dropped into the above solution. After 4 h, the resulting mixture was redissolved in the ethyl acetate and washed saturated sodium chloride two times, respectively. The collected organic solvent was dried using Na_2SO_4 and evaporated in vacuum, then the residue was purified by silica column (DCM: MeOH = 300:1, *v/v*) to afford **TPTYR** as a slight yellow solid. ^{1}H-NMR, ^{13}C-NMR, and high-resolution mass spectra of **TPTYR** are shown in Fig. S1-S3 in the Supporting Information, respectively. Yield: 102 mg (31.8 %). ^{1}H-NMR (300 MHz, 298 K, DMSO-d_6): δ 2.39 (s, 3H), 5.15 (s, 2H), 6.21 (s, 1H), 6.74-6.75 (d, 1H), 6.86-6.89 (d, 2H), 7.00-7.04 (m, 2H), 7.16-7.19 (t, 1H), 7.69 (d, 1H), 9.49 (s, 1H). ^{13}C-NMR (75 MHz, 298 K, DMSO-d_6): δ 161.81, 160.58, 157.96, 155.12, 153.86, 138.18, 130.02, 126.95, 118.65, 115.4, 114.87, 113.71, 113.16, 111.68, 102.13, 70.20, 18.59. HR-EI-MS: m/z calcd for **TPTYR** ($C_{17}H_{14}O_4$ $[M]^+$), 282.0892; found: m/z 282.0894.

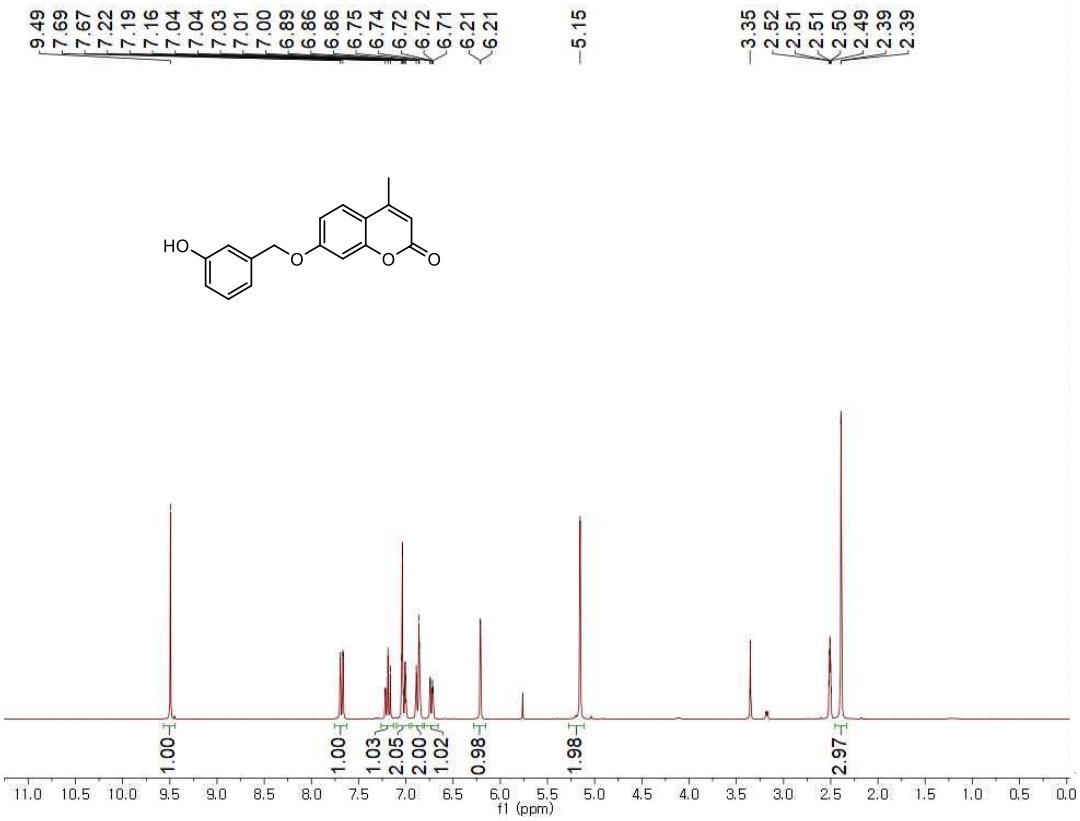


Fig. S1 ^{1}H- NMR spectrum of **TPTYR** (300 MHz, 298 K, DMSO-d_6).

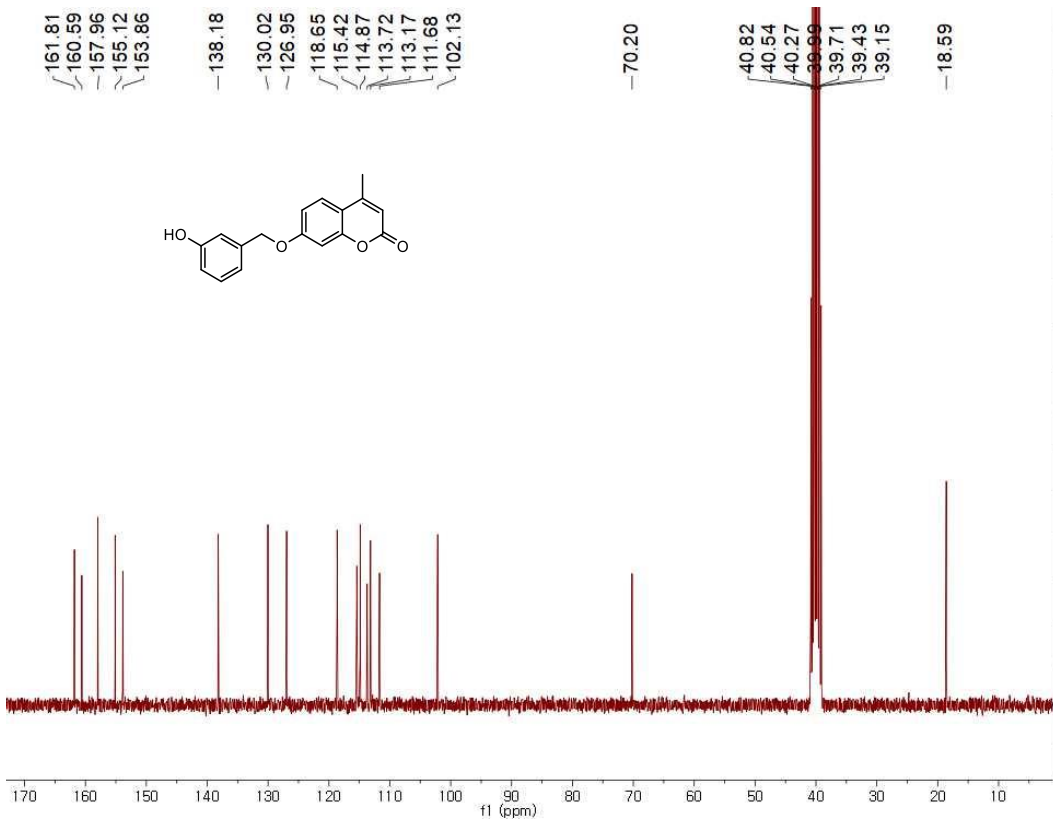


Fig. S2 ^{13}C-NMR spectrum of **TPTYR** (75 MHz, 298K, DMSO-d_6).

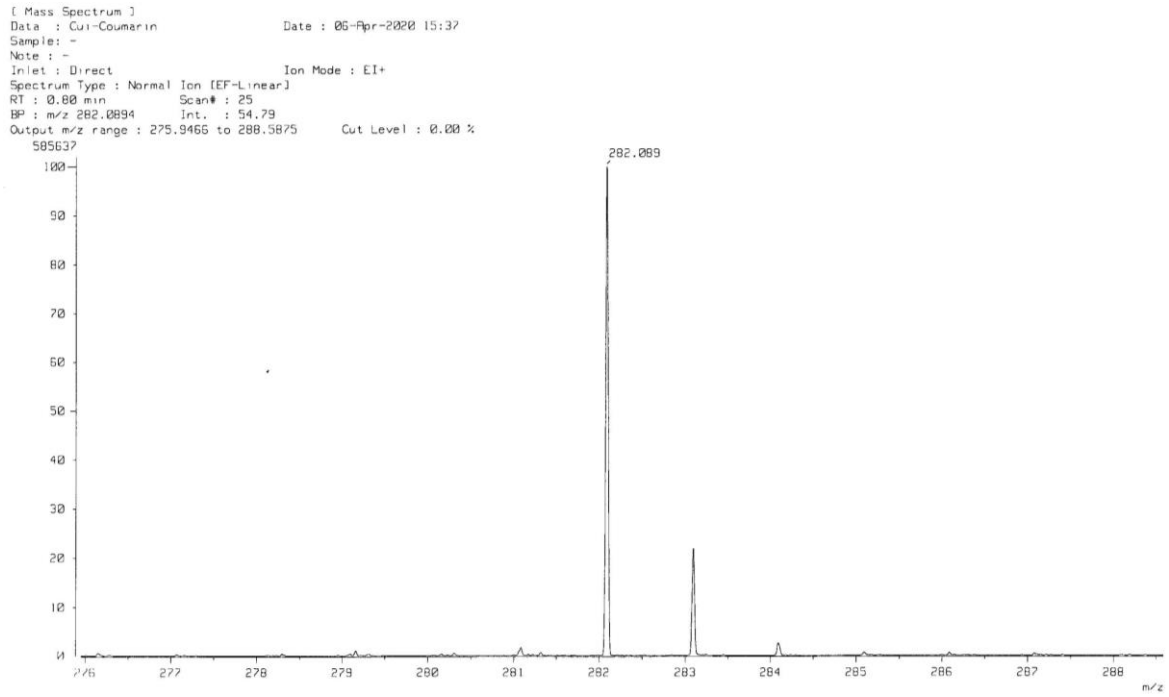


Fig. S3 High-resolution EI-Mass spectrum of **TPTYR**.

3. General procedure for analyses

Unless otherwise specified, the analyses were made according to the following procedure. In a tube, 10 μL of 1 mM **TPTYR** were mixed, followed by the addition of an appropriate volume of tyrosinase solution or other species. The final volume was adjusted to 2 mL with PBS and the reaction solution was mixed well. After incubation at 37 °C for 6 h, a 3-mL portion of the reaction solution was transferred to a quartz cell of 1-cm optical length to measure absorbance or fluorescence with $\lambda_{ex}/_{em}$ = 320/454 nm (both excitation and emission slit widths were set to 2.5 nm). Under the same conditions, a blank solution containing no tyrosinase (control) was prepared and measured for comparison.

4. Measurement of two-photon cross section

The two-photon action cross section ($\Phi\delta$) was determined using rhodamine 6G as the reference. **TPTYR** (5 μM) was dissolved in PBS buffer (10 mM, pH = 7.4) and two-photon induced fluorescence intensity was measured at 690-880 nm. Two-photon action values of the probe were calculated according to the following equation: $\Phi_s\delta_s = \Phi_r\delta_r(S_s/S_r)(C_r/C_s)(\varphi_r/\varphi_s)$. Subscripts s and r indicated sample and reference. $\Phi\delta$ denoted the TPA value. Capital S and φ represented the fluorescence signal and fluorescence collection efficiency of equipment, respectively. C indicated the concentration of the solution.

5. Test of fluorescence quantum yield

The fluorescence quantum yield (Φ) was tested using 4-methyl-7-hydroxylcoumarin as standard compound following the reported procedure.[1,3]

6. Two-Photon fluorescence microscopy

Two-photon fluorescence microscopy images were obtained with multiphoton microscopes (Leica TCS SP8MP) with ×40 oil objectives with numerical aperture (1.30) by exciting the probe with a mode-locked titanium-sapphire laser source set at a wavelength of 740 nm and output power of 2.49 W, which corresponded to 7.57×10^5 W cm^{-2} average power in the focal plane.

7. Cell Culture

All the cells were cultured on glass-bottomed dishes and incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C for 2 days before imaging. The cells were treated with **TPTYR** and incubated for 3 h, then they were washed using serum-free media. Each cell culture medium was supplemented with 10% FBS (WelGene), penicillin (100 units Ml^{-1}), and streptomycin (100 μg mL^{-1}). The culture medium of B16 cells is Roswell Park Memorial Institute 1640 (RPMI 1640) and HeLa cells is Dulbecco's Modified Eagle Medium (DMEM).

8. Cytotoxicity measurement

To evaluate cell viability of **TPTYR** to cells, MTT methods were performed in B16 and HeLa cells. Cells were cultured in 96-well plate for 24 h, then each concentration of **TPTYR** was added (0-50 μM). After incubation for 8 h, cell culture medium was replaced using MTT solution and further incubation for 4 h. Absorbance was measured at 450 nm using microplate reader.

9. Photostability test of TPTYR in cells

Photostability of **TPTYR** was determined by monitoring the changes in fluorescence intensity of **TPTYR**-labeled B16 cells. The intensities were recorded for 1 h at 2 sec intervals. The fluorescence intensity was maintained for 1 h, indicating that the probe has a high photostability.

10. Preparation and staining of rat skin tissue

Rat tissue slices were prepared from the dorsal skin of a 14 days old SD rat. The skin slices were cut into 800 μm thicknesses using a vibrating-blade microtome in Dulbecco's phosphate-buffered saline (DPBS) buffer. Slices were incubated with **TPTYR** and incubated for 3 h, then they washed using DPBS buffer. Slices were transferred to glass-bottomed dishes and observed in a two-photon fluorescence microscopy.

Supporting figures

11. Absorption and fluorescence spectra of fluorophore

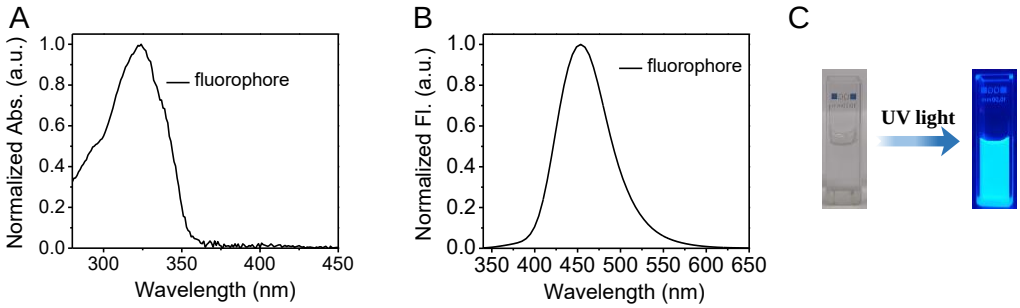


Fig. S4 (A) Absorption and (B) fluorescence spectra of fluorophore (coumarin, 5 μM) as well as (C) the color change in fluorescence under 365 nm hand lamp. $\lambda_{ex/em}$ = 320/454 nm.

12. Optimization of reaction conditions

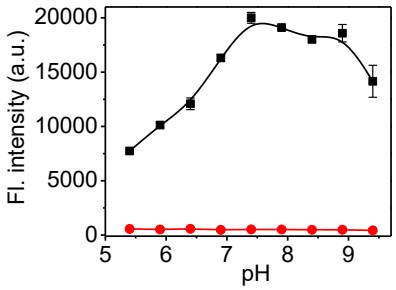


Fig. S5 Effect of pH on the fluorescence intensity of **TPTYR** (5 μM) before (red dot) and after (black dot) reaction with tyrosinase (200 U/mL) at 37 ºC in PBS buffer (10 mM). $\lambda_{ex/em}$ = 320/454 nm. The results are expressed as the mean ± standard deviation (n = 3).

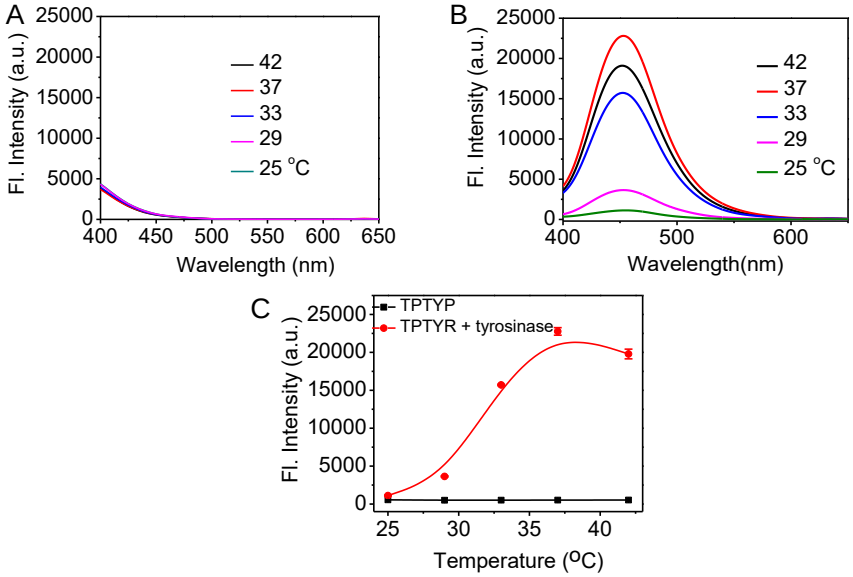


Fig. S6 The fluorescence spectra of **TPTYR** (5 μM) before (A) and after reaction (B) with tyrosinase (200 U/mL) at different temperature (25, 29, 33, 37 and 42 ºC) in PBS buffer (10 mM, pH 7.4) with $\lambda_{ex/em}$ = 320/454 nm. (C) The plots of fluorescence intensity versus temperature of **TPTYR** before (black) and after reaction (red) with tyrosinase (200 U/mL). The results are expressed as the mean ± standard deviation (n = 3).

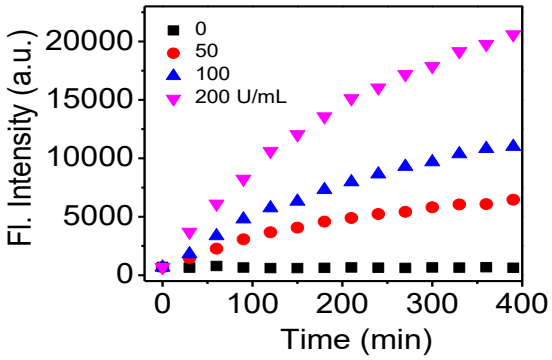


Fig. S7 Plots of fluorescence intensity vs. the reaction time of **TPTYR** (5 μM) with varied concentrations of tyrosinase (0-200 U/mL). The measurements were performed at 37 °C in PBS (pH 7.4) with $\lambda_{ex/em}$ = 320/454 nm.

13. Selectivity test

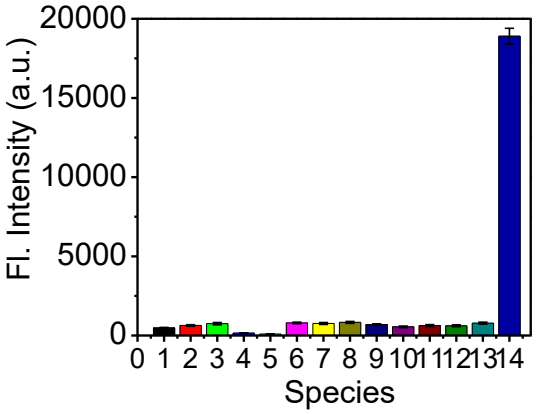


Fig. S8 Fluorescence response of **TPTYR** (5 μM) to various species: (1) blank; (2) $MgCl_2$ (2.5 mM); (3) $CaCl_2$ (2.5 mM); (4) $FeCl_3$ (2.5 mM); (5) $FeCl_2$ (2.5 mM); (6) glucose (10 mM); (7) Vc (1 mM); (8) glycine (1 mM); (9) glutamic acid (1 mM); (10) tyrosine (1 mM); (11) cysteine (1 mM); (12) glutathione (1 mM); (13) urea (20 mM); (14) tyrosinase (200 U/mL). The measurements were performed at 37 °C in PBS (pH 7.4) with $\lambda_{ex/em}$ = 320/454 nm. The results are expressed as the mean ± standard deviation (n = 3).

14. Linear relationship

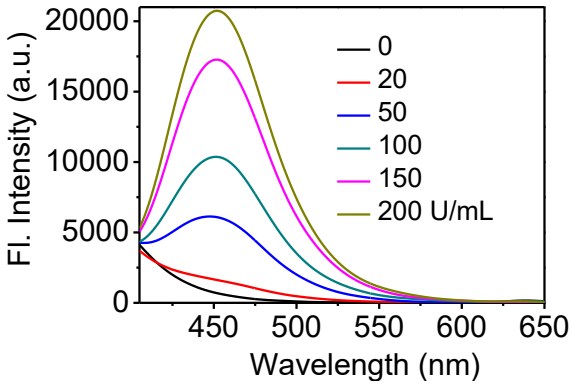


Fig. S9 (A) Fluorescence response of **TPTYR** (5 μM) to tyrosinase at various concentrations: (0, 20, 50, 100, 150 and 200 U/mL). The measurements were performed at 37 °C with $\lambda_{ex/em}$ = 320/454 nm.

15. Compared with other known tyrosinase fluorescent probes

Authors	Excitation/Emission	Detection limit	Biological application	One/Two Photon	Reference
Sidhu et al.	425/467 and 535 nm	0.2 U/mL^{-1}	A375 cells	One	Ref.4
Wu et al.	550/586 nm	0.04 U/mL^{-1}	B16, HepG2, MCF-7 cells	One	Ref.5
Ding et al.	670/706 nm	0.36 U/mL	Apple slices and B16 cells	One	Ref.6
Sidhu et al.	340/440 and 540 nm	1.2 U/mL	B16 cells	One	Ref.7
Bobba et al.	740/452 nm	0.025 U/mL	B16 cells	Two	Ref.8
Wu et al.	670/708 nm	2.76 U mL^{-1}	B16, Hela cells, Zebrafish	One	Ref.1
Yan et al.	770/503 nm	/	B16 and HeLa cells	Two	Ref.9
Li et al.	550/583 nm	0.5 U mL^{-1}	B16 and HepG2 cells	One	Ref.10
Li et al.	450/548 and 660 nm	0.6 U/mL	B16 and HeLa cells	One	Ref.11
Peng et al.	500/675 nm	0.5 U/mL	B16, HepG2, A549, HeLa, HPF, HSF, OCM-1A, and M619 cells	One	Ref.12
Cui	740/454 nm	0.34 U/mL	B16, HeLa and rat skin tissue	Two	This work

Table S1 Comparison of properties and biological applications of other known tyrosinase probes.

16. Effect of inhibitor on one-photon fluorescence intensity of the reaction system

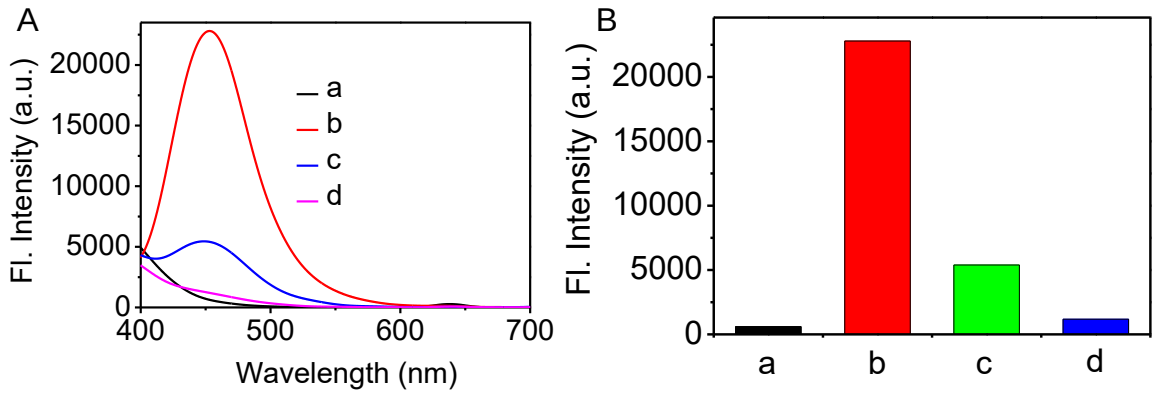


Fig. S10 (A) and (B) Fluorescence intensity of different reaction systems. (a) The fluorescence of **TPTYR** (5 μM) in PBS of pH 7.4); (b) system (a) + tyrosinase (200 U/mL); (c) system (b) + kojic acid (100 μM); (d) system (b) + kojic acid (200 μM). $\lambda_{ex/em}$ = 320/454 nm.

17. Effect of inhibitor on two-photon action and fluorescence intensity of the reaction system

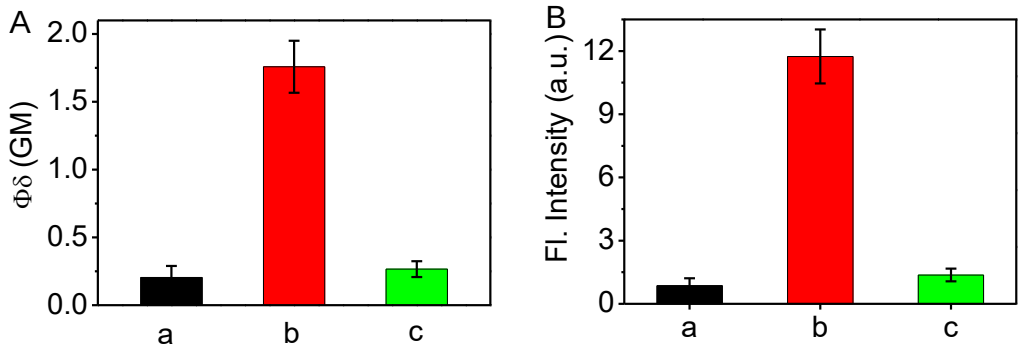


Fig. S11 (A) Two-photon action and (B) fluorescence intensity of different reaction systems**.** (a) **TPTYR** (5 μM) in PBS of pH 7.4); (b) system (a) + tyrosinase (200 U/mL); (c) system (b) + kojic acid (200 μM). $\lambda_{ex/em}$ = 740/454 nm. The results are expressed as the mean ± standard deviation (n = 3).

18. High-resolution mass spectrum of the solution of TPTYR with tyrosinase

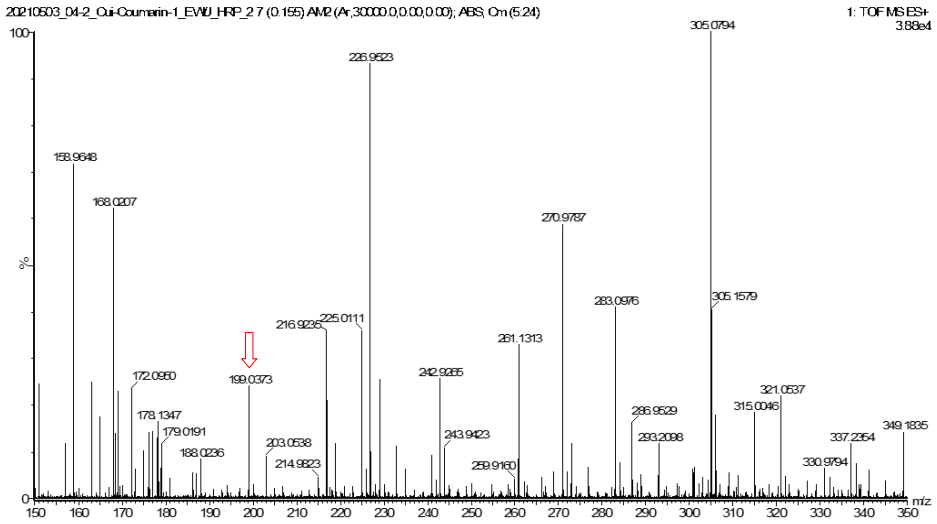


Fig. S12 High-resolution mass spectrum of reaction of **TPTYR** (20 μM) with tyrosinase (200 U/mL). The red arrow pointed at the peak of 199.0373 ($C_{10}H_8NaO_3^+$), indicating that the generation of coumarin.

19. Evaluation of the cytotoxicity of TPTYR to B16 and HeLa cells

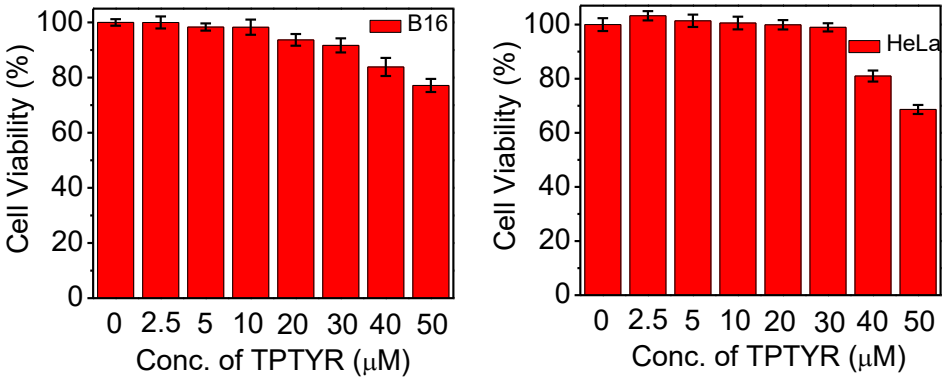


Fig. S13 Effects of **TPTYR** at varied concentrations (0-50 μM) on the viability of B16 and HeLa cells.

The viability of the cells without **TPTYR** is defined as 100%. The results are expressed as the mean ± standard deviation of five separate measurements (n = 5).

20. References

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