Supporting Information

Rational Engineering of Highly Sensitive and Specific Tyrosinase probe for Guiding Melanoma Surgical Resection

Yifeng Jian[a], Yongnan Luo[c], Ke Li[a]*, Minghui Yang[a]*, Xiang Chen[b]*

a. College of Chemistry & Chemical Engineering, Central South University, Changsha, Hunan 410083, China.

b. The Department of Dermatology, Xiangya Hospital, Central South University, Changsha, Hunan 410000, China.

c. Yellow River Conservancy Commission Hydrology and Water Resources Bureau of Henan

Corresponding Authors

Ke Li − College of Chemistry & Chemical Engineering, Central South University, Changsha, Hunan 410083, China. Email: kelicsu@csu.edu.cn.

Minghui Yang − College of Chemistry & Chemical Engineering, Changsha, Hunan 410083, China. Email: yangminghui@csu.edu.cn.

Xiang Chen − The Department of Dermatology, Xiangya Hospital, Central South University, Changsha, Hunan 410000, China. E-mail: chenxiangck@126.com.

Authors

Yifeng Jian − College of Chemistry & Chemical Engineering, Central South University, Changsha, Hunan 410083, China.

Yongnan Luo – Yellow River Conservancy Commission Hydrology and Water Resources Bureau of Henan, Zhengzhou, Henan 450004, China.

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1. Materials and Instruments

Reagents and solvents were obtained from Sigma-Aldrich, Bide Pharmatech Ltd, and Beyotime Biotechnology, Shanghai Alad-din Biochemical Technology Co. Ltd, and were used as received unless otherwise noted. NMR spectra were performed using a Bruker DRX-400 spectrometer at 400 MHz or 500 MHz for 1H NMR and 126 MHz for 13C NMR. Fluorescence spectra in the experiment were recorded using a Hitachi F-7000 fluorescence spectrophotometer (Tokyo, Japan). Absorption spectra were measured with a Shimadzu UV-2600i spectrophotometer (Kyo-to, Japan). Confocal images were acquired using an Olympus FV3000 full-spectrum near-infrared laser scanning confocal microscope (Tokyo, Japan).

2. Spectral Measurements

The probes SCy-con and SCy-tyr were dissolved in DMSO to prepare 1 mM stock solutions (stored at 4 °C). All in vitro spec-tral measurements were performed in a mixed system contain-ing 25 mM HEPES buffer (pH 7.4) and DMSO at a volume ratio of 4:1 (v/v). Unless otherwise specified, all analyte solu-tions were incubated at 37 °C for 2.5 hours prior to spectral acquisition. The excitation and emission slit widths were set to 5.0 nm each.

3. Molecular Docking Simulation

The three-dimensional structures of the probes were constructed using ChemDraw, and their geometries were energy-minimized. The crystal structure of tyrosinase (PDB code: 2Y9X) was retrieved from the Protein Data Bank. The binding modes between TYR and the probes were analyzed using AutoDock, and the resulting data were processed and visualized with PyMOL. All parameters were calculated following standard procedures

4. Live cell Imaging

All experiments were conducted in living cells. B16-F10, HepG-2, 4T1, and HeLa cells were seeded in confocal dishes and cultured for 24 hours (37 °C, 5% CO_2) in an incubator. After cell adhesion, the probes were added and incubated for 1 hour, fol-lowed by imaging under a fluorescence confocal microscope. For inhibitor experiments, B16-F10 cells were first incubated with 200 μM kojic acid for 2 hours, then treated with 10 μM SCy-tyr for another 1 hour before fluorescence imaging. λex = 640 nm, λem = 650-700 nm.

5. Apple Slice Staining and Imaging

Square-shaped apple slices (10 mm side length, 2 mm thickness) were placed in an incubator at 37 °C or 4 °C. The control group was pretreated with 10 mM PBS for 2 h, while the inhibitor group was pretreated with 200 μM kojic acid for 2 h. Digital images of the apple slices were captured using a smartphone at various time points. Small tissue samples from the apple slices at the 0 h and 3 h time points were collected, stained with 10 μM SCy-tyr, and imaged under a fluorescence confocal microscope. λ_{ex} = 640 nm, λ_{em} = 650-700 nm.

6. Tumor Imaging and Image-Guided Resection Studies

BALB/c nude mice (female, 6–8 weeks old) were purchased from Hunan SJA Laboratory Animal Co., Ltd. (Hunan, China). All animals were housed under specific pathogen-free conditions at a temperature of 22 ± 2 °C, relative humidity of 50 ± 5%, and a 12 h light/dark cycle, with 3–5 mice per cage. Food and water were provided ad libitum. For anesthesia, mice received an intraperitoneal injection of tribromoethanol (250 mg/kg body weight); the depth of anesthesia was

confirmed by loss of the righting reflex, muscle relaxation, and absence of response to painful stimuli. To establish the tumor model, each mouse was subcutaneously injected in the right dorsal region with 25 μL of PBS containing 1×10^7 B16-F10 cells. After one week, when the melanoma tumors had grown to approximately soybean size, the tumor-bearing mice were administered the probe (experimental group) or an equal volume of PBS (control group) via intratumoral injection or topical spray, and then imaged using a small animal imaging system (excitation wavelength: 640 nm; emission detection: 695–770 nm). At the end of the experiments, mice were euthanized by cervical dislocation.

7. Cell Culture

B16-F10 (Mouse melanoma cells), 4T1 (mouse breast cancer cells), HepG-2 (Human hepatocellular carcinoma cell line), and Hela (Human cervical cancer cell line) were obtained from Xiangya Hospital, China. Cells were maintained in a RPMI-1640 (B16-F10 and 4T1) or DMEM (HepG-2 and Hela) supplemented with fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin) and cultured under 37°C in a 5% CO2/95% air humidified incubator. When the cell density in the culture flask reached 80–90%, the cells were passaged into confocal dishes and further cultured for 24 hours. Imaging experiments were performed after cell adhesion was achieved.

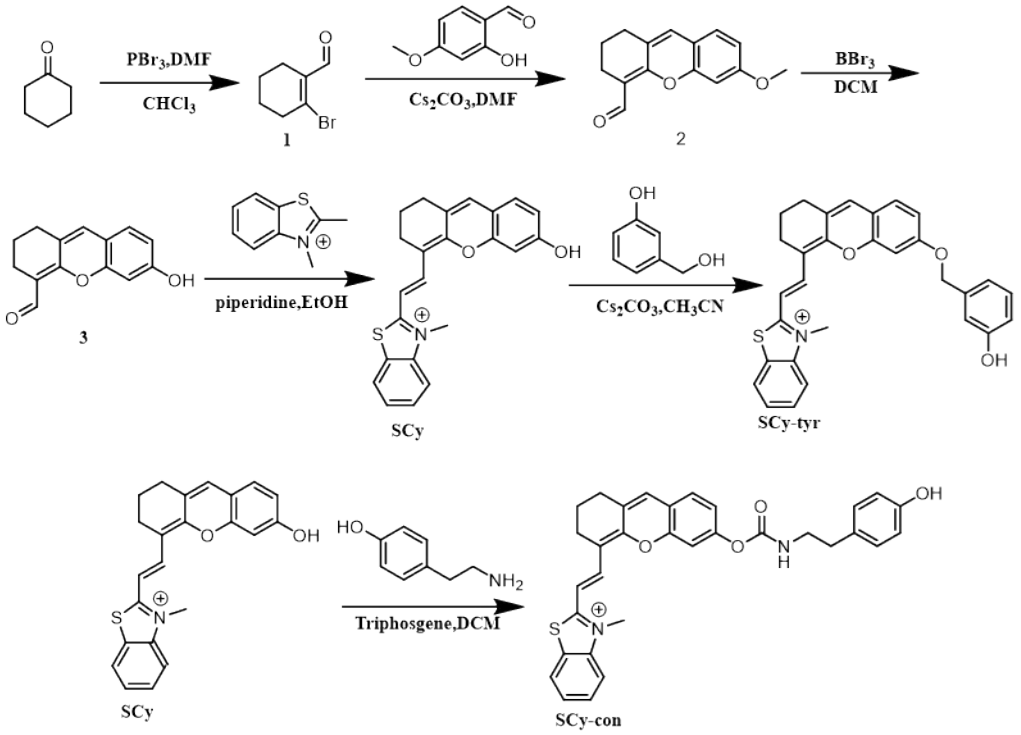
8. Flow Cytometry Analysis

Cells to be analyzed were seeded in 6-well plates and cultured at 37 °C under 5% CO_2 for 24 hours. Unless otherwise specified (e.g., for time-dependent analysis in Figure 3c), all treatments involved incubation with 10 μM SCy-tyr for 1 hour. Subsequently, the cells were washed twice with PBS and detached using 0.25% trypsin. For the inhibitor group, cells were pre-treated with 200 μM kojic acid for 2 hours prior to the above procedure. The trypsinized cells were then analyzed by flow cytometry (CytoFLEXS, Beckman Coulter Life Sciences, USA).

9. Cytotoxicity Assay

Cytotoxicity was assessed using the CCK-8 (Cell Counting Kit) assay. Four cell types (B16-F10, HepG-2, 4T1, HeLa) were seeded into 96-well cell culture plates at a density of 5×10^3 cells per well. Each well was treated with 200 μL of culture medium containing SCY-tyr at concentrations of 1, 3, 5, 10, 15, and 20 μM. After incubation at 37 °C under 5% CO_2 for 24 hours, the old medium was aspirated, and 200 μL of fresh medium containing 10% CCK-8 reagent was added to each well. The plates were further incubated for 2–3 hours. Absorbance was measured at 450 nm using a microplate reader. Cell viability was calculated according to the following formula: Cell viability (%) = [(Mean absorbance of treated group − Mean absorbance of blank group (medium with CCK-8, no cells)] / [(Mean absorbance of control group (cells with CCK-8, no drug) − Mean absorbance of blank group)] × 100%.

10. Synthesis



Scheme S1. The synthetic routes of SCy-tyr and SCy-con.

Synthesis of SCy. Compound 3 was synthesized according to the reported method.[1] Piperidine (1.0 mL) was added to the solution of the compound 3 (150.0 mg, 0.7 mmol) and 2,3-dimethylbenzothiazolium iodide (382.0 mg, 1.3 mmol) in anhydrous ethanol (20.0 mL) dropwise. The resulting solution was refluxed under stirring for 6 h, and then the solvent was removed by evaporation under reduced pressure. The crude product was further purified by silica column chromatography to produce a blue solid. Yield: 204.0 mg (62%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.59 (s, 1H), 8.25 (dd, J = 20.5, 11.2 Hz, 2H), 8.02 (d, J = 8.2 Hz, 1H), 7.73 (s, 1H), 7.63 (d, J = 7.9 Hz, 1H), 7.32 (d, J = 8.1 Hz, 1H), 7.22 (s, 1H), 6.91 – 6.78 (m, 2H), 6.74 (d, J = 8.4 Hz, 1H), 4.12 (s, 3H), 2.74 – 2.57 (m, 4H), 1.80 (s, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 169.85, 160.80, 157.72, 153.73, 142.06, 141.76, 130.88, 128.77, 128.62, 127.05, 125.60, 123.61, 115.54, 113.47, 111.76, 105.37, 101.87, 43.66, 35.04, 28.48, 24.24, 22.15, 20.07. HRMS (ESI) m/z calcd for $C_{23}H_{20}NO_2S^+$ $[M]^+$: 374.1209, found: 374.1179.

Synthesis of Probe SCy-tyr. SCy (100.0 mg, 0.2 mmol) and Cs_2CO_3 (130.0 mg, 0.4 mmol) were dissolved in anhydrous CH3CN (20.0 mL). The mixture was stirred for 0.5 h at room temperature. The 3-bromomethylphenol was added, and the reaction mixture was then stirred for 12 h at 25 ℃ under nitrogen atmosphere. After that, the reaction mixture was then concentrated under reduced pressure using a rotary evaporator to obtain a crude solid residue. Purification was achieved through column chromatography to produce a blue solid. Yield:40.0 mg (32%). 1H NMR (500 MHz, DMF-d_7) δ 10.14 (s, 1H), 8.89 (d, J = 14.7 Hz, 1H), 8.74 (d, J = 8.1 Hz, 1H), 8.57 (d, J = 8.5 Hz, 1H), 8.23 (dd, J = 8.5, 7.2, 1.2 Hz, 1H), 8.11 (t, J = 7.7 Hz, 1H), 7.89 (d, J = 2.2 Hz, 1H), 7.86 (d, J = 2.5 Hz, 1H), 7.73 (d, J = 2.0 Hz, 1H), 7.67 (d, J = 3.2 Hz, 3H), 7.44 (d, J = 2.6 Hz, 1H), 7.38 (d, J = 7.6 Hz, 1H), 7.27 (dd, J = 8.0, 2.5 Hz, 1H), 5.65 (s, 2H), 4.76 (s, 3H), 3.32 (p, J = 1.9 Hz, 2H), 3.15 (p, J = 1.9 Hz, 2H), 3.11 (dd, J = 12.5, 6.3 Hz, 2H). 13C NMR (126 MHz, DMF-d_7) δ 142.91, 139.52, 138.62, 130.02, 129.46, 128.90, 127.78, 125.00, 124.93, 124.08, 118.74, 116.08, 115.50, 115.07, 114.43, 113.20, 106.51, 102.33, 70.75, 35.52, 35.47, 35.36, 35.26,

35.23, 35.19, 35.10, 29.19, 24.89, 22.87, 14.04. HRMS (ESI) m/z calcd for $C_{30}H_{26}NO_3S^+$ $[M]^+$: 480.163, found: 480.281.

Synthesis of Probe SCy-con. SCy (100.0 mg, 0.2 mmol), N, N-Diisopropylethylamine (DIPEA, 1.0 mL) and triphosgene (178.0 mg, 0.6 mmol) were dissolved in absolutely anhydrous CH2Cl2 (20.0 mL). The mixture was stirred for 3 h at 25 ℃ under nitrogen atmosphere. The solvent was removed by evaporation under reduced pressure. After that, 4-2-aminoethylphenol (55.0 mg, 0.4 mmol) dissolved in CH2Cl2 (20.0 mL) and DIPEA (1.0 mL) were added, and then the reaction mixture was stirred for 12 h at 25 ℃ under nitrogen atmosphere. The reaction mixture was then concentrated under reduced pressure using a rotary evaporator to obtain a crude solid residue. Purification was achieved through column chromatography to produce a blue solid. Yield:46.0 mg (35%). 1H NMR (400 MHz, DMSO-d_6) δ 10.67 (s, 1H), 8.36 – 8.21 (m, 3H), 8.03 (s, 1H), 7.78 – 7.72 (m, 1H), 7.64 (t, J = 7.6 Hz, 1H), 7.59 – 7.51 (m, 1H), 7.37 – 7.30 (m, 2H), 7.23 (s, 1H), 7.19 (d, J = 8.4 Hz, 1H), 7.08 – 6.98 (m, 1H), 6.91 – 6.80 (m, 2H), 6.76 (dd, J = 8.4, 2.3 Hz, 1H), 4.13 (s, 3H), 2.65 (dt, J = 10.8, 6.0 Hz, 6H), 1.83 – 1.78 (m, 2H), 1.24 – 1.13 (m, 2H). 13C NMR (126 MHz, DMSO- d_6) δ 169.88, 160.83, 157.75, 153.72, 151.21, 142.10, 142.07, 141.79, 130.90, 129.84, 129.29, 128.77, 128.62, 127.06, 126.50, 125.60, 123.61, 121.60, 120.93, 115.54, 113.94, 113.49, 111.75, 105.38, 101.87, 64.88, 35.01, 28.49, 24.24, 20.08, 18.61, 15.14. HRMS (ESI) m/z calcd for $C_{32}H_{29}N_2O_4S^+$ $[M]^+$: 537.184, found: 537.311.

11. Supplemental Figures

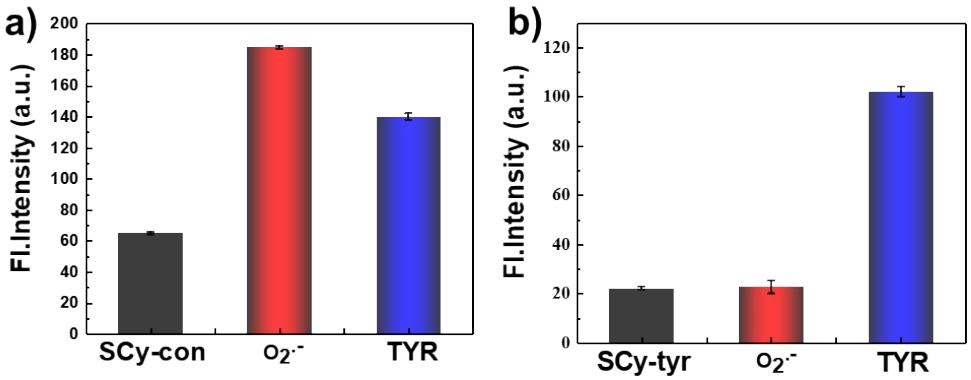


Figure S1. Fluorescence intensity of (a) 10 μM SCy-con and (b) 10 μM SCy-tyr at 730 nm incubated with 200 μM $O_2^{\cdot-}$ or 200 U/mL TYR (λ_{ex} = 680 nm). Data are presented as mean values ± s.d. (n= 3 independent experiments).

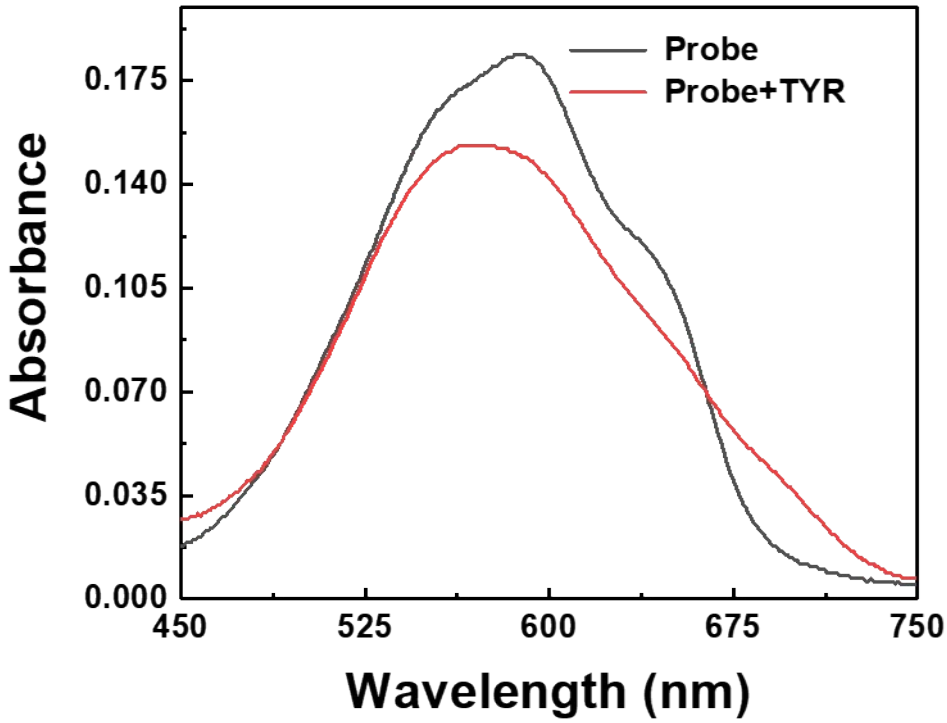


Figure S2. Absorbance spectra of SCy (10 μM) and SCy-tyr (10 μM) before and after the addition of TYR (200 U/mL).

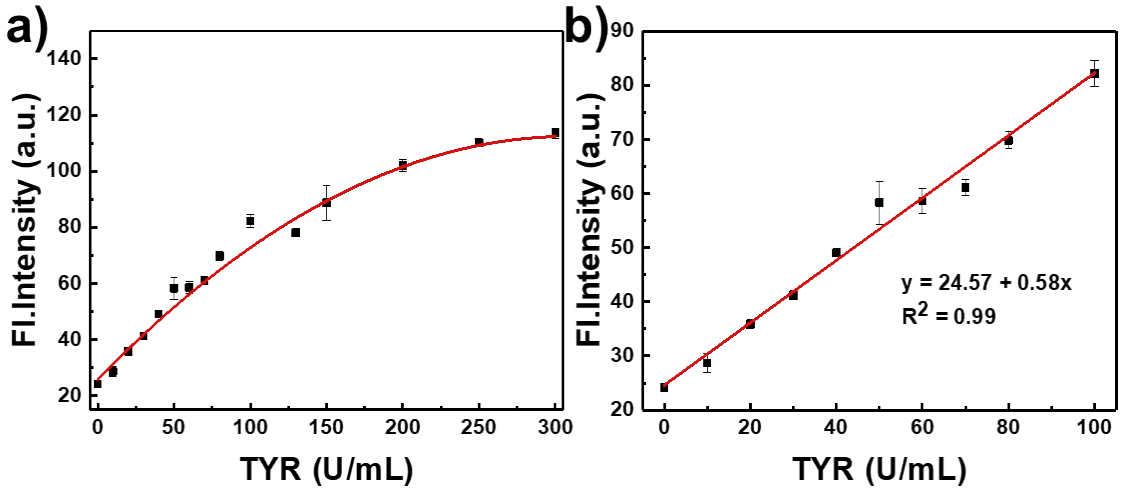


Figure S3. (a) Linear curve toward the concentration of TYR from 0 to 300 U/mL. (b) The linear fitting curve of the fluorescence enhancement towards the concentration of TYR between 0 and 100 U/mL..

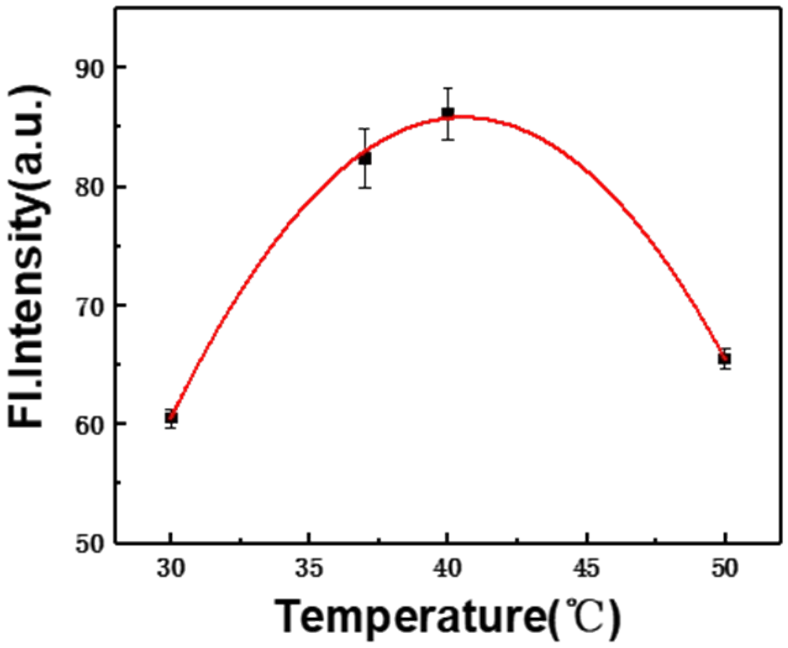


Figure S4. Fluorescence intensity of 10 μM SCy-tyr after incubation with 200 U/mL TYR for 2.5 h at different temperatures. $\lambda_{ex/em}$ = 680/730 nm.

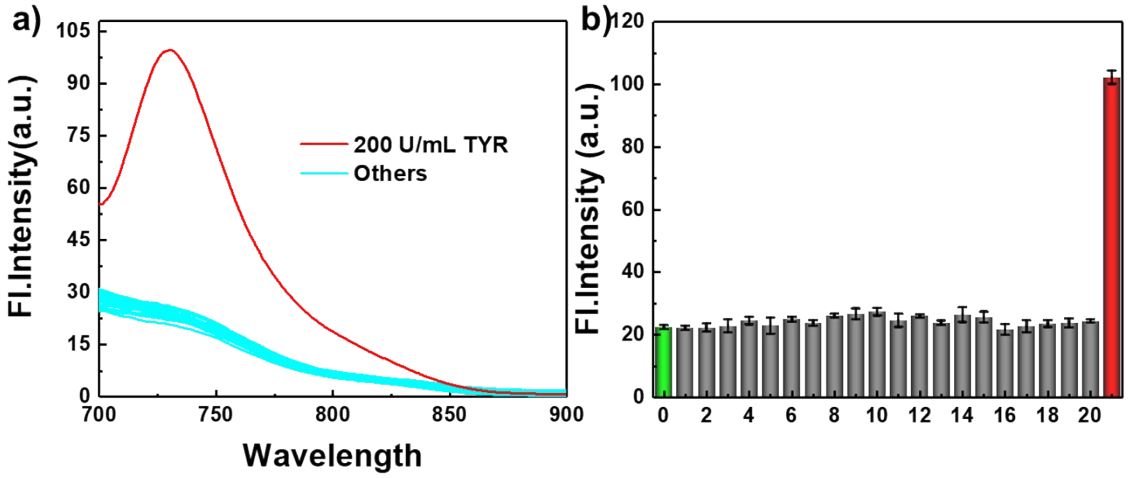


Figure S5. (a) Fluorescence spectra of SCy-tyr (10 μM) after adding various substances. (b) Fluorescence intensity at 730 nm in (a). (0) none, (1) $CuSO_4$, (2) $CaCl_2$, (3) L-valine, (4) DL-arginine, (5) L-threonine, (6) glutathione, (7) L-serine, (8) L-leucine, (9) DL-Aspartic Acid, (10) L-phenylalanine, (11) Dl-aspartic alkali, (12) L-cysteine, (13) $FeCl_3$, (14) L-proline, (15) L-histidine, (16) DL-tyrosine, (17) L-isoleucine, (18) L-glutamic acid, (19) β -alanine, (20) glycine, (21) TYR (200 U/mL). $\lambda_{ex/em}$ = 680/730 nm.

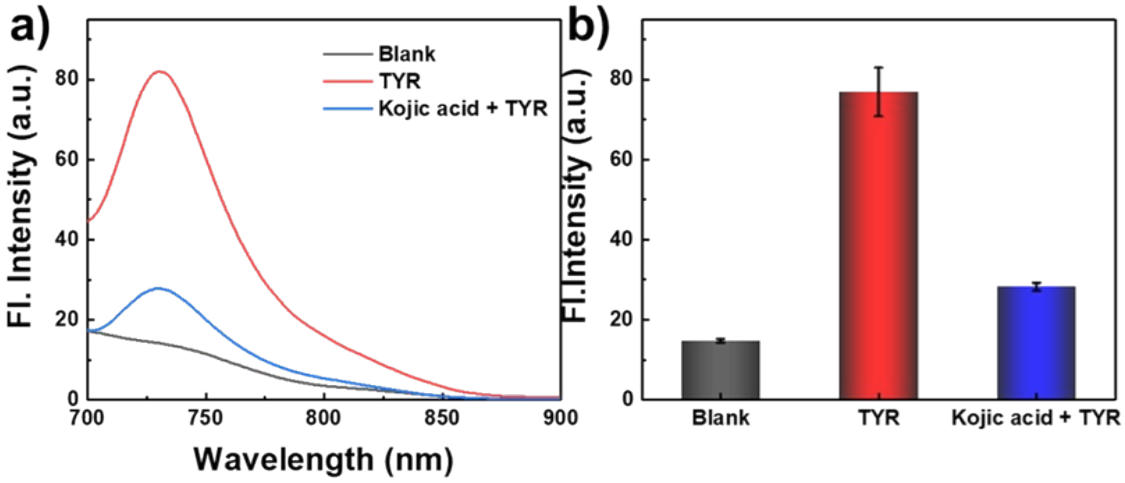


Figure S6. (a) Fluorescence spectra of 10 μM SCy-tyr incubated with 200 U/mL TYR. Kojic acid group: TYR was pretreated with 200 μM kojic acid for 2 hours, followed by incubation with SCy-tyr for 2.5 hours. (b) Fluorescence intensity at 730 nm in (a). λ_{ex} = 680 nm.

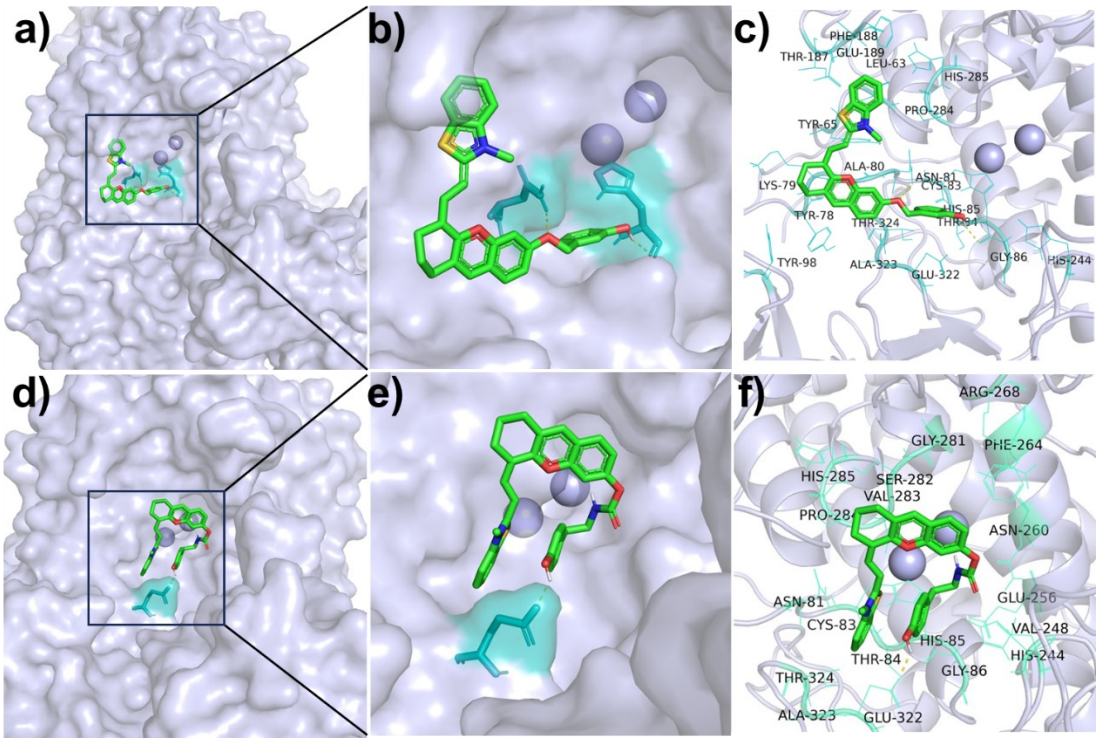


Figure S7. Molecular docking results of SCy-tyr (the first raw) and SCy-con (the second raw) in the binding pocket of tyrosinase displayed as the surface (PDB code: 2Y9X). (a) and (d): Binding mode of the probes to the TYR active site; (b) and (e): Local enlargements of (a) and (d); (c) and (f): docking simulation of the local interactions between probes and TYR, where the hydrogen bonds are illustrated as yellow dashed lines. Carbons are in green, nitrogens in yellow, oxygens in red, and amino acid residues that form hydrogen bonds with the probes in cyan.

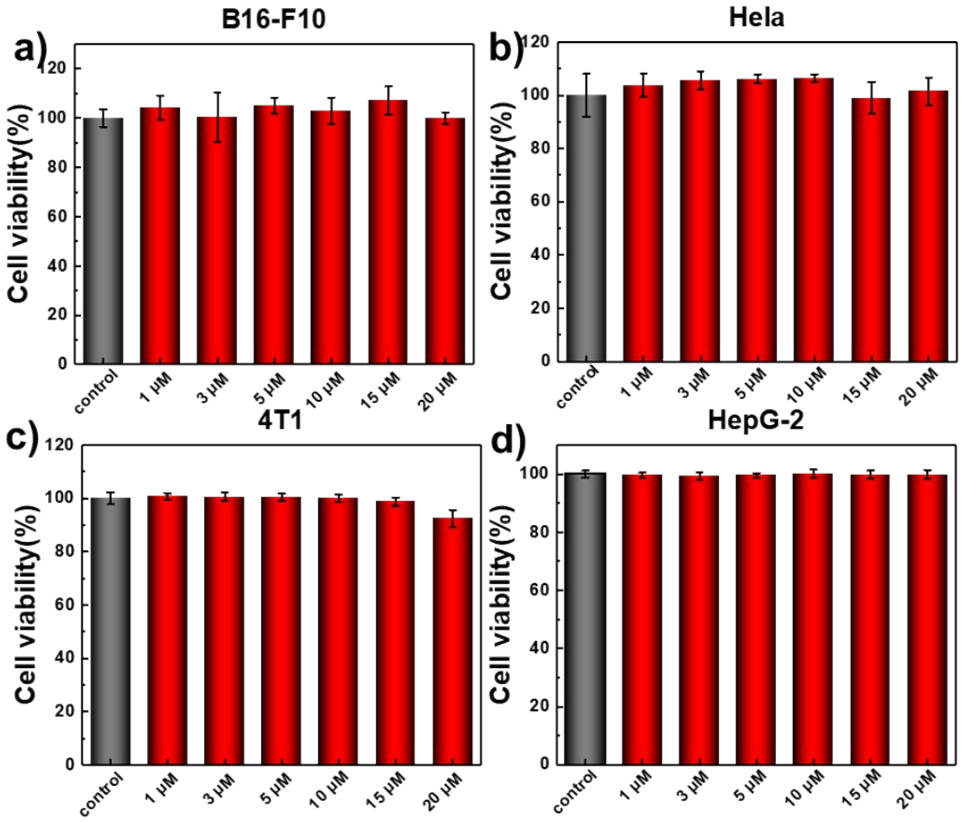


Figure S8. Cytotoxicity analysis of SCy-tyr toward (a) B16-F10, (b) Hela, (c) 4T1, and (d) HepG-2.

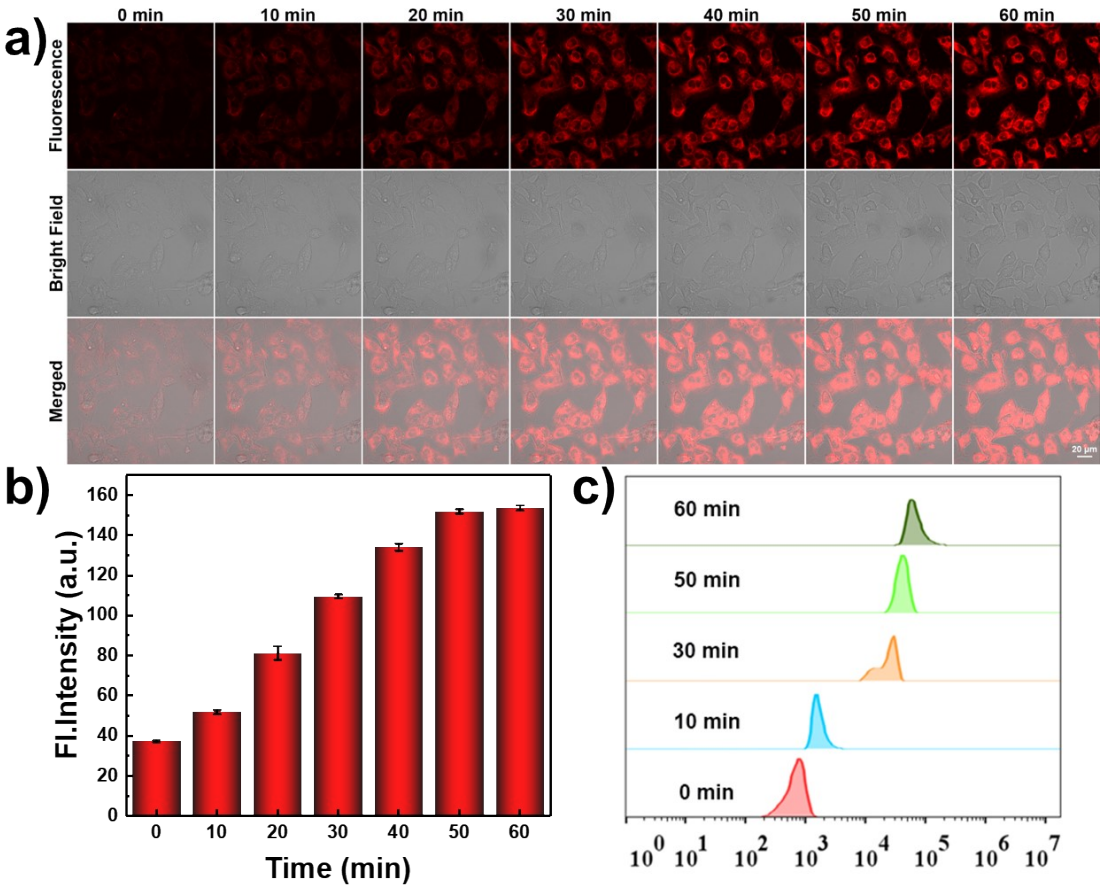


Figure S9. (a) Real-time imaging of B16-F10 cells incubated with SCy-tyr (10 μM) for 60 min, with CLSM images obtained at 10 min intervals. (b) Average fluorescence intensity in panel (a). Data represent mean ± standard error (n = 3 independent experiments). (c) Flow cytometric analysis of the cell samples used in (a). λ_{ex} = 640 nm, λ_{em} = 650-700 nm; scale bar: 20 μm.

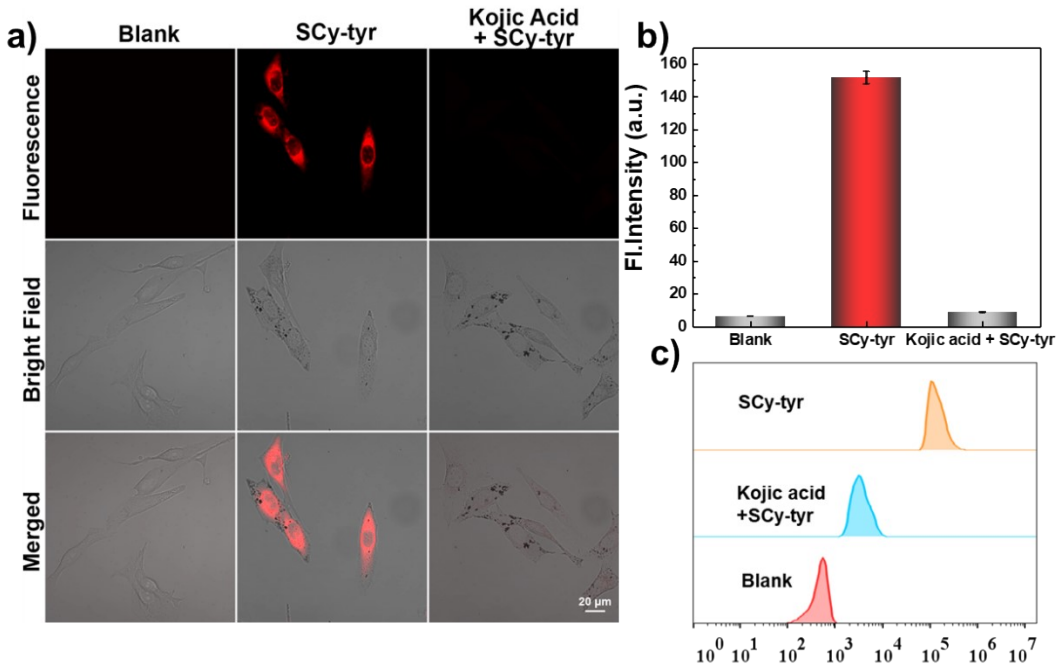


Figure S10. (a) B16-F10 cells were pre-treated with or without kojic acid for 2 h, and then incubated with SCy-tyr (10 μM) for 60 min, followed by obtaining CLSM images. (b) Average fluorescence intensity in panel (a). Data represent mean ± standard error (n = 3 independent experiments). (c) Flow cytometric analysis of the cell samples used in (a). λ_{ex} = 640 nm, λ_{em} = 650-700 nm; scale bar: 20 μm.

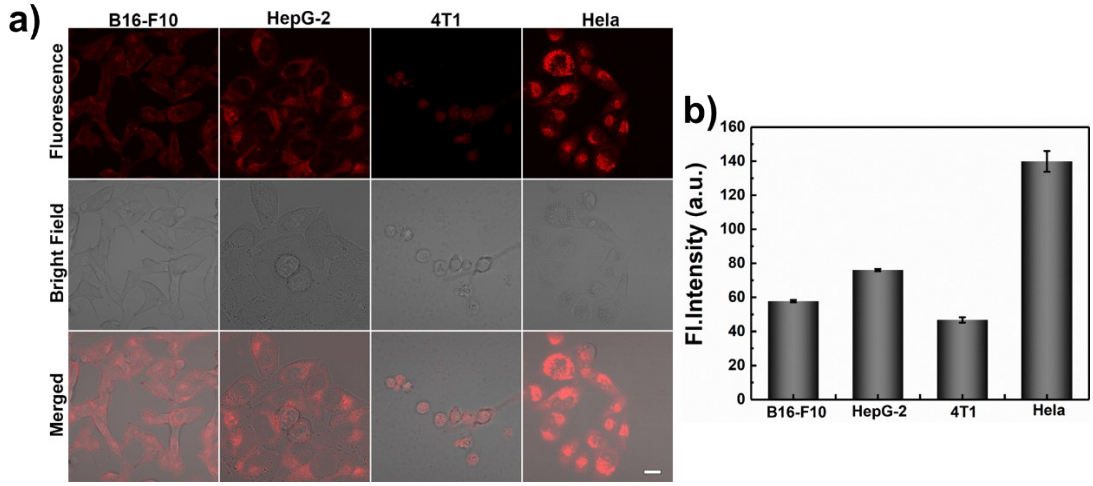


Figure S11. (a) B16-F10, HepG-2, 4T1 and Hela cells were incubated with SCy-con (10 μM) for 60 min, respectively, followed by obtaining CLSM images. (b) Average fluorescence intensity in panel (a), scale bar: 20 μm.

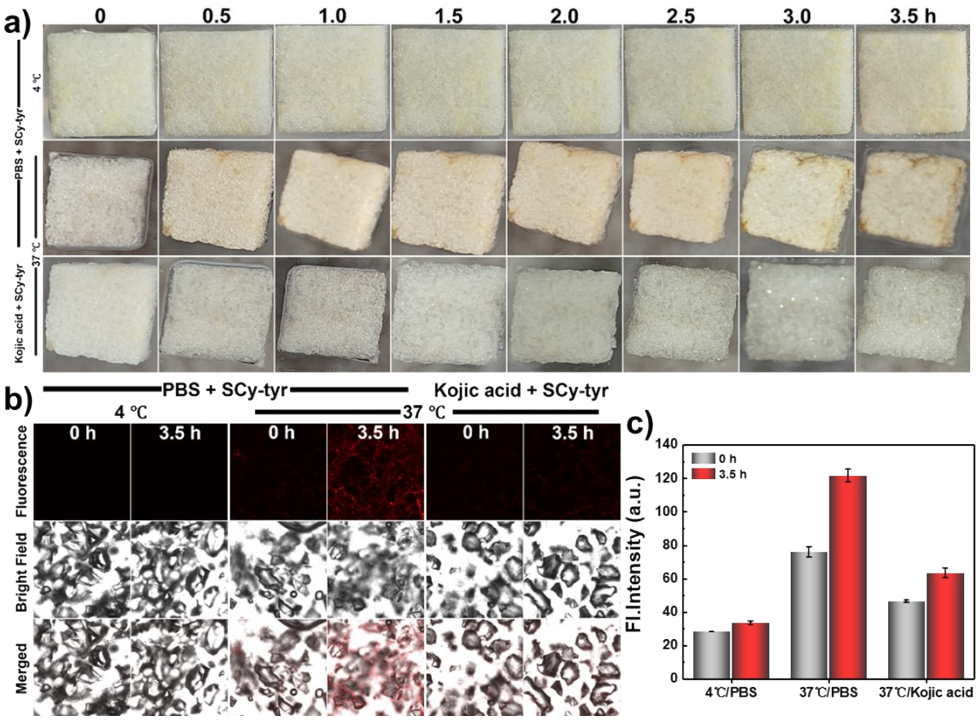


Figure S12. (a) Digital images of apple slices (10 mm-side length) at 0, 0.5, 1, 1.5, 2, 2.5, 3 and 3.5 h respectively. (b) Fluorescence images of apple slices at 0 and 3.5 h. Apple slices were pretreated with PBS (10 mM) or kojic acid (200 μM) for 2 h, and then incubated with SCy-tyr for 3.5 h, followed by obtaining CLSM images. scale bar: 200 μm. (c) Average fluorescence intensity in panel (b).

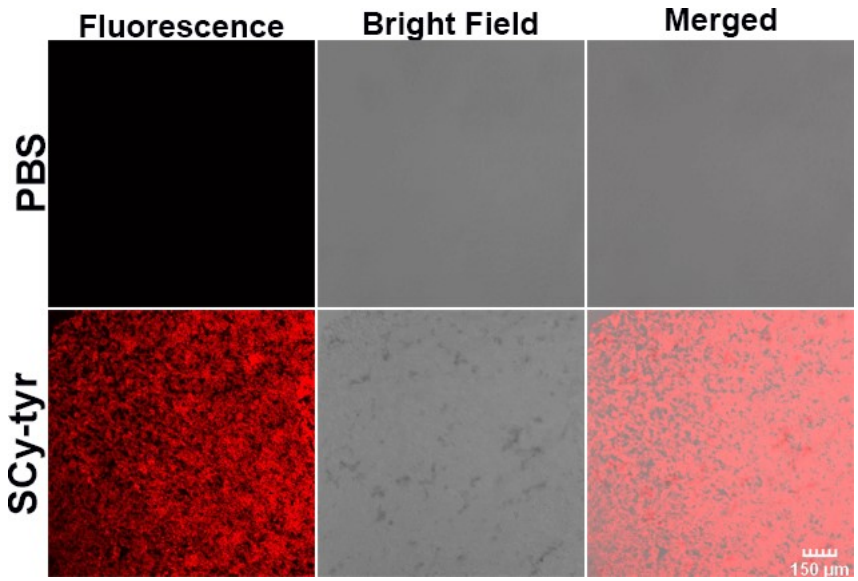


Figure S13. CLSM images of melanoma tissue incubated with SCy-tyr (10 μM) or PBS (10 mM) for 60 min.

12. ^{1}H NMR, ^{13}C NMR and HRMS Spectra

Figure S14. ^{1}H NMR spectrum of SCy in DMSO-d_6.

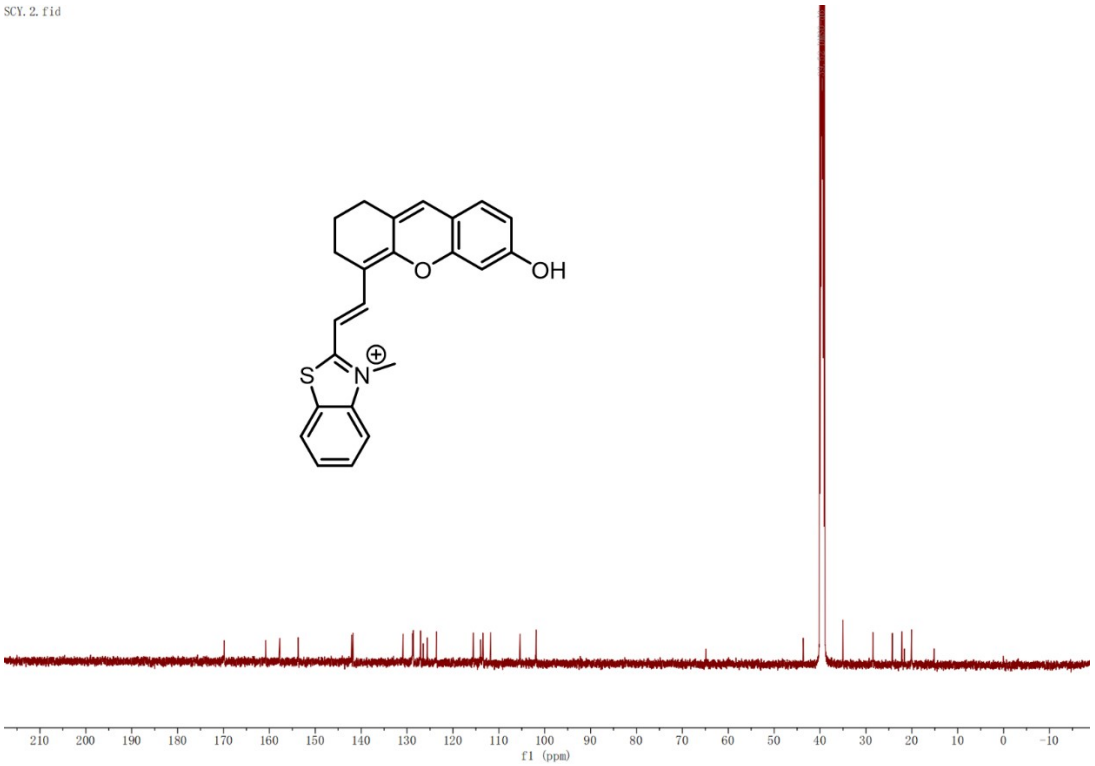


Figure S15. ^{13}C NMR spectrum of SCy in DMSO-d_6.

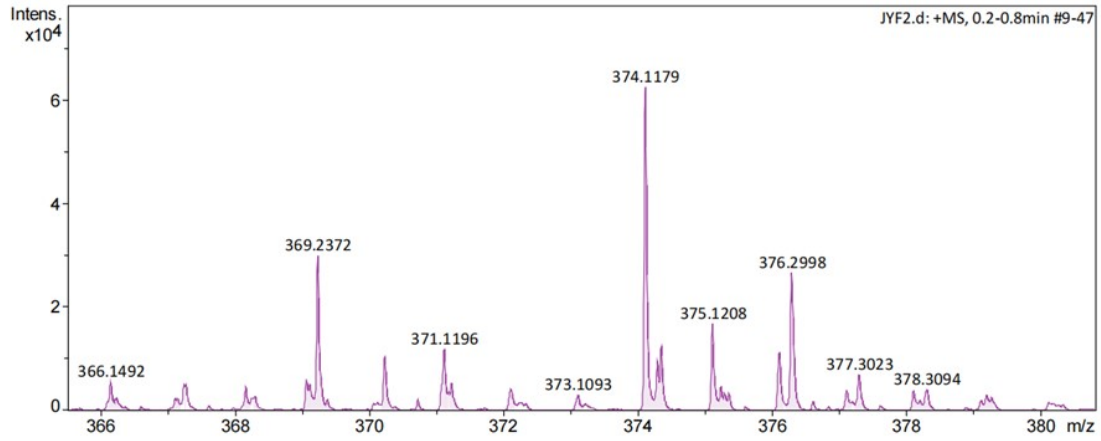


Figure S16. HRMS spectrum of SCy.

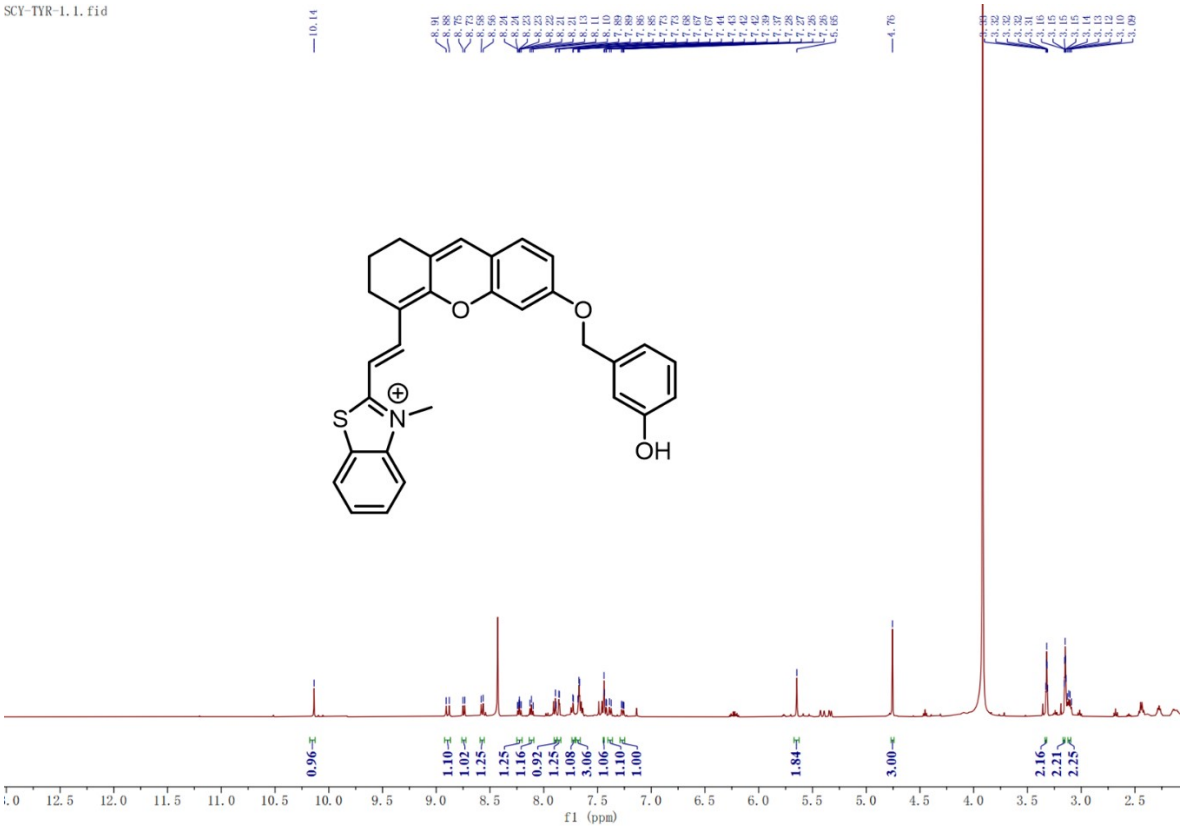


Figure S17. ^{1}H NMR spectrum of SCy-tyr in DMF-d_7.

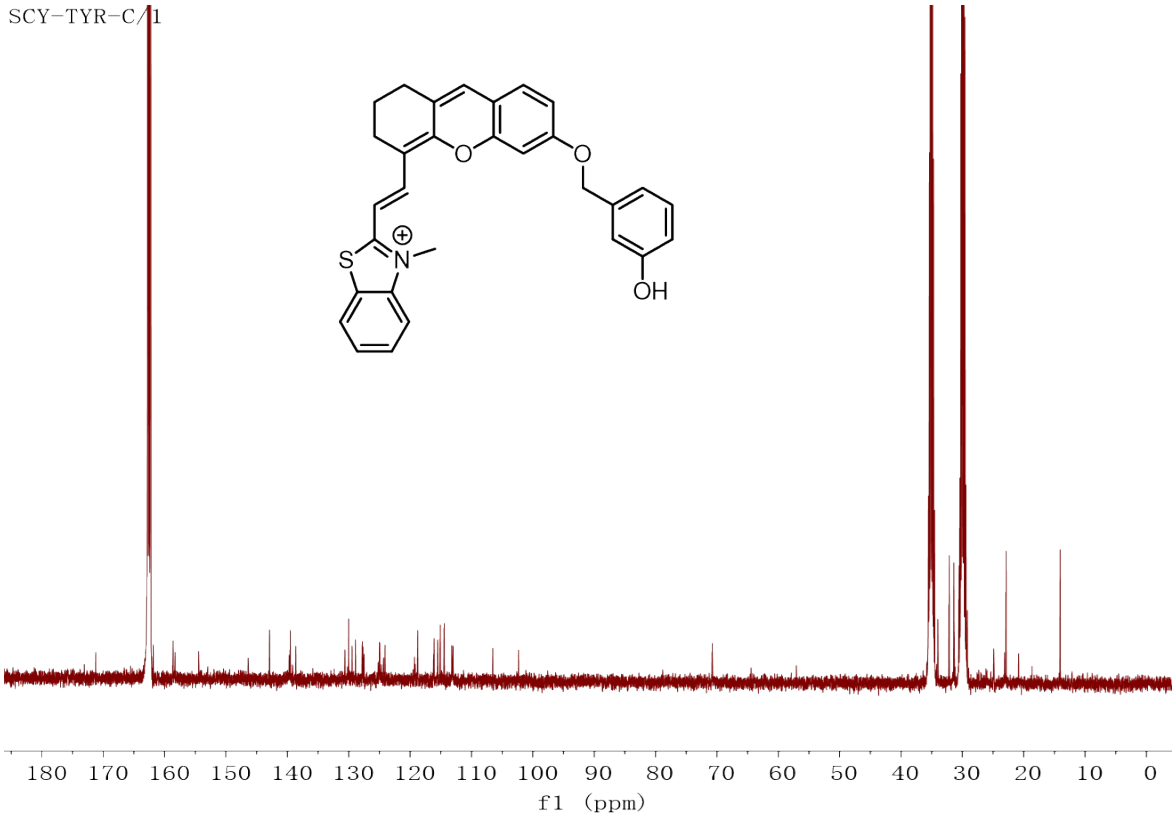


Figure S18. ^{13}C NMR spectrum of SCy-tyr in DMF-d_7.

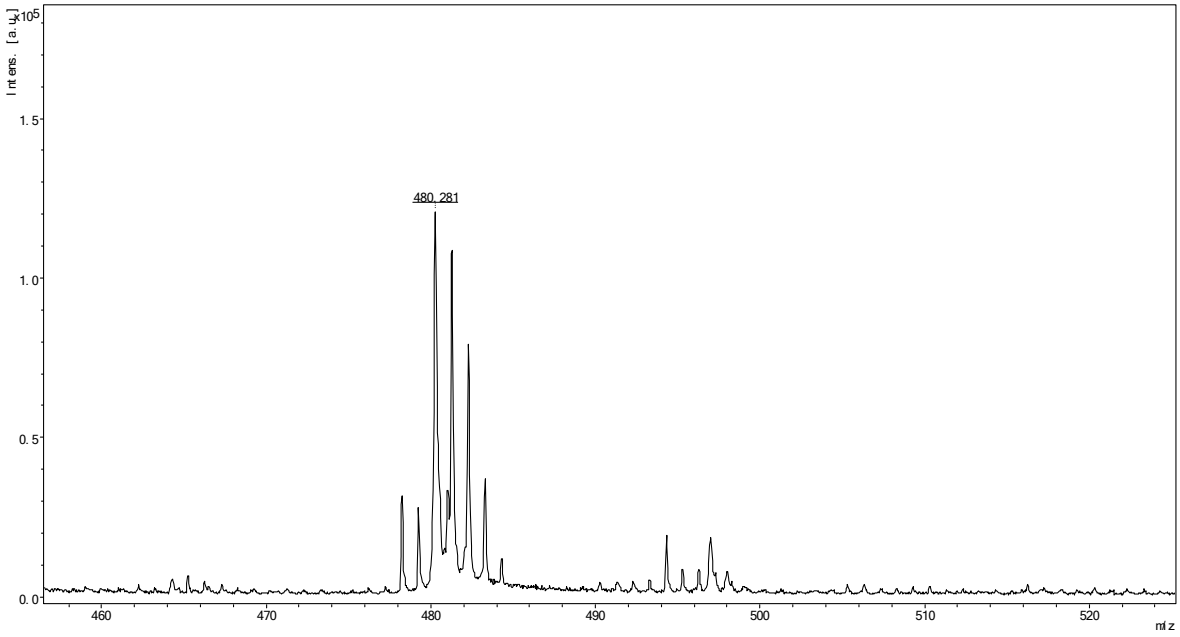


Figure S19. HRMS spectrum of SCy-tyr.

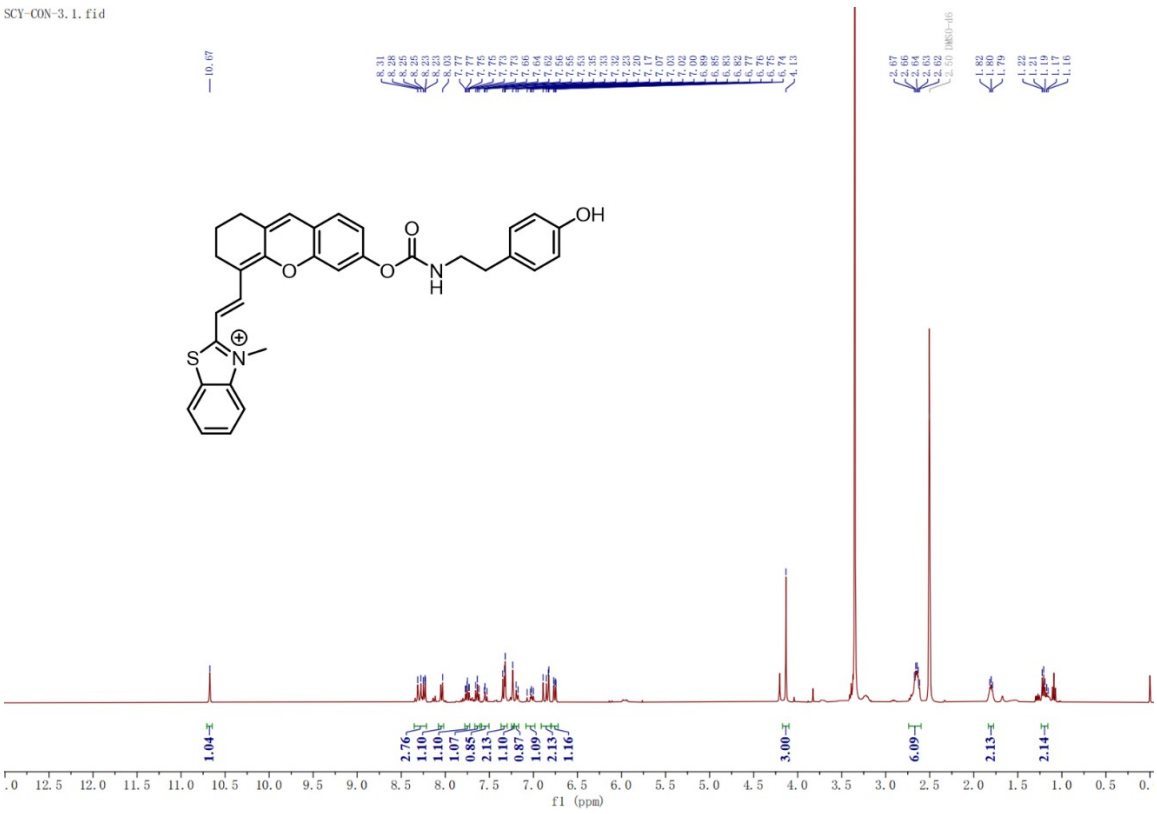


Figure S20. ^{1}H NMR spectrum of SCy-con in DMSO-d_6.

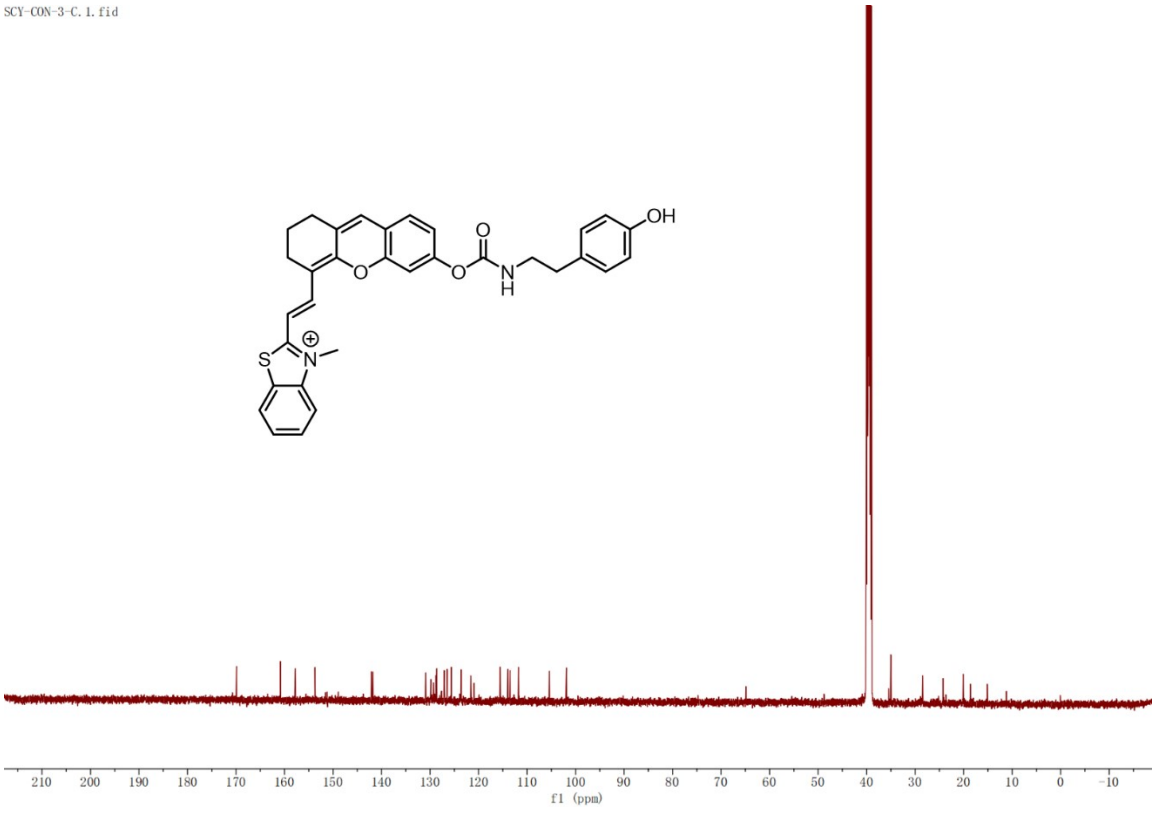


Figure S21. ^{13}C NMR spectrum of SCy-con in DMSO-d_6.

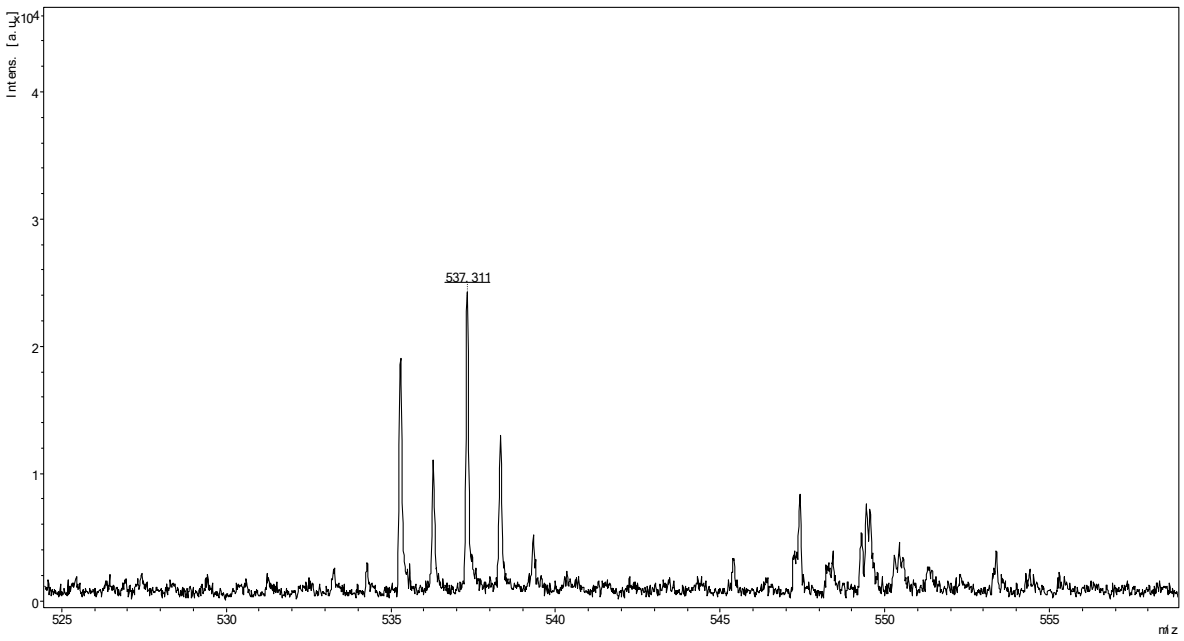


Figure S22. HRMS spectrum of SCy-con.

Reference

1. M. Liu, Y. Lv, X. Jie, Z. Meng, X. Wang, J. Huang, A. Peng and Z. Tian, *Sensors and Actuators B: Chemical*, 2018, **273**, 167-175.