A Cyclometallated Iridium(III) Complex with Multi-photon Absorption Properties as an Imaging-Guided Photosensitizer

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Experimental and methods

Materials and apparatus

All chemicals were obtained commercially and solvents were purified with conventional methods before using. The ^{1}H (400 MHz or 600 MHz) NMR spectra were collected on a Bruker Avance 400 spectrometer at 25°C (TMS as internal standard in NMR). Mass Spectrometer was recorded using LTQ Orbitrap XL and Bruker Autoflex III TOF/TOF. UV-vis absorption spectra were recorded on a UV-265 spectrophotometer. Fluorescence measurements were carried out on a Hitachi F-7000 fluorescence spectrophotometer. Quantum yield was determined by FLUORMAX-4P. Confocal microscopy imaging was acquired with a Leica SP8 confocal microscopy and 100/63 × oil-immersion objective lens. In vivo fluorescence imaging was conducted on IVIS Lumin2 III.

Two-photon excited fluorescence (2PEF) spectroscopy and two-photon absorption (2PA) cross-section

2PEF spectra were obtained by the two-photon excited fluorescence (2PEF) method with a femtosecond laser pulse and a Ti: sapphire system (680-1080 nm, 80 MHz, 140 fs) as the light source. The reference sample is Rhodamine B with a concentration of 1.0×10^{-3} M in ethanol. The concentration of Ir-Biotin was 1.0×10^{-3} M. 2PA cross-section was calculated by using the following equation:

$$\delta = \delta_{ref} \frac{\Phi_{ref} c_{ref} n_{ref} \quad F}{\Phi \quad c \quad n \quad F_{ref}}$$

Here, ref stand for reference sample, δ is the two-photon absorption cross section, Φ is quantum yield, c is the concentration of the sample, n is refractive index, F is two-photon fluorescence integral area. The absolute value of the two-photon absorption cross-section of the reference sample is derived from the literature.

Three-photon excited fluorescence (3PEF) spectroscopy and three-photon absorption (3PA) cross-section

3PEF spectra were obtained by the multi-photon excited fluorescence method with Coherent Astrella+TOPAS Prime (1200-2600 nm, 1 kHz, 120 fs) as the light source. The reference sample is Rhodamine 6G (1.0×10^{-3} M). The concentration of Ir-Biotin was 1.0×10^{-3} M. The multi-

photon absorption cross-section were calculated by using the following equation:

$$\sigma = \frac{\gamma}{N_A \times d_0 \times 10^{-3}} \cdot \left(\frac{hc}{\lambda}\right)^2$$

Here, γ is three photon absorption coefficients, λ is wavelength of incident light, N_A is the Avogadro constant, d_0 is the concentration of the sample (1.0×10^{-3} M).

ROS generation detection

DCFH was used as the ROS-monitoring agent. 10 μL of DCFH stock solution (1.0 mM) and 5 μL of Ir-Biotin (1.0 mM) were added to 2 mL PBS solution, and LED light (400-700 nm, 40 mW/cm^2) was employed as the light source. The emission of DCFH at 525 nm was recorded under various irradiation times.

Electron spin resonance (ESR) assay

The EPR measurements were carried out with a Bruker Nano X-band spectrometer at 298 K. The spin traps 2,2,6,6-tetramethylpiperidine (TEMP for trapping $^1O_2$) and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO for trapping O_2-, OH-) were used to verify the formation of reactive oxygen species (ROS) generated by Ir-Biotin. The ESR signals of the Ir-Biotin (5 μM) before and after LED light (400-700 nm, 40 mW/cm^2) irradiation were recorded.

Singlet oxygen ($^1O_2$) generation detection

The singlet oxygen was measured by a singlet oxygen indicator named 1,3-diphenylisobenzofuran (DPBF). Air-equilibrated DMSO solutions containing the Ir-Biotin and DPBF (10 mM) was exposed to LED light (400-700 nm, 40 mW/cm^2) irradiation for different time. The absorbance of DPBF at 418 nm was recorded every 2 s. Methylene blue (MB) was used as a reference for ROS sensitization (Φ_Δ= 0.52). A DMSO solution of DPBF without the probes was examined to assess its photostability under identical irradiation conditions. The following equation was used for the determination of Φ_Δ:

$$\Phi_{\Delta}^{unk} = \Phi_{\Delta}^{ref} \times \left(m^{unk} \times F^{ref}\right) / \left(m^{ref} \times F^{unk}\right)$$

where m is the slope of a linear fit of the change of absorbance at 418 nm against the irradiation

time (s) and F is the absorption correction factor, which is given by F = 1–10−AF (A = absorbance at 365 nm and L = path length of the cell).

Cell culture

Cells (including cancer cell and normal cell) were cultured in 25 cm^2 culture flasks in DMEM, supplemented with fetal bovine serum (10 %), penicillin (100 units per mL) and streptomycin (50 units per mL) at 37 ℃ in a CO_2 incubator (95 % relative humidity, 5 % CO_2). Cells were seeded in 35 mm glass bottom cell culture dishes, at a density of 1×10^5 cells and were allowed to grow when the cells reached more than 60 % confluence.

Cytotoxicity assay

The cytotoxicity of the Ir-Biotin toward adherent cells was studied by MTT assay. Hela cells were detached with trypsin, and seeded into 96-well plates (100 μL per well), incubated in a humidified incubator at 37℃ for 24 h. Then adherent Hela cells were treated with different concentrations of the Ir-Biotin in the growth medium at 37℃ in 96-well plates. After 24 h incubation, MTT (5 mg mL^{-1}, 10 μL) was added to each well and incubated for additional 4 h. The supernatant was then removed and 100 μL of DMSO was added to dissolve the formazan crystals. And the cell culture plate was shaken for 10 min until no particulate matter was visible. Absorbance in each well was measured at 570 nm using a microplate reader (Biotek, USA). The cell viability (%) was calculated according to the following equation: cell viability (%) = A/B × 100, where A represents the optical density of the wells treated with various concentration of Ir-Biotin and B represents that of the wells treated with medium.

Mitochondrial co-localization assays

For co-localization experiments, HeLa cells are incubated with Ir-Biotin (2 μM) for 30 min. Then the cells are treated with Mitotracker Deep Red (0.5 μM) for 20 min. At last, samples are washed three times by PBS solution and 1 mL of PBS solution is added into each well. The fluorescent images of the cells are collected by confocal laser scanning microscopy. The red fluorescence of Mito-Tracker Red is collected between 660-680 nm upon excitation at 633 nm.

The green fluorescence of Ir-Biotin is collected between 550-600 nm upon excitation at 405 nm. The co-localization coefficient and mean fluorescence intensity of the images are determined by the software with image J.

Cell uptake analysis

QSG cells (Biotin receptor negative overexpressing cells) and Hela cells (Biotin receptor-positive overexpressing cells) were seeded onto cell culture dishes and grown to about 70% confluency. QSG cells and HeLa cells were treated with Ir-Biotin (2 μM) respectively. In order to further verify the cancer-specific targeting of Ir-Biotin, Hela cells were precultured with biotin (100 μM, 2 h) before incubation with Ir-Biotin, and after 1 h incubation, the cellular uptake ability of Ir-Biotin were analyzed using CLSM. 10^5 HeLa cells and QSG cells were seeded on 35 mm confocal dishes and allowed to adhere overnight. One of the dishes of HeLa cells was pre-incubated with biotin (100 μM) for 2 h. Cells were then incubated with Ir-Biotin (2 μM) for 1 h at 37℃. After this time, the cells were detached with trypsin, harvested and centrifuged. The number of cells was accurately counted. The cell pellet was resuspended in lysis buffer and the cells were lysed. The cell organelles were separated using a ultracentrifuge the supernatant solution was separated, Each sample was the digested using a 60% HNO_3 solution and was diluted to a solution of 2% HNO_3 in water. The Ir content was determined using an ICP-MS apparatus and the Ir content was then associated with the number of cells.

ROS generation under two-photon laser *in vitro*

HeLa cells were treated with 2 μM of Ir-Biotin in the dark. After the cells were incubated with 10 μM of DCFH-DA at 37℃ for 30 min, the cells were subjected to light irradiation (760 nm, 100 mW/cm^2). Then, confocal fluorescence imaging was performed (DCFH-DA: Ex: 488 nm, Em: 500-540 nm).

The species of ROS generation under two-photon laser *in vitro*

HeLa cells were incubated with 2 μM Ir-Biotin, followed by 1 μM singlet oxygen sensor green (SOSG) and 1 μM dihydroethidium (DHE) for 30 minutes, respectively. Cells were washed with

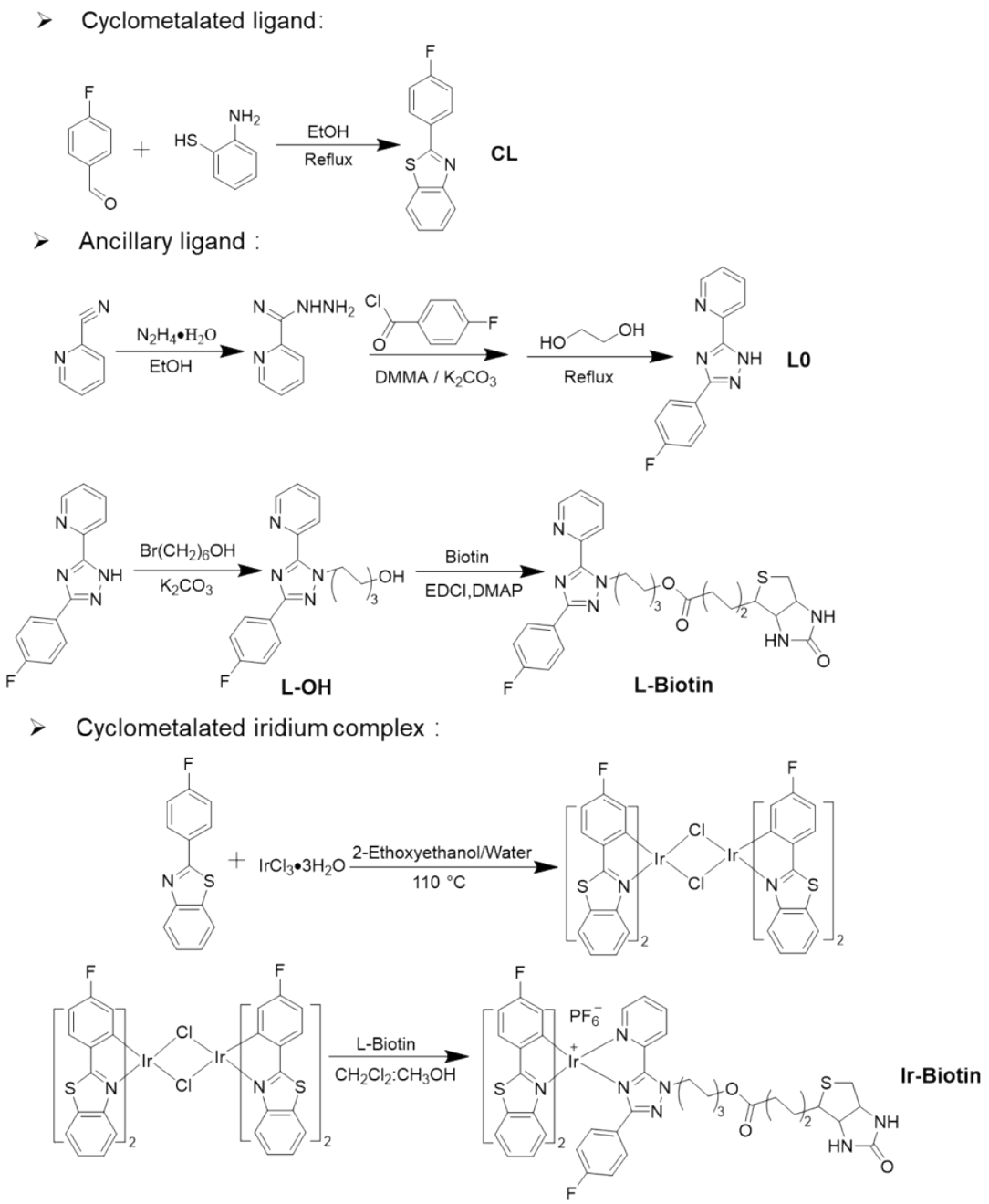
PBS and then irradiated with 760 nm light at a power density of 100 mW/cm^2 for 10 minutes. Intracellular fluorescence was observed using CLSM (SOSG: Ex: 504 nm, Em:500-550 nm. DHE: Ex: 480 nm, Em: 590-630 nm).

Flow cytometry study

Cells seeded into the 6-well plates were cultured for 24 h. Next, the medium was replaced with medium containing Ir-Biotin (2 μM), at 37 °C for 30 min. After irradiated by light (Concentration: 2 μM, Light: 760 nm laser, irradiation time: 5 min), the cells were incubated for another 4 h, then collected by centrifugation and resuspended in binding buffer containing Propidium Iodide (PI, 10 μL) and Annexin-V FITC (5 μL) for 15 min in darkness. The signal was collected by a BD FACS Calibur flow cytometer (Beckaman/Gallios).

Live/Dead assay with calcein AM/PI

Hela cells were incubated with Ir-Biotin (2 μM) for 30 min. Calcein AM and PI were used to confirm the viability of cell lines. Fluorescence images were collected by confocal laser scanning microscopy after laser irradiation.



Scheme S1. The synthetic routes of ligand and Ir-Biotin.

Synthesis of 2-(4-fluorophenyl)benzo[d]thiazole (CL)

The ligands were synthesis base on previous work with minor modifications. [Small 2018, 14, 1802166] 4-fluorobenzaldehyde (1.24 g, 10 mmol) and 2-aminobenzenethiol (1.25 g, 10 mmol) were dissolve in dry ethanol, then the solution was heated to reflux for 6 h. When cooled to temperature, solid was obtained by suction filtration and washed with EtOH (1 mL×3). The white solid was gained in 80% (1.83 g) yield. ^{1}H NMR (400 MHz, d_6-DMSO) δ(ppm): 8.16 (dd, J = 8.4,

5.0 Hz, 3H), 8.07 (d, J = 8.2 Hz, 1H), 7.60–7.52 (m, 1H), 7.49 (d, J = 8.0 Hz, 1H), 7.47–7.39 (m, 2H). ESI-MS: m/z, $[M+H]^+$ = cal: 230.04 found: 230.04.

Synthesis of 2-(3-(4-fluorophenyl)-1H-1,2,4-triazol-5-yl)pyridine (L0)

L0 were synthesis according to previous work without modifications and a white crystalline compound were obtained. [**Inorganic Chemistry, Vol. 46, No. 26, 2007]**. ^{1}H NMR (400 MHz, d_6-DMSO) δ(ppm): 14.86 (s, 1H), 8.73 (d, J = 4.6 Hz, 1H), 8.21 – 8.09 (m, 3H), 8.03 (dd, J = 13.6, 6.0 Hz, 1H), 7.55 (s, 1H), 7.55 (s, 1H), 7.35 (t, J = 8.6 Hz, 2H). ESI -MS: m/z, $[M+H]^+$ = cal: 241.08 found: 241.08.

Synthesis of 6-(3-(4-fluorophenyl)-5-(pyridin-2-yl)-1H-1,2,4-triazol-1-yl)hexan-1-ol (L-OH)

L0 (1.20 g, 5 mmol), K_2CO_3 (3.45 g, 25 mmol) and TBAB (1.61 g, 0.5 mmol) was added MeCN (50 mL) under N_2 atmosphere. The mixture was stirred at 60℃ for 30 min, then 6-Bromo-1-hexanol of MeCN solution (30 mL) was added. The resultant mixture was heated to 70 ℃ for 6 h. when cooled the room temperature. The solvent removed and the residue separated by column chromatograph (using silica gel, $Rf_{(PE: EA=10:1)}$ =0.4) giving a yield of 1.19 g (70 %). ^{1}HNMR (400 MHz, CD_3CN) δ(ppm): 8.85–8.72 (m, 1H), 8.40 – 8.29 (m, 1H), 8.25–8.14 (m, 2H), 8.08–7.99 (m, 1H), 7.63–7.48 (m, 1H), 7.33–7.13 (m, 2H), 4.94– 4.77 (m, 2H), 3.62–3.44 (m, 2H), 3.39–3.29 (m, 1H), 1.57–1.28 (m, 8H). ESI-MS: m/Z, $[M+H]^+$=cal: 341.17 found: 341.18.

Synthesis of 6-(3-(4-fluorophenyl)-5-(pyridin-2-yl)-1H-1,2,4-triazol-1-yl)hexyl 5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate (L-Biotin)

L-Biotin were synthesis base on previous work with minor modifications[2016-Scientific RepoRts 6，21459]. ^{1}H NMR (400 MHz, CD_2Cl_2) δ(ppm):8.69 (d, J = 4.4 Hz, 1H), 8.28 (d, J = 7.9 Hz, 1H), 8.14 (dd, J = 8.0, 5.8 Hz, 2H), 7.88 (t, J = 7.7 Hz, 1H), 7.49–7.29 (m, 1H), 7.15 (t, J = 8.6 Hz, 2H), 5.25 (s, 1H), 4.97 (s, 1H), 4.84 (t, J = 7.3 Hz, 2H), 4.54–4.35 (m, 1H), 4.34–4.22 (m, 1H), 4.02 (t, J = 6.5 Hz, 2H), 3.14 (dd, J = 11.7, 7.1 Hz, 1H), 2.89 (dd, J = 12.7, 4.6 Hz, 1H), 2.68 (d, J = 12.8 Hz, 1H), 2.28 (t, J = 7.4 Hz, 2H), 1.63 (dt, J = 14.4, 7.1 Hz, 6H), 1.41 (d, J = 3.8 Hz, 6H). ESI-MS: m/z, $[M+H]^+$=cal: 567.25 found: 567.42.

Synthesis of Ir-Biotin

(4-fluorophenyl)benzo[d]thiazole (0.504 g, 2.19 mmol) and IrCl3•3H_2O (0.353 g, 1.00 mmol)

were dissolved in in 2-ethoxyethanol (15 mL) and H_2O (5 mL) at a flask. subsequently, the solution in dark was refluxed for 24 h under nitrogen atmosphere, and then the solution was cooled down to room temperature. The yellow solid of $[(C^N)_2\ Ir(m\text{-}Cl)_2]$ was obtained by suction filtration and was dry in a vacuum drying oven. A mixture of $[(C^N)_2\ Ir(m\text{-}Cl)_2]$ (0.136 g, 0.0100 mmol) and L4 in CH_2Cl_2/CH_3OH (1:1, v / v %) was refluxed under an inert condition of nitrogen for 12 h. After the reaction was finished and cooled to room temperature. The excess NH_4PF_6 was added and stirred overnight. Then the bright yellow solid precipitates were obtained. The crude product was purified by column chromatograph (using silica gel, $Rf_{\text{(DCM: MeOH=20:1)}}$ =0.3) giving a bright yellow solid (500 mg 37.1%). ^1H NMR (400 MHz, CD_2Cl_2) δ(ppm): δ 8.30 (dd, J = 15.7, 7.7 Hz, 2H), 8.02 (d, J = 5.0 Hz, 1H), 7.92 (d, J = 8.0 Hz, 1H), 7.84 (t, J = 8.4 Hz, 2H), 7.52 (t, J = 6.2 Hz, 1H), 7.41 (s, 1H), 7.38 – 7.27 (m, 3H), 7.20 – 6.98 (m, 4H), 6.78 (t, J = 8.6 Hz, 2H), 6.59 (t, J = 8.1 Hz, 1H), 6.46 (t, J = 8.6 Hz, 1H), 6.02 (t, J = 13.1 Hz, 1H), 5.95 (d, J = 9.2 Hz, 1H), 5.72 (d, J = 9.4 Hz, 1H), 4.71 – 4.51 (m, 2H), 4.41 (s, 1H), 4.22 (s, 1H), 3.93 (t, J = 6.1 Hz, 2H), 3.33 (d, J = 1.7 Hz, 3H), 3.09 (s, 1H), 2.83 (dd, J = 12.7, 4.7 Hz, 1H), 2.61 (d, J = 12.9 Hz, 1H), 2.24 (t, J = 7.2 Hz, 2H), 1.46 – 1.27 (m, 9H), 1.19 (d, J = 11.0 Hz, 3H), 0.87 (d, J = 56.1 Hz, 3H). ^{13}C NMR (600 MHz, d_6-DMSO), δ 180.78, 180.16, 174.95, 173.44, 164.62, 164.40, 164.09, 163.30, 162.93, 162.70, 162.45, 160.09, 159.38, 156.71, 152.62, 151.52, 150.82, 149.74, 149.05, 148.13, 148.09, 144.82, 141.58, 138.33, 137.51, 137.09, 131.68, 131.61, 130.38, 129.36, 128.48, 127.00, 126.63, 126.61, 125.29, 125.23, 124.30, 123.06, 119.12, 118.78, 118.67, 116.98, 116.20, 115.42, 115.28, 111.45, 111.29, 110.45, 110.29, 110.45, 110.29, 100.00, 61.63, 61.10, 61.05, 59.77, 56.36, 55.91, 32.79, 32.69, 28.57, 28.38, 25.63, 25.36, 25.22, 25.05, 24.95. ESI-MS: m/z, $[M\text{-}PF6]^+$ = cal: 1215.27 found: 1215.50.

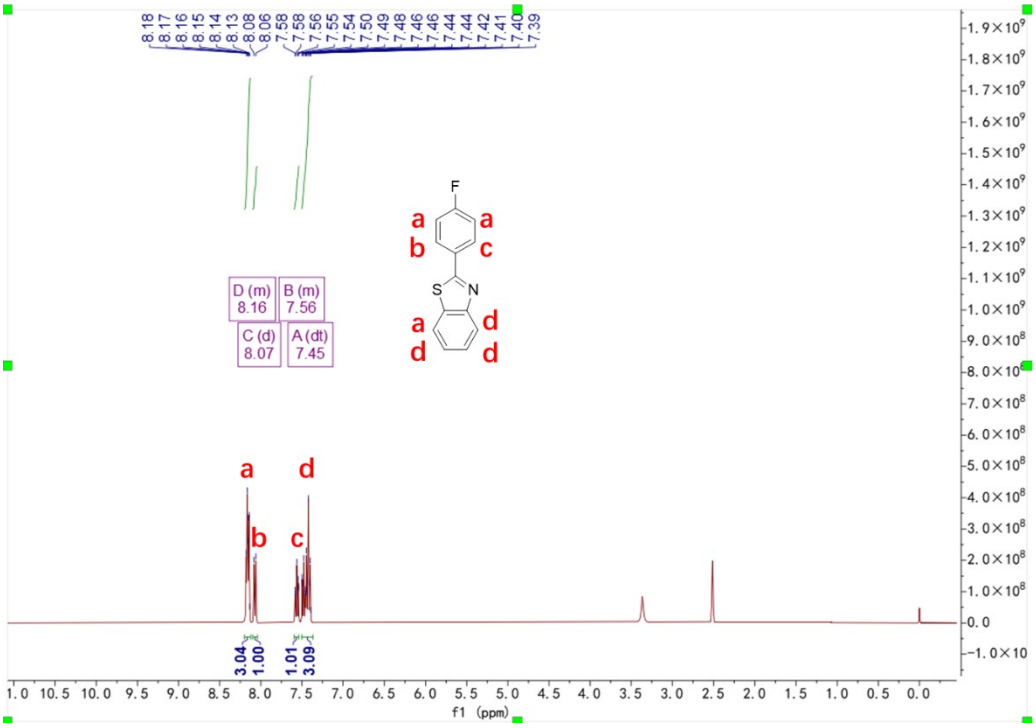


Figure S1 ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of **CL**.

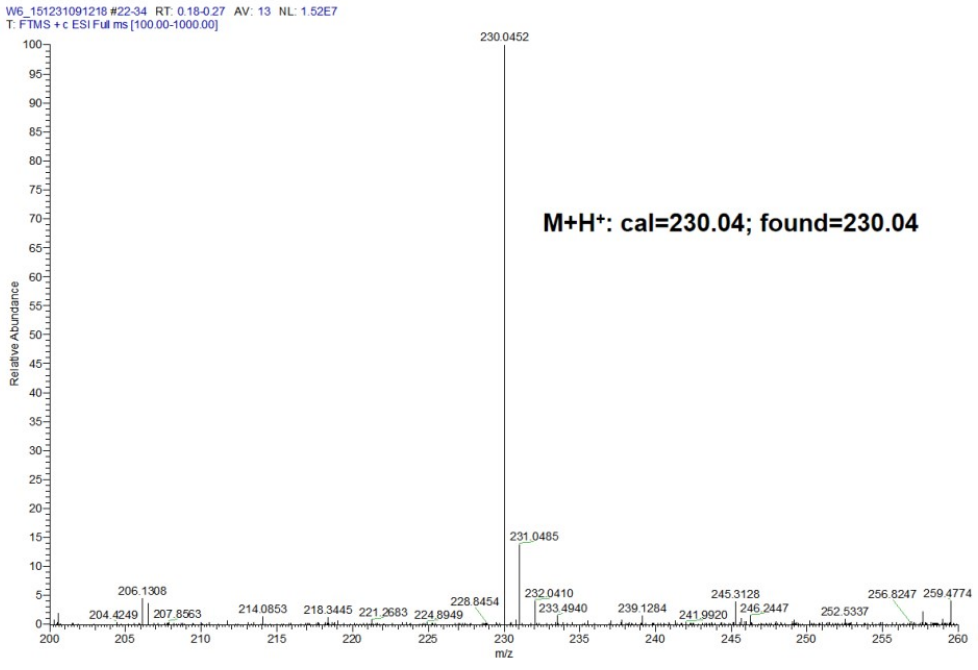


Figure S2 Mass spectrum of **CL**.

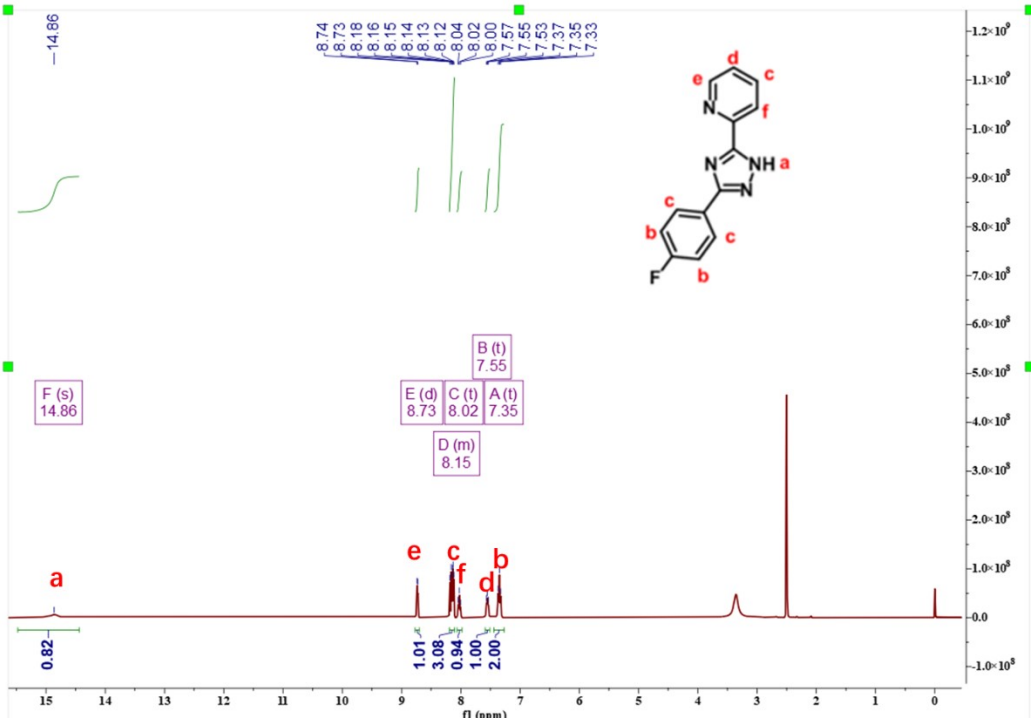


Figure S3 ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of **L0.**

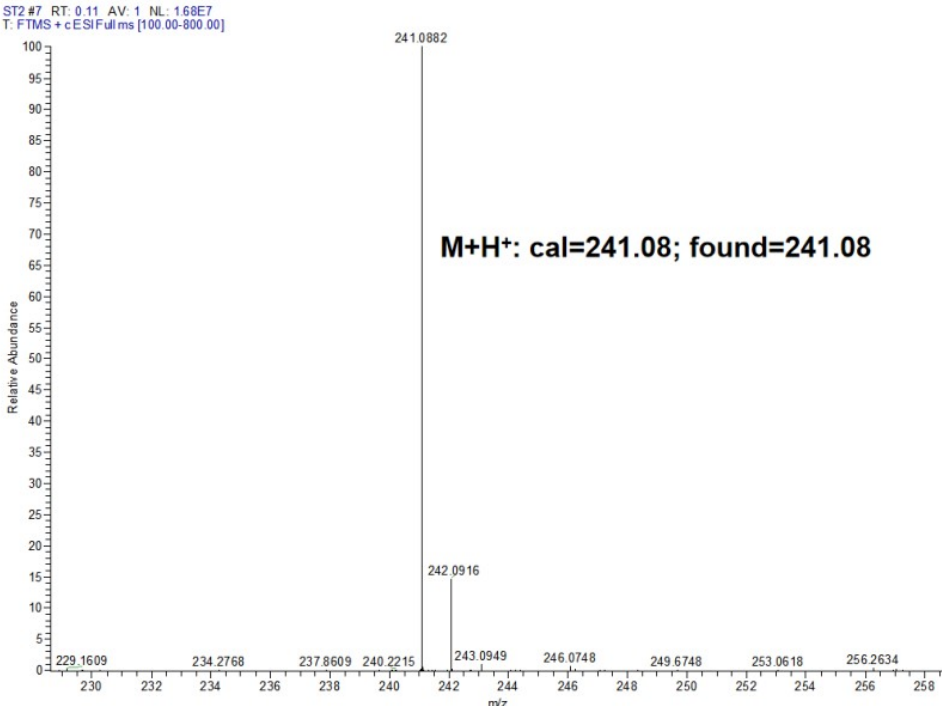


Figure S4 Mass spectrum of **L0.**

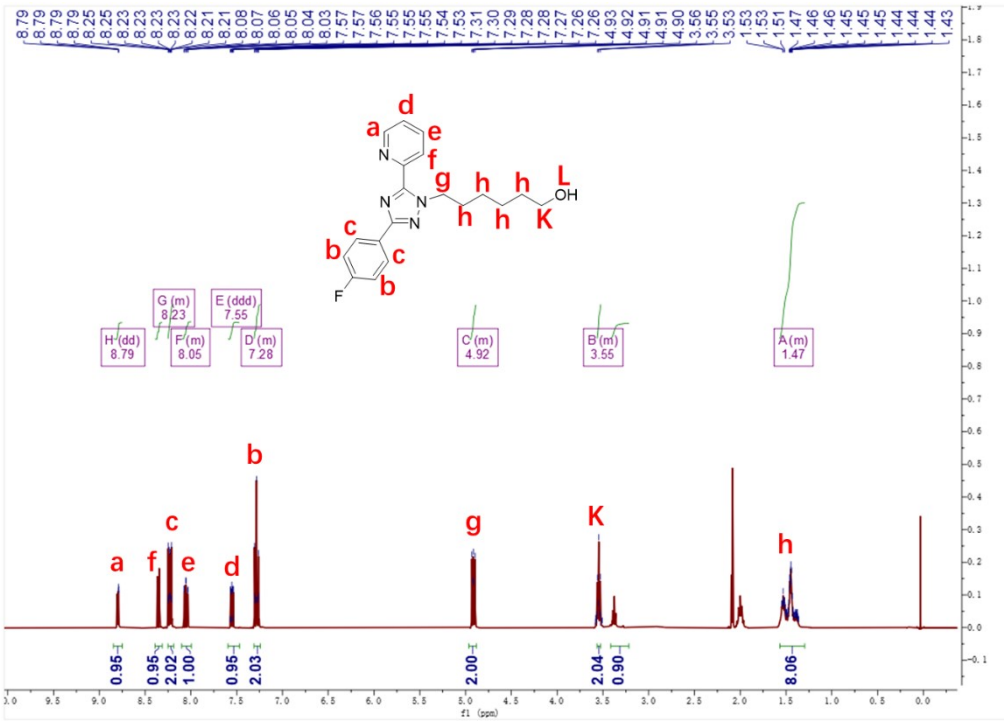


Figure S5 ^{1}H NMR spectrum (400 MHz, CD_3CN) of **L-OH.**

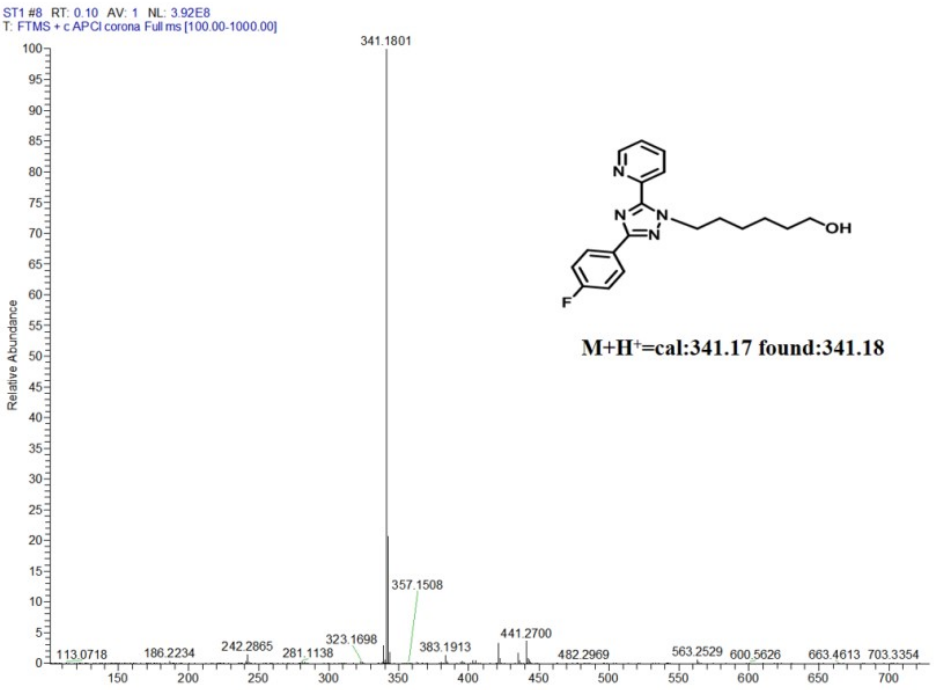


Figure S6 Mass spectrum of **L-OH.**

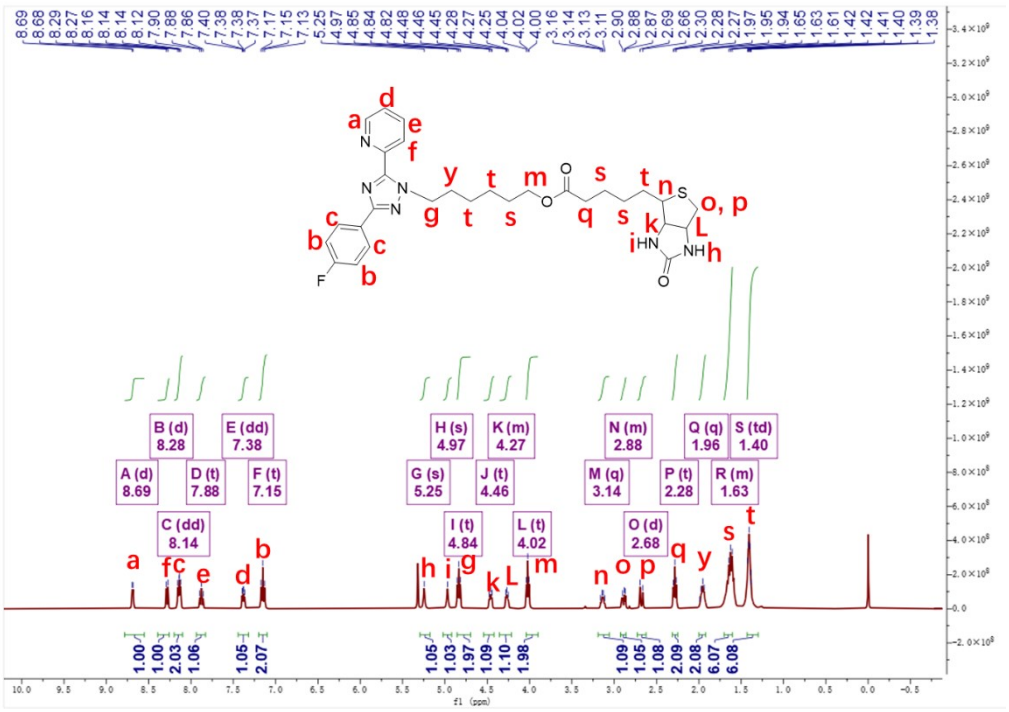


Figure S7 ^{1}H NMR spectrum (400 MHz, CD_2Cl_2) of **L-Biotin.**

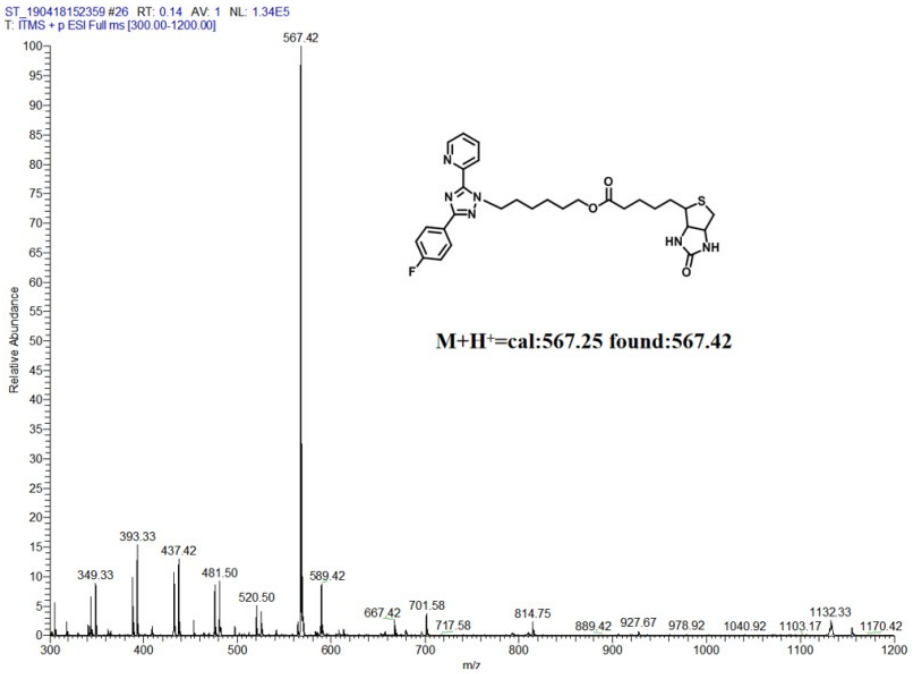


Figure S8 Mass spectrum of **L-Biotin.**

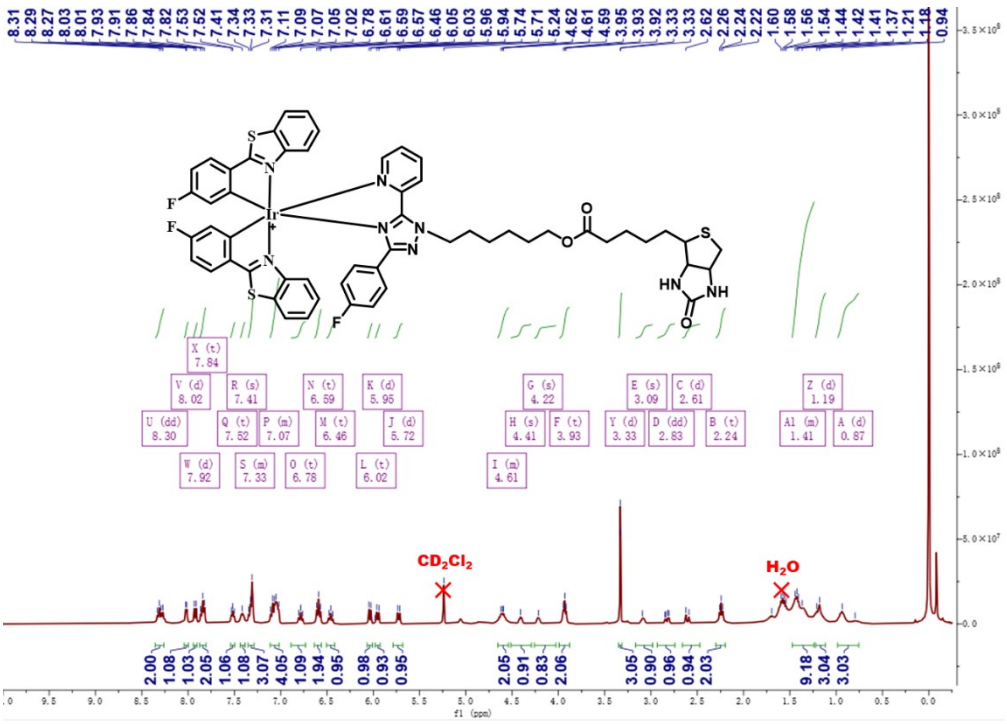


Figure S9 ^{1}H NMR spectrum (400 MHz, CD_2Cl_2) of **Ir-Biotin.**

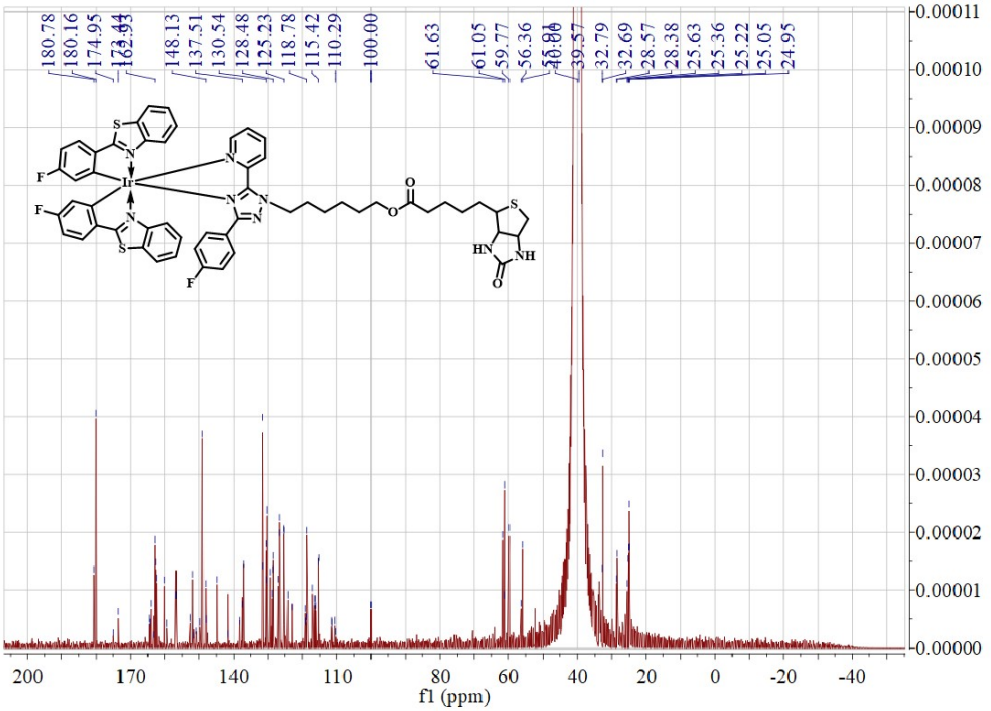


Figure S10 ^{13}C NMR spectrum (600 MHz, DMSO-d_6) of **Ir-Biotin.**

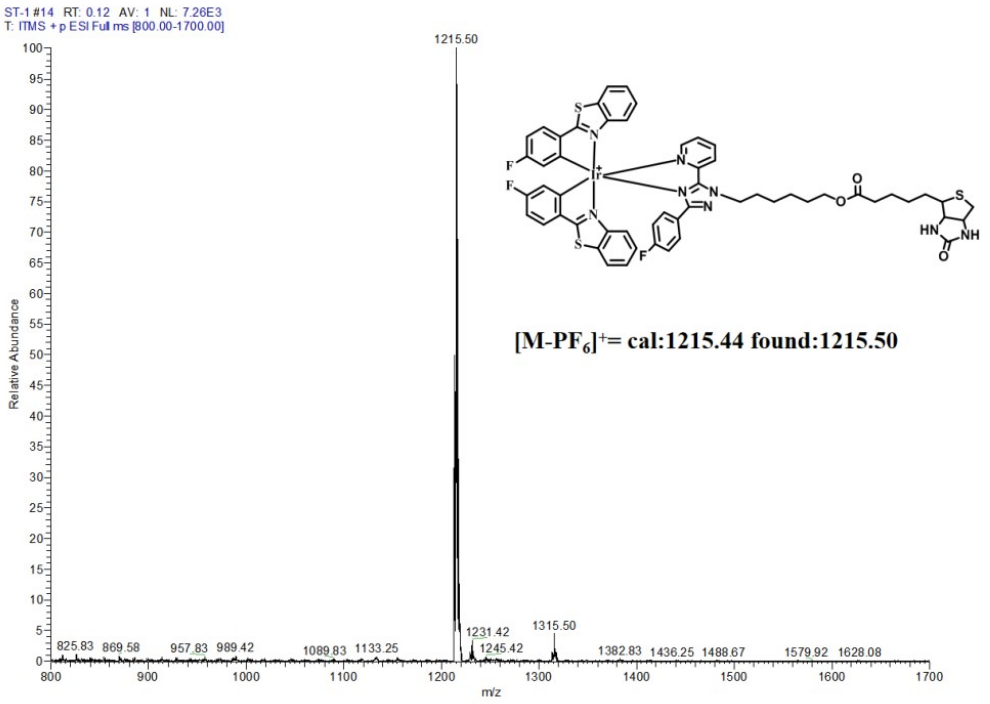


Figure S11 Mass spectrum of **Ir-Biotin.**

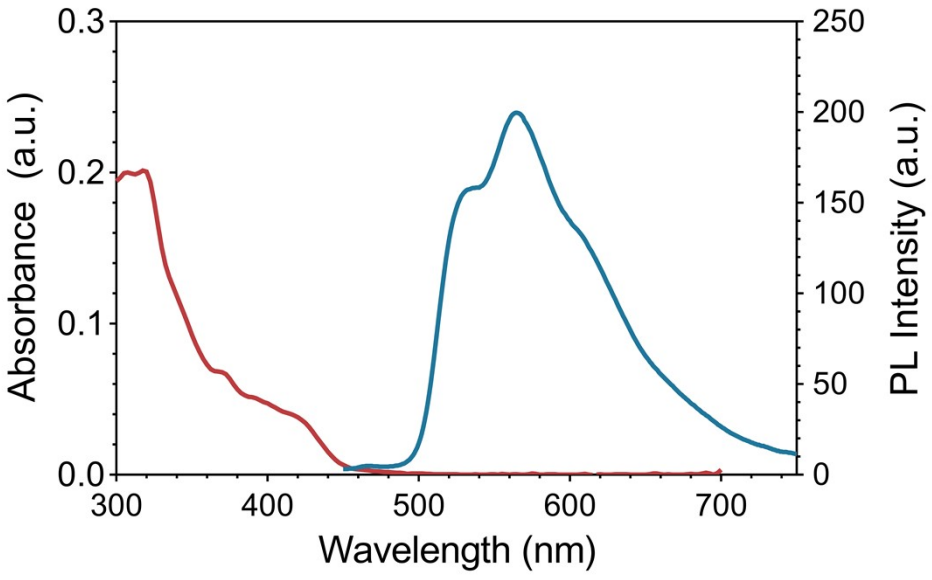


Figure S12 UV-Vis absorption and fluorescence spectra of **Ir-Biotin** in DMSO (excited at 320 nm, c = 1.0×10^{-5} mol/L).

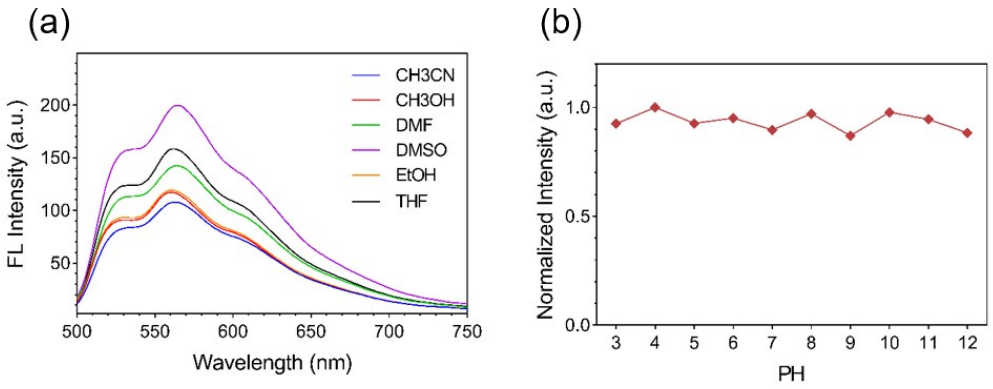


Figure S13 Effects of different solvents (a) and pH (b) on the fluorescence intensity of **Ir-Biotin**.

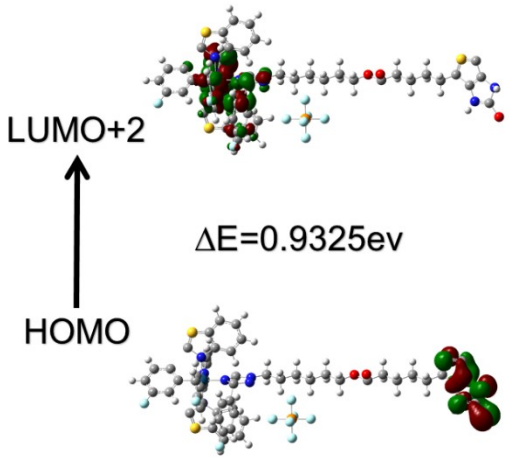


Figure S14 HOMO and LUMO distributions of **Ir-Biotin**.

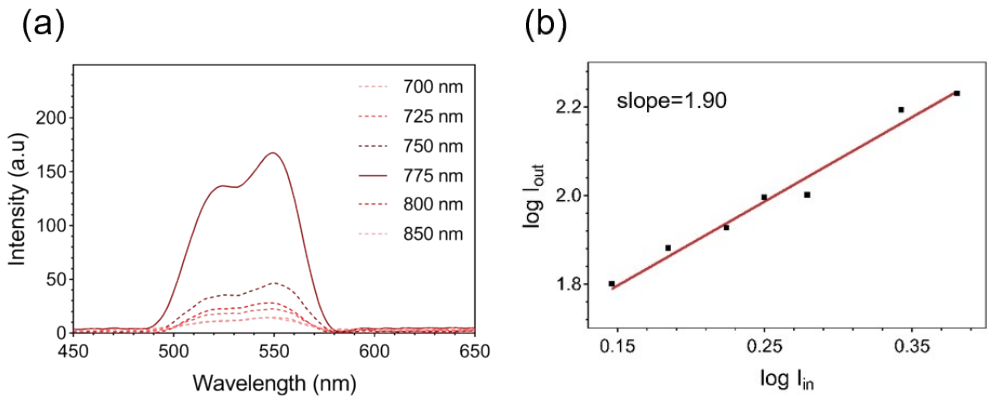


Figure S15 (a) Two-photon fluorescence intensity of **Ir-Biotin** in DMSO (c = 1×10^{-3} mol/L). (b) The square relationship of two-photon excited fluorescence intensity (I_{out}) of **Ir-Biotin** with the addition of input power.

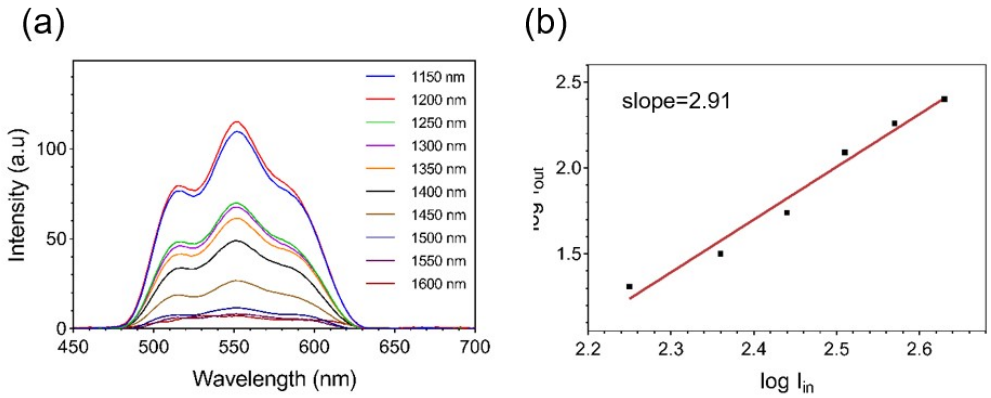


Figure S16 (a) Three-photon fluorescence intensity of **Ir-Biotin** in DMSO (c = 1×10^{-3} mol/L). (b) The square relationship of three-photon excited fluorescence intensity (I_{out}) of **Ir-Biotin** with the addition of input power.

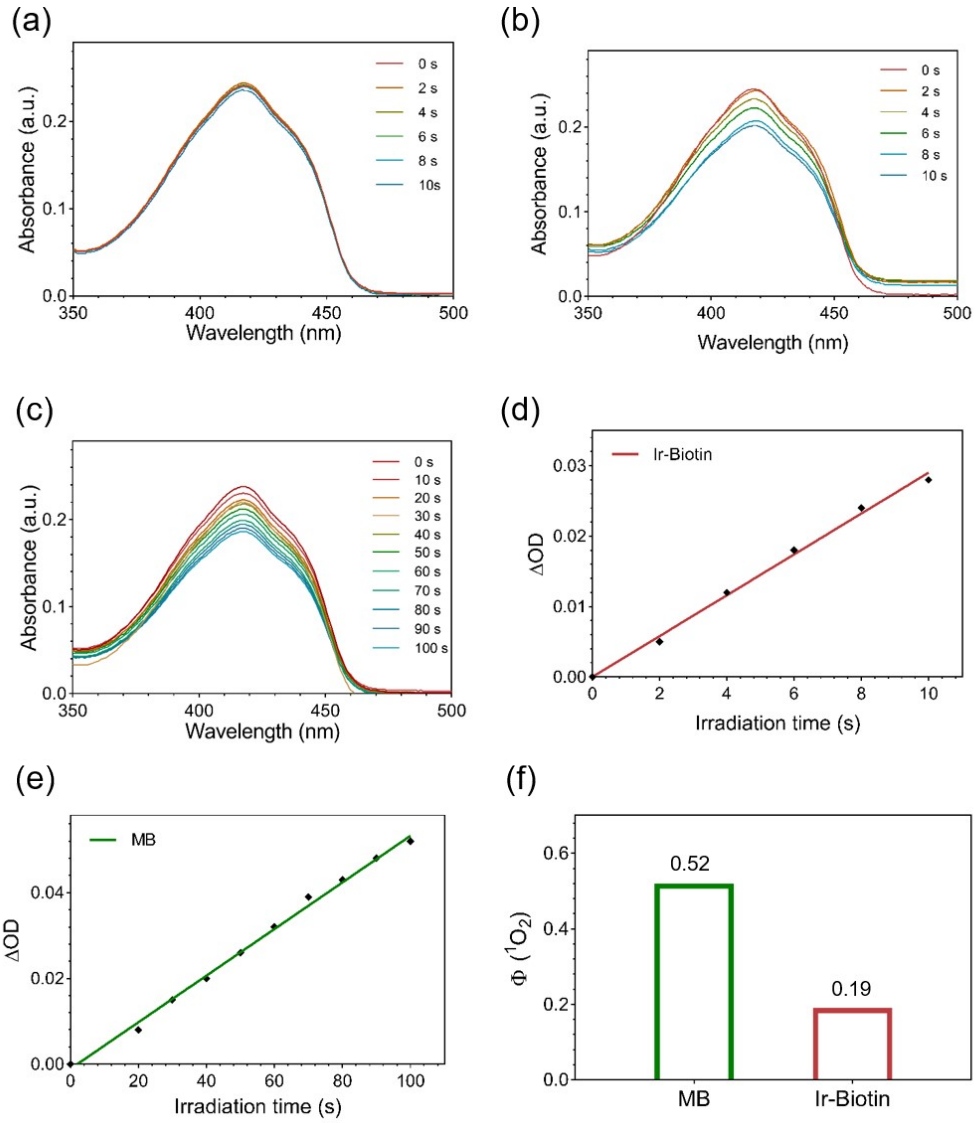


Figure S17 (a) Changes in the DPBF absorption spectrum after 10 s of irradiation. (b) Absorption spectra of DPBF irradiated for 10 s in the presence of **Ir-Biotin**. (c) Absorption spectra of DPBF irradiated for 100 s in the presence of MB. Plot of change in absorbance of DPBF at 418 nm vs irradiation time in the presence of **Ir-Biotin** (d) and MB (e). (f) Singlet oxygen quantum yields of MB and **Ir-Biotin**.

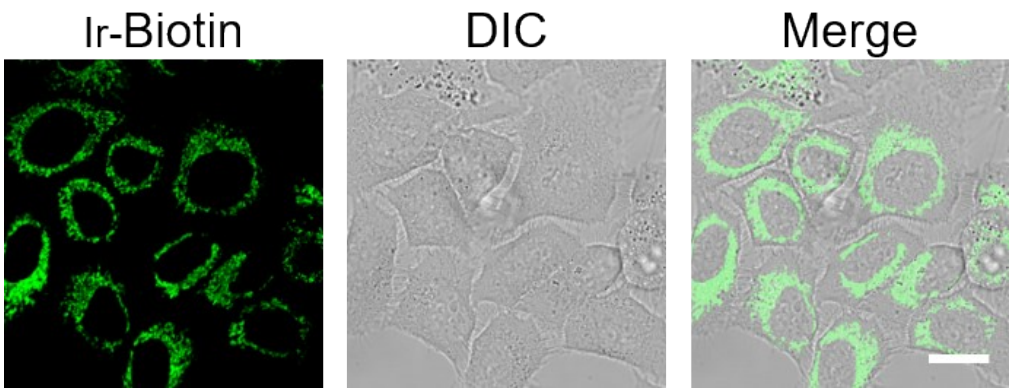


Figure S18 CLSM images of HeLa cells treated with **Ir-Biotin** (Concentration: 2 μM. Scale bar: 20 μm).

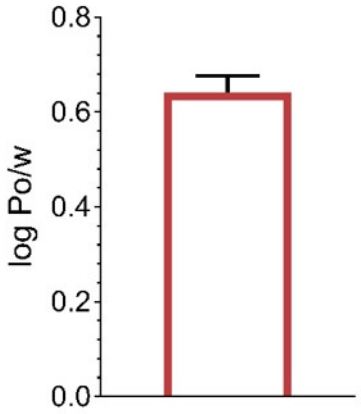


Figure S19 Octanol/water partition coefficient of **Ir-Biotin**.

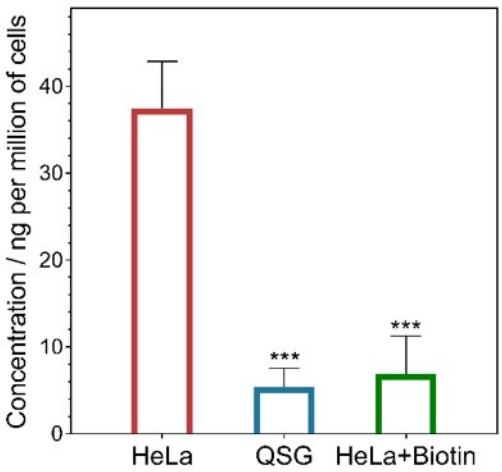


Figure S20 Comparison of **Ir-Biotin** uptake in HeLa cells and QSG cells. (*P < 0.05, **P < 0.01, ***P < 0.001 determined by One-way ANOVA).

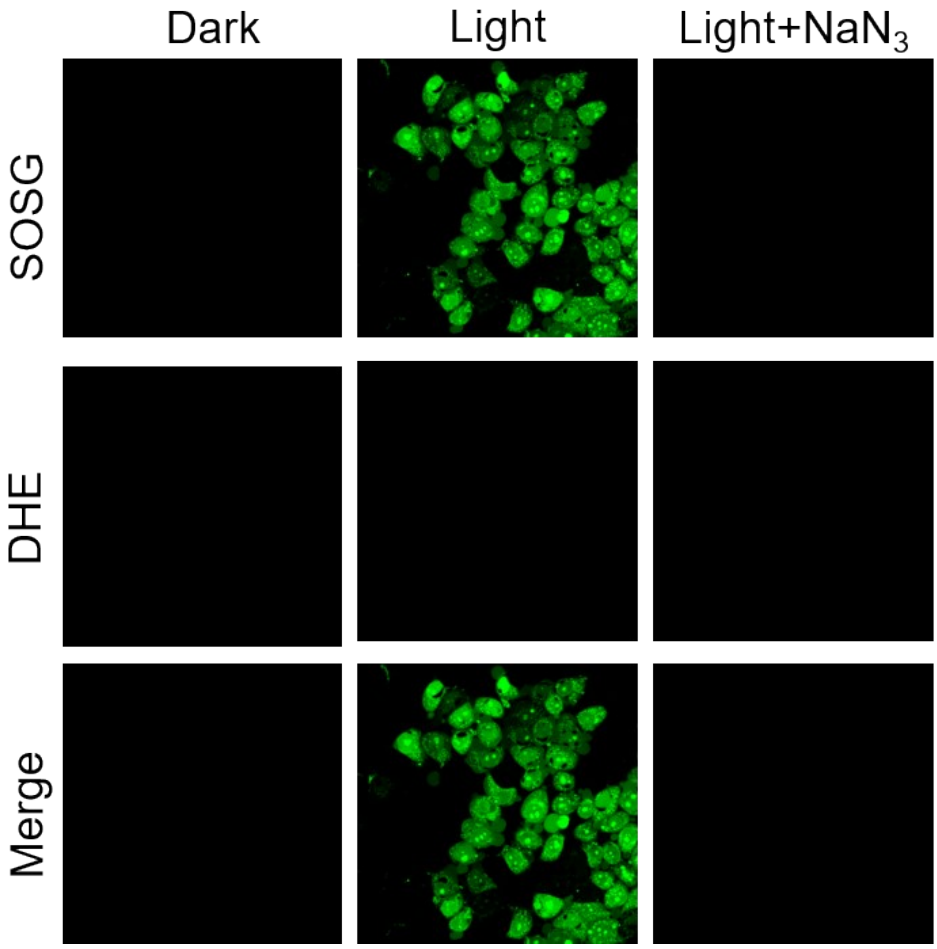


Figure S21 Detection of ROS species in HeLa cells using DHE and SOSG as indicators for $O_2^{\cdot-}$ and $^1O_2$. NaN_3 acts as a specific scavenger for $^1O_2$ (Concentration: 2 μM. Light: 760 nm laser. irradiation time: 10 min Scale bar: 40 μm).

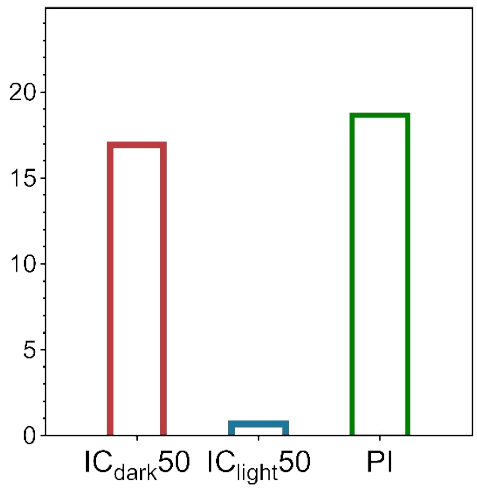


Figure S22 IC50 value and phototoxicity index (PI) of **Ir-Biotin** under light and dark.

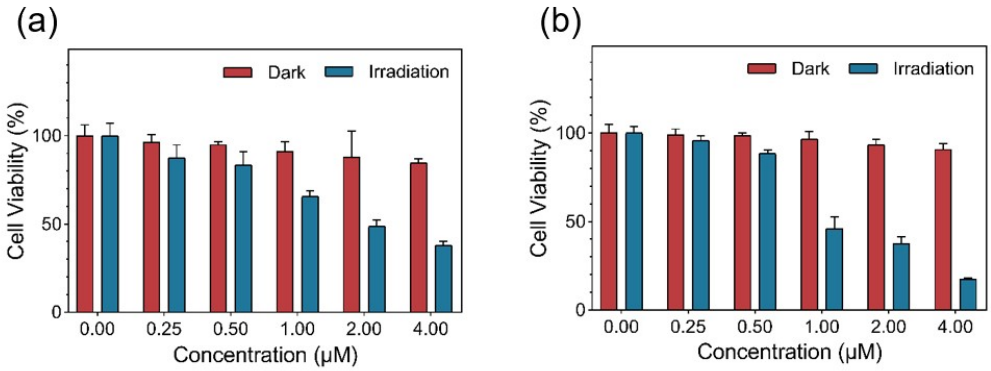


Figure S23 Detection dark toxicity and phototoxicity of **Ir-Biotin** against HepG2 (a) and 4T1 (b) cells.

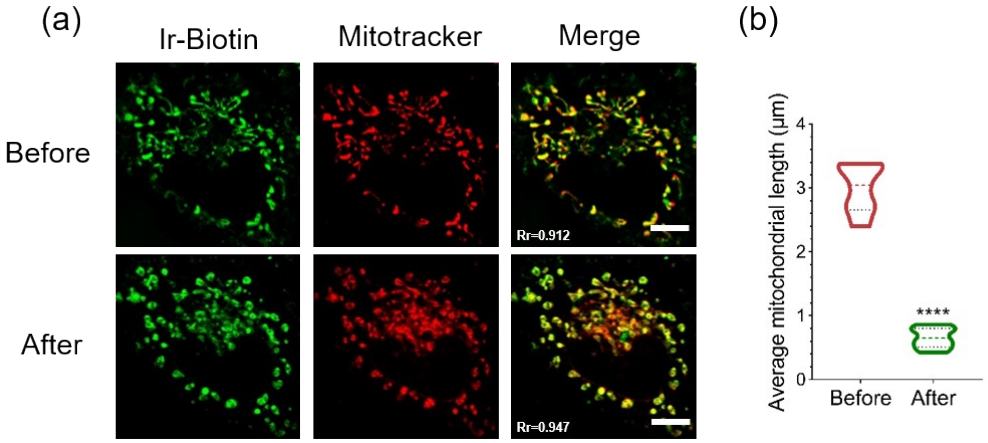


Figure S24 Laser irradiation 30 S mitochondrial length change (a) and quantization (b) (Concentration: 2 μM. Light: 760 nm laser. Scale bar: 10 μm. *P < 0.05, **P < 0.01, ***P < 0.001 determined by Student's t-test).