Support Information

Anticancer activity and mechanism studies of photoactivated iridium (III) complexes toward lung cancer A549

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Section I Experimental procedures

Material and methods

$IrCl_3 \cdot 3H_2O$ and other chemical reagents were provided by Beijing HWRK Chemical Co., Ltd (Beijing, China). Tumor cells lines: human lung carcinoma (A549), human hepatocellular carcinoma BEL-7402, non-cancer cells (NIH3T3) were obtained from Sun Yat-Sen University (Guangzhou). MitoTracker® Deep Red FM, 2′,7′-dichlorodihydro-fluorescein diacetate (DCHF-DA), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1), Annexin V-FITC were provided by

Beyotime Biotechnology (Shanghai, China). HRMS spectra were measured by direct injection in the Waters Xevo G2-XS QT of the mass analyzer. Fetal bovine serum (FBS) and Dulbecco's modified eagle medium (DMEM) were obtained from the Gibco company. A Varian-500 spectrometer was used to measure ^{1}H and ^{13}C NMR spectra.

Cell culture

Human lung carcinoma (A549), human cervical carcinoma (HeLa), mouse melanoma (B16), human hepatocellular carcinoma (BEL-7402), human colon cancer (HCT116), and human normal liver (LO2) cells were obtained from Sun Yat-Sen University (Guangzhou, China). A549, HeLa, B16, HCT116 and LO2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), BEL-7402 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium, all mediums were supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 U/mL penicillin and 0.1 mg/mL streptomycin. All cells were cultured in a humidified incubator containing 5% CO_2 at 37 °C.

HPLC determination of compound purity

The compounds were dissolved in chromatographic methanol and filtered through a filter membrane, using water containing 0.1% trifluoroacetic acid (TFA) as mobile phase A and methanol containing 0.1% TFA as mobile phase B at a flow rate of 3 mL/min. The detection wavelength was 251 nm.

UV detection of complexes

The complex was prepared as a 20 mM masterbatch with DMSO and diluted with PBS to form a 20 μM solution, and the UV absorption of the complex solutions were detected at 0 h and 24 h.

In vitro cell activity assay

The anti-tumor activity of the three complexes was assayed using MTT (thiazolyl blue) colorimetric assay. HepG-2, HeLa, BEL-7402, A549, HCT-116, and LO2 were chosen. The well-grown cells were spread in 96-well plates and placed in an incubator at 37 °C and 5% CO_2 for 24 h. Different concentration gradients of the complexes prepared by the half dilution method were added, and the control group was treated with an equal amount of DMSO, and the 96-well plates were light-activated for 30 min after 4 h. After that, the culture medium in the plate was discarded, and 90 μL of serum-free culture medium and 10 μL of MTT solution (5 mg/mL, prepared in PBS) were added to each well and incubated for 4 h. The solution in the wells was discarded, 100 μL of DMSO solution was added to each well and shaken on a micro-volume shaker. Finally, the absorbance was measured at 490 nm using a multifunctional enzyme marker, and the data was analyzed and the concentration of complex was calculated when the cell viability was 50%, thus obtaining the IC_{50} value.

Wound healing experiment

The well-grown A549 cells were added in 6-well plates and cultivated in incubator for

24 h. A straightedge and the tip of a 200 μL pipette gun were used to draw a line evenly and vertically through the wells to create a trauma, and the culture medium was removed and the cells were washed with PBS for 2-3 times, and the width of the scratches was recorded at 0 h. Subsequently, the IC_{50}-concentrated complex was added and the cells were incubated for 4 h under light. The width of the scratches in the wells was recorded under an inverted light microscope after 24 h.

Cellular uptake assay

A certain number of A549 cells with good growth status were spread in a 12-well plate and placed in the incubator for 24 h. After adding $2IC_{50}$ concentration of the complex for 4 h, the 6-well plate was light-activated for 30 min, and the culture medium was discarded, and the residue was washed with PBS, and then the cells were fixed with 75% ethanol. After 25 min, the cells were washed twice with PBS, added DAPI solution to avoid light staining for 30 min and then washed with PBS to remove the residual dye, added an appropriate amount of PBS to soak the cells, and photographed under a smart cell imager with 20 × magnification.

Co-localization at the mitochondrial

Mitochondrial localization can be analyzed using the Mito-Tracker Deep Red probe. Well-grown A549 cells were spread in 12-well plates and placed in an incubator. We added the IC_{50} concentration of the complex after 24 h. After 4 h, the 6-well plate was light-activated for 30 min. The culture medium in the wells was removed, and the

prepared Mito-Tracker Deep Red staining solution was added to the wells and placed in a 37 °C oven for staining, and the cells were washed twice with PBS after 30 min, and the cells were photographed under the Smart Cell Imager with 20× magnification.

Intracellular reactive oxygen species

A549 cells were inoculated in 6-well plates, and after 24 h the complex was added, and after 4 h the plates were taken to light exposure for 30 min. The cells were collected in Ep tubes by adding trypsin digestion, centrifuged to remove the supernatant, washed with PBS, and then stained in Ep tubes by adding DCFH-DA probe solution (300 μL) for 30 min, then centrifuged to remove the staining solution and washed with PSB, and finally blown up in PBS and transferred to flow tubes for detection on flow cytometry.

Quantitative analysis of intracellular Ca^{2+} content

A549 cells were inoculated into 6-well plates, and when the cells reached a certain number, compound at IC_{50} concentration was added. After 4 h, the 6-well plates were exposed to light for 30 min, and then the cells were washed twice with PBS and digested with trypsin and collected in Ep tubes. After centrifugation to remove the supernatant and washing with PBS, 200 μL of Fluo-3AM Fluorescent Probe Dye was added, incubated at 37 °C in dark, centrifuged for 30 min to remove the dye, finally washed with PBS and transferred to flow-through tubes for assay analysis.

Mitochondrial permeability transformation pore opening

Preparation of Calcein AM staining solution: Dissolve Calcein AM (1.5 μL) and the agonist (15 μL) in 1500 μL assay buffer and mix well. Add 15 μL of $CoCl_2$ (100×) to the prepared Calcein AM staining solution (1500 μL) and mix well to form a fluorescence quenching working solution. Take 300 μL of this working solution and add 1.5 μL of Ionomycin (200×) to make a positive control. The well-grown A549 cells were spread in 12-well plates, and the IC_{50} concentration of complex was added, with the cells illuminated for 30 min after 4 h. After 24 h, the culture medium was removed from the plate and the cells were washed with PBS, incubated with the staining solution for 30 min, followed by qualitative analysis on a Smart Cell Imager with 20 × magnification. The cells were spread in 6-well plates, and the IC_{50} concentration of the complex was added, and the cells were exposed to light for 30 min after 4 h. After 24 h, the cells were digested with trypsin and collected in Ep tubes, and the culture medium was removed by centrifugation, while dye was added and the cells were incubated in the oven at 37 °C for 30 min. After the staining medium was removed by centrifugation and the cells were washed, it was transferred the cell suspensions to flow tubes and quantified on flow cytometer.

Analysis of mitochondrial membrane potential

A549 cells in good growth condition were spread in 12-well plates, and IC_{50} concentration of the compound was added when the cells grew to 85%, followed by light exposure for 30 min after 4 h. After 24 h, the plates were removed and the cells were washed twice with PBS, and the cells were stained with JC-1 solution (300 μL)

and protected from light for 30 min at 37 °C, then the wells were washed cleanly by PBS (300 μL), immersed into a Smart Cell Imager and photographed with a 20× magnification lens. For quantitative analysis, we spread the cells in 6-well plates and incubated them in an incubator, added the IC_{50} concentration of the complex for 4 h, and then lightened the plates. After 24 h, the cells were digested with trypsin and collected in Ep tubes and washed with PBS to remove the upper layer of culture medium. JC-1 staining solution (300 μL) was added and the cells were stained at 37 ℃ for 30 min, centrifuged to remove the staining solution and washed with PBS. Finally, the cell suspension was transferred to flow tubes and detected on flow cytometer.

Detection of intracellular MDA level

Cells were spread in a 6-well plate, and when the cells grew to about 85%, the complex was added, incubated for 4 h. After 24 h, the culture medium was removed from the plate and the cells were washed with PBS. 100 μL of lysate was added to each well and the cells were scraped on ice and placed in Ep tubes and lysed in the refrigerator at 4 °C for 15 min. After centrifugation, the supernatant was collected in another Ep tube and 10 μL of each sample was placed in a 96-well plate and the absorbance at 570 nm was detected by BCA workup, and the concentration of the proteins was calculated.

Detection of intracellular ROS

Qualitative analysis: A549 cells with good growth status were spread in 12-well plates, IC_{50} concentration of complex was added when the cells reached a certain number, and

the cells were illuminated for 30 min in 4 h. After continuing incubation for 24 h, the culture medium was removed from the wells, and the cells were washed with PBS twice, and 300 μL of BODIPY 581/591 C11 working solution was added to each well and incubated in an oven for 30 min at 37 °C. Remove the stain solution and PBS wash the cells to analyze on a smart cell imager. Quantitative analysis: A549 cells were spread in 6-well plates, digested with trypsin and collected in Ep tubes, centrifuged to remove the culture medium and then stained with BODIPY 581/591 C11 working solution for 30 min. Finally, centrifugation was performed to remove the staining solution, the cells were transferred to flow tubes and analyzed by flow cytometry.

Release of cytochrome c

A549 cells were evenly spread in a 12-well plate, cultured for 24 hours in a suitable environment, and then added IC_{50} concentration of complex, during which the cells needed to be illuminated for 0.5 h. After 24 hours, the residual complex was washed away with PBS, and the cells were fixed with immune fixation solution for 30 min, and then the cells were incubated with Cyt-C antibody at low temperature for 12 h. The next day, the cells were washed with immune cleaning solution for 3 times, incubated by FITC antibody and was stained by DAPI for 30 minutes, and then after cleaning with PBS, it could be filmed under the smart cell imager.

Detection of LDH

A549 cells with good growth status were spread in 96-well plates, and the control

group, and drug treatment group were set up respectively. After incubation for 24 h, the cells were washed by the culture medium, and 200 μL of low serum culture medium was added, which were placed in the incubator at 37 ℃. For detection, the 96-well plate was placed in a micro-oscillator and oscillated for 5 min. Add 60 μL of super clear solvent

from each hole to the new 96-well plate, and mix the 30 μL LDH test solution for each hole and incubate at room temperature for 30 min. After completion, the 96-well plate was placed on the enzyme-labeled instrument at 490 nm to determine OD value.

Calculation for HOMO and LUMO

Geometry optimization with orbital calculations on compounds 5a-5c was performed using Gaussian 09 package at the level of B3LYP density functional in conjunction with the 6-31G(d) basis sets for C, H, N, O, Br, atoms, LanL2DZ for Ir atom. And the Time-dependent density functional theory (TD-DFT) calculations were employed to further explain the generation mechanism of 5a-5c.

Section II Supplementary materials for figures

Figure S1 Purity determination using methanol and H_2O as mobile phase, the purity is 97.06% for 5a, 98.73% for 5b, 97.58% for 5c

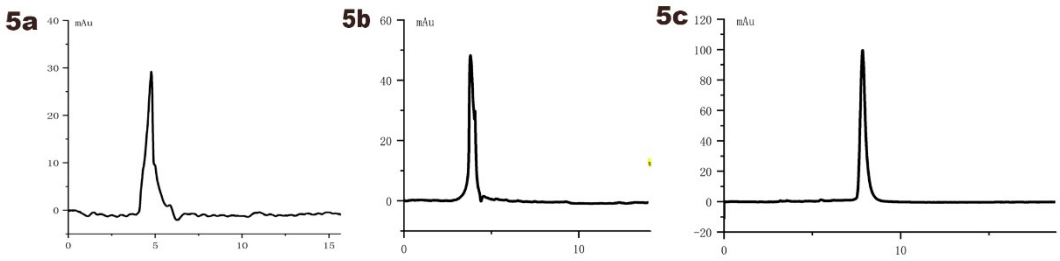

Figure S2 Stability determination of **5a**, **5b** and **5c** in PBS solution at 0 and 48 h

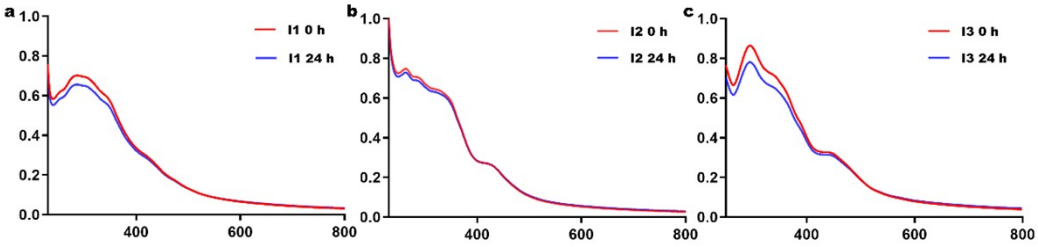

Figure S3 (a) Cellular uptake, (b) Co-location of the complexes at mitochondria, (c) Mitochondrial permeability transition core open, (d) green fluorescence intensity.

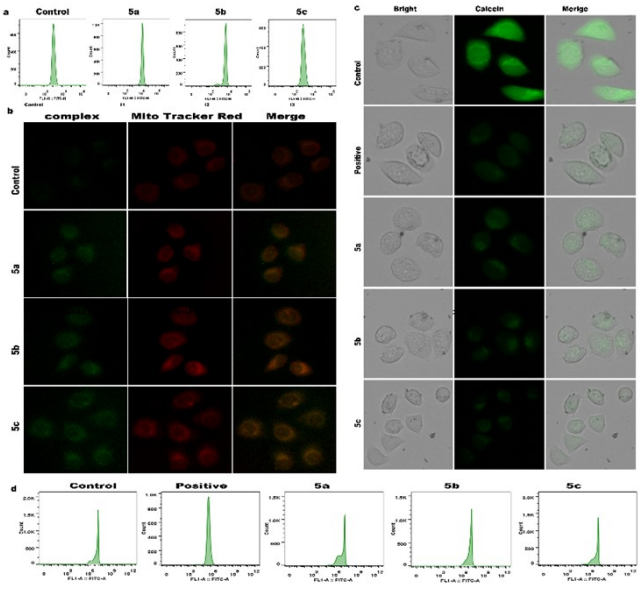

Figure S4 (a) Change of mitochondrial membrane potential, (b) determination of ratio of red versus green fluorescence, (c) the release of cytometry C

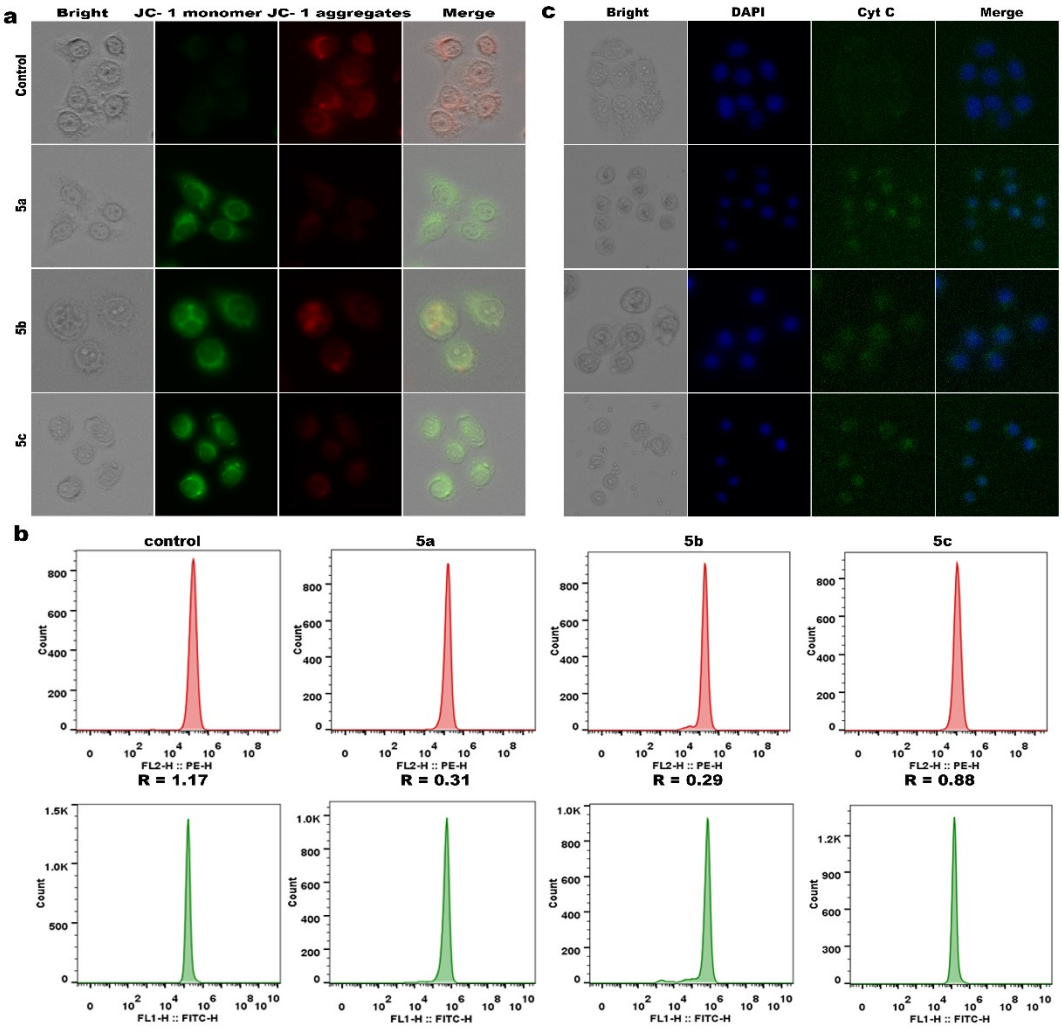

Figure S5 (a) Intracellular ROS level, (b) western blotting assay of p38, (c) Intracellular Ca^{2+} content

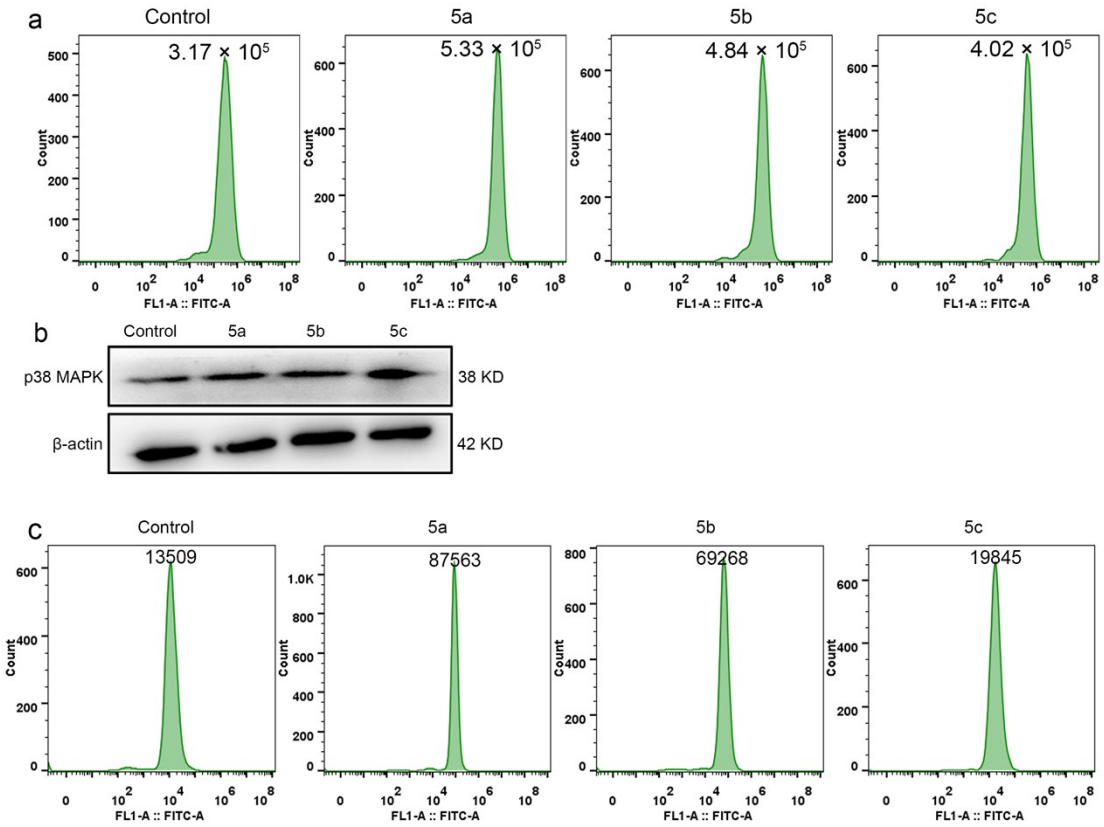

Figure S6 Wound healing experiments while A549 cells were treated with IC_{50} concentration of 5a, 5b, 5c for 24 h

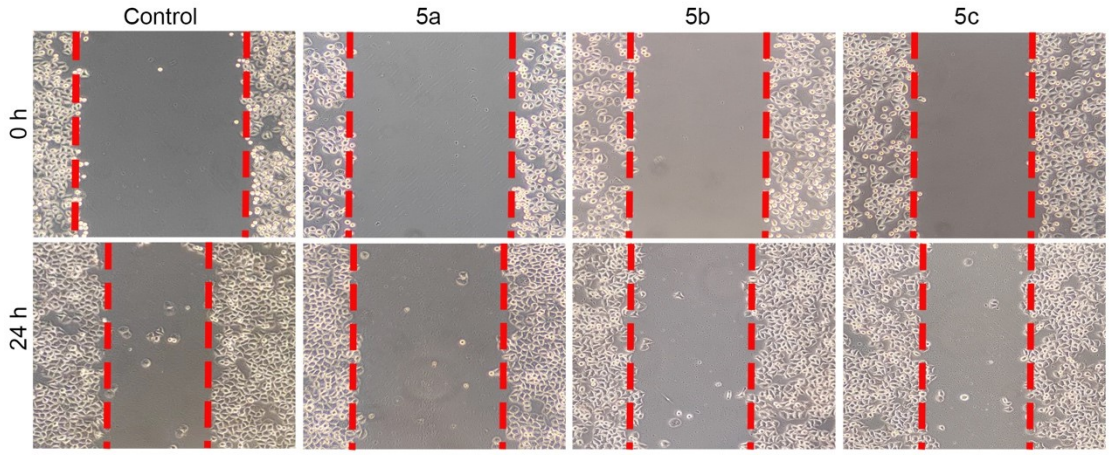

Figure S7 HRMS spectra of 5a

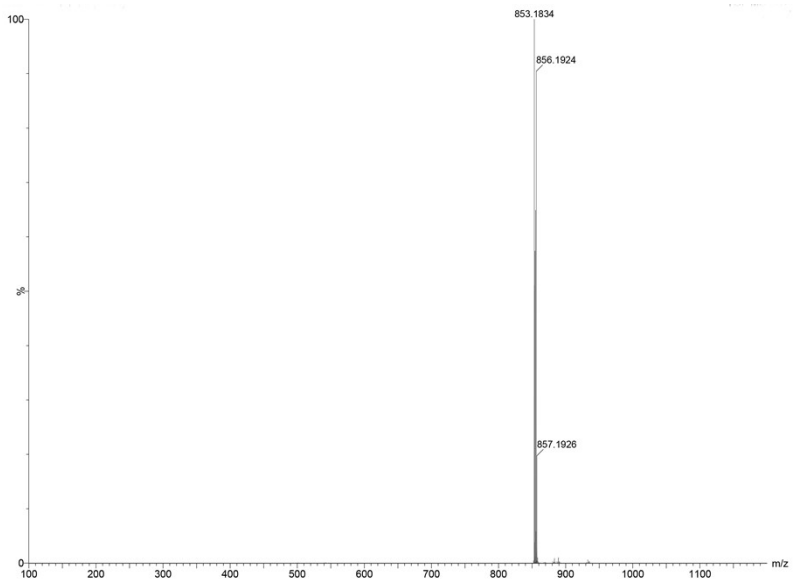

Figure S8 ^{1}H NMR spectra of 5a

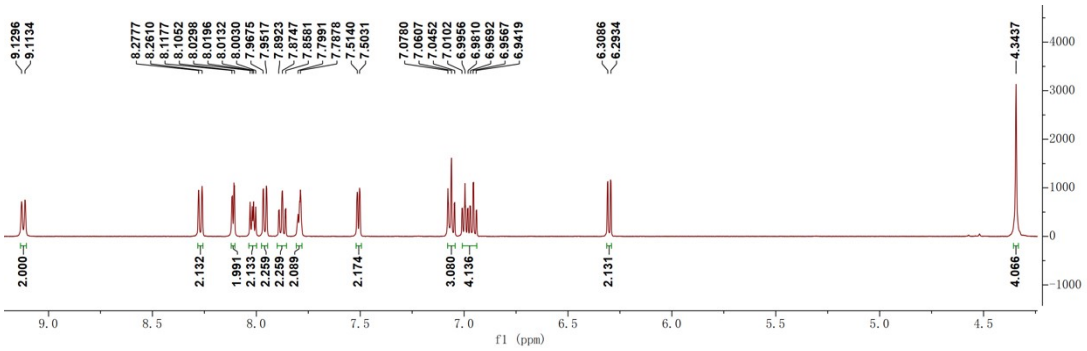

Figure S9 ^{13}C NMR spectra of 5a

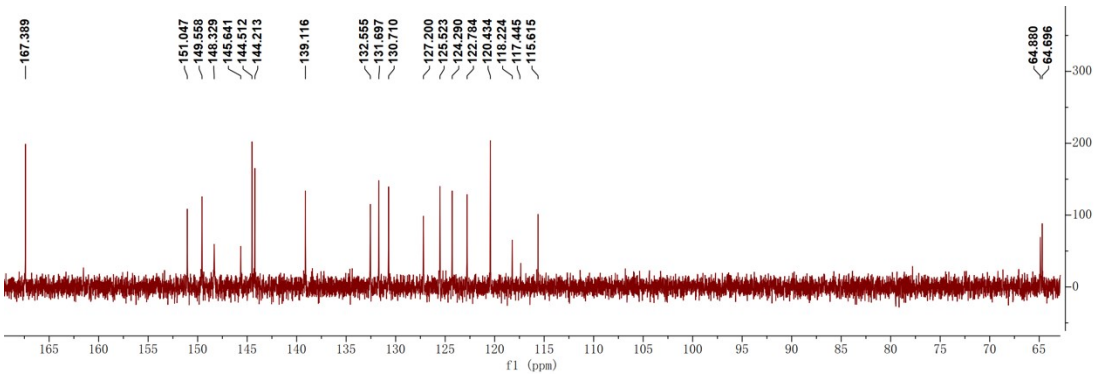

Figure S10 HRMS spectra of 5b

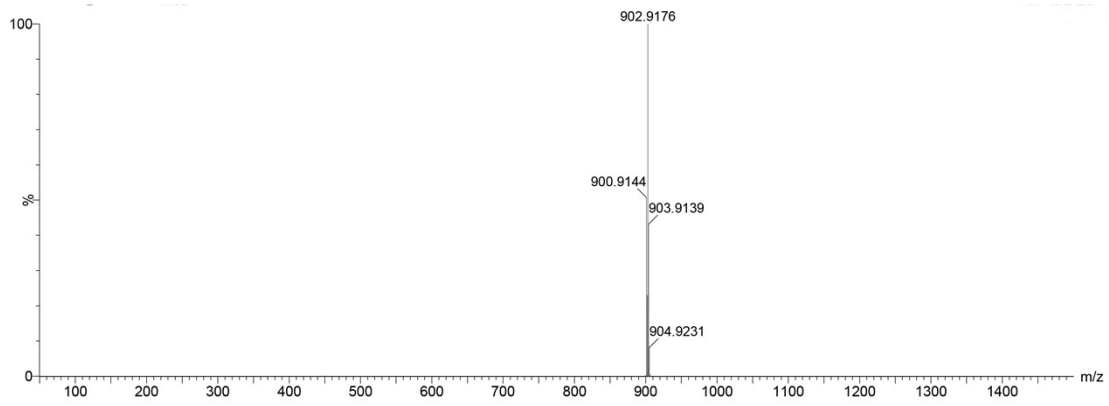

Figure S11 ^{1}H NMR spectra of 5b

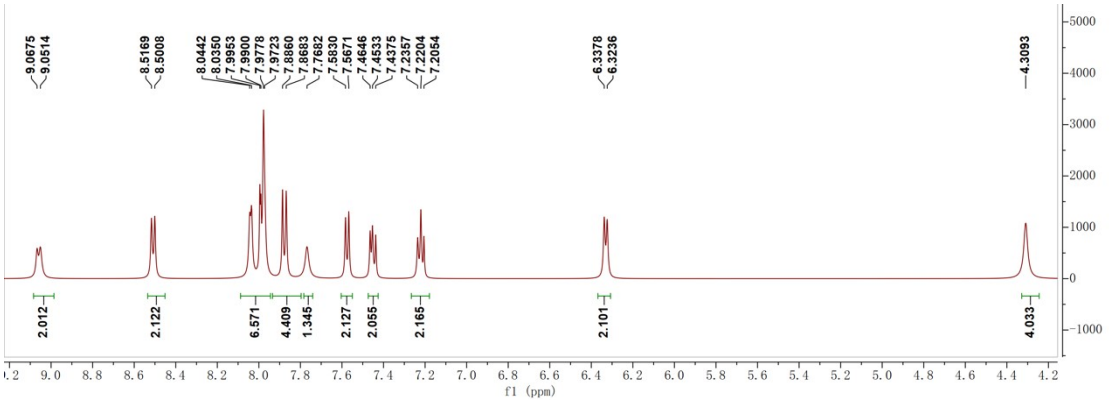

Figure S12 ^{13}C NMR spectra of 5b

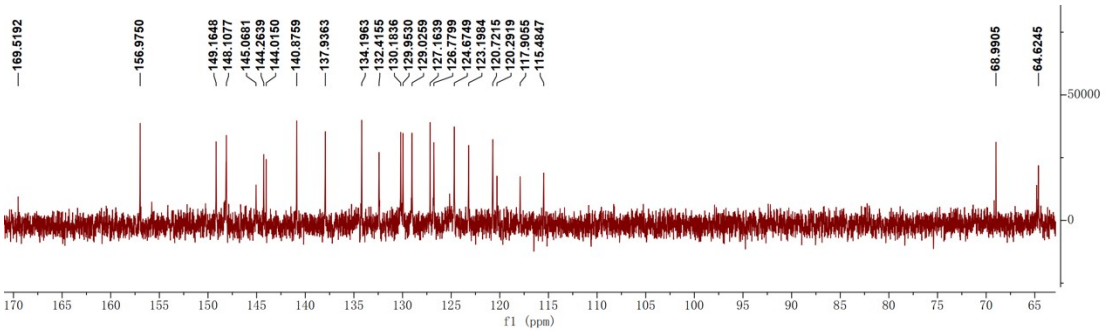

Figure S13 HRMS spectra of 5c

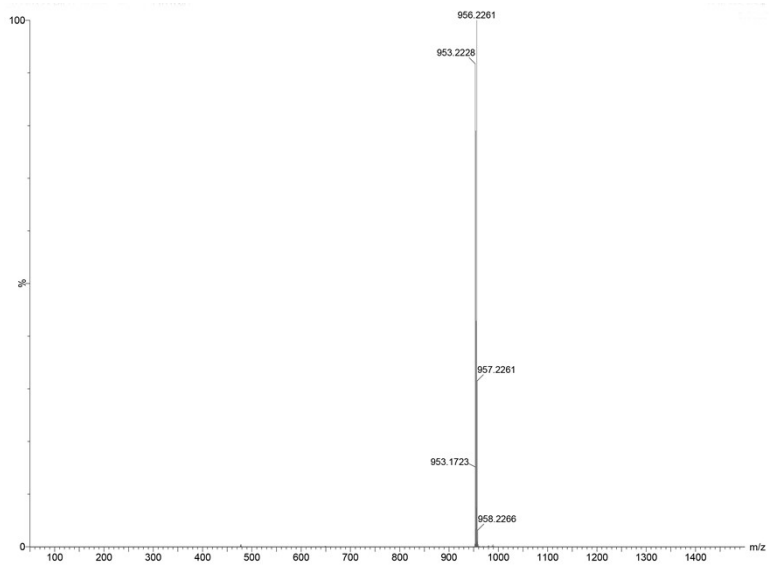

Figure S14 ^{1}H NMR spectra of 5c

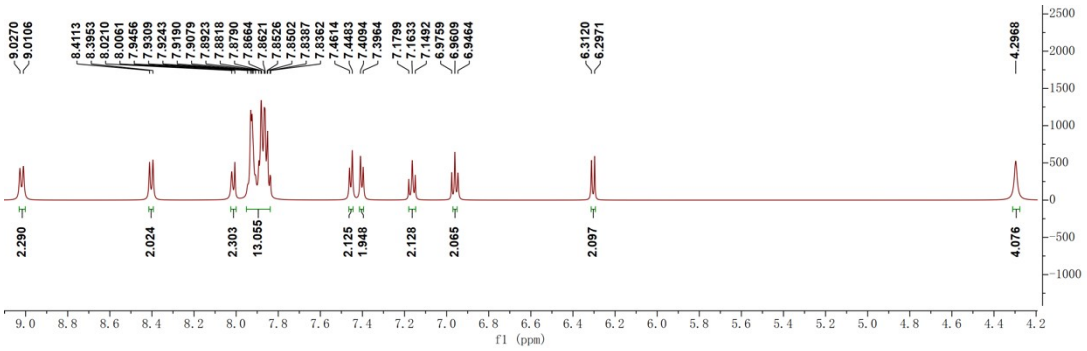

Figure S15 ^{13}C NMR spectra of 5c