Electronic Supplementary Information

A mitochondria-targeted iridium(III)-based photoacid generator induces dual-mode photodynamic damage within cancer cells

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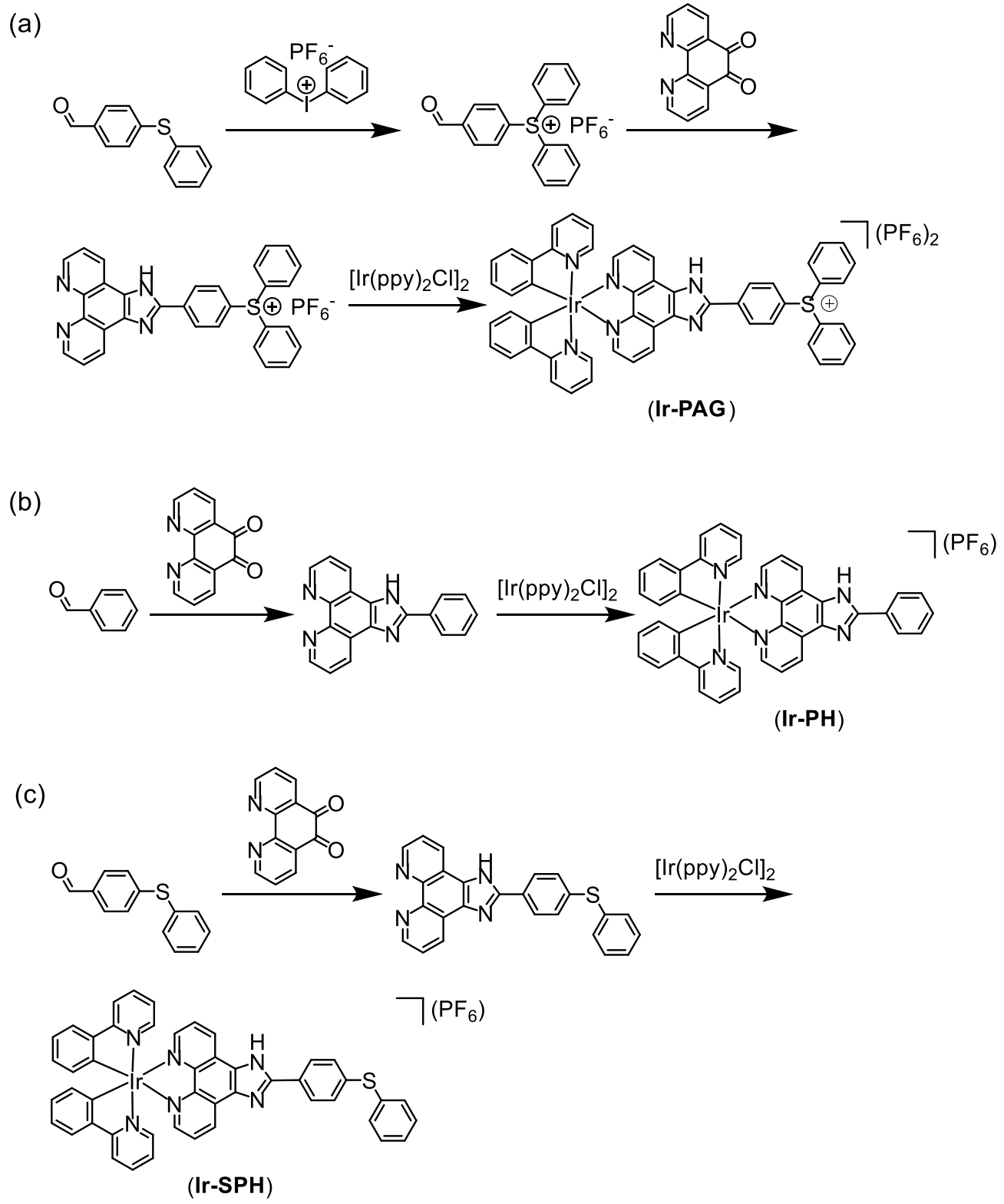
Experimental section

Materials and measurements

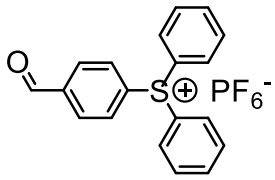
Materials. $IrCl_3 \cdot nH_2O$ (Alfa Aesar, USA), 2-phenylpyridine (ppy, Sigma Aldrich, USA), diphenyliodonium hexafluorophosphate (Sigma Aldrich, USA), 1,10-phenanthroline-5,6-dione (Sigma Aldrich, USA), 4-phenylthiobenzaldehyde (Sigma Aldrich, USA), Benzaldehyde (Sigma Aldrich, USA), DMSO (Sigma Aldrich, USA), NH_4PF_6 (Alfa Aesar, USA), PBS (phosphate buffered saline, Sigma Aldrich, USA), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, H_2DCF-DA (Sigma Aldrich, USA), MTDR (Life Technologies, USA), LTDR (Life Technologies, USA) and rhodamine B base (Sigma Aldrich, USA) were used as received. Annexin V-FITC/PI apoptosis detection kit was purchased from Sigma Aldrich (USA) and used as recommended by the manufacturer. The tested compounds were dissolved in DMSO, and the final concentration of DMSO was kept at 1% (v/v).

Measurements. ESI-MS: LCMS-2010A liquid chromatography-mass spectrometer and Thermo LCQ-DECA-XP liquid chromatography-mass spectrometer; ^{1}H and ^{13}C NMR: Bruker Avance 400 spectrometer; Elemental Analysis (EA): elemental Vario EL elemental analyzer from Germany Element; UV-Vis spectra: American Varian Cary 300 UV/Vis spectrophotometer; Fluorescence spectra and lifetime measurements: Edinburgh FLS920 spectrometer; Microplate reader: iMark absorbance microplate reader (Bio-Rad, USA); Laser confocal fluorescence microscope: Carl Zeiss LSM 710, Germany.

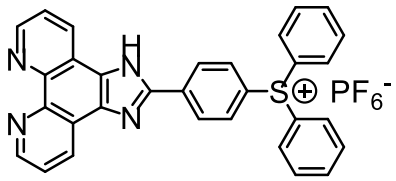
Synthesis and characterization



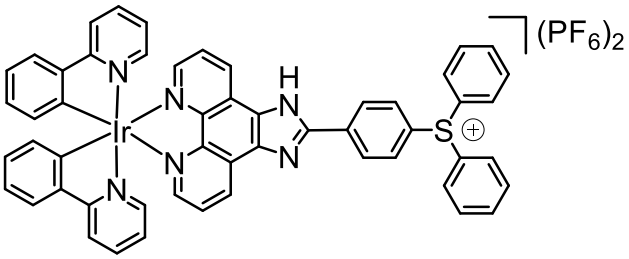
Scheme S1. Synthetic procedures of (a) **Ir-PAG**, (b) **Ir-PH** and (c) **Ir-SPH**.



(4-formylphenyl)diphenylphosphonium hexafluorophosphate. This compound was synthesized according to a modified literature method.[1] A mixture of diphenyliodonium hexafluorophosphate (1278 mg, 3 mmol), 4-phenylthiobenzaldehyde (642.1 mg, 3 mmol) and copper acetate (18 mg, 0.15 mmol, 5%), dissolved in 17 mL chlorobenzene was heated to reflux at 125 ℃ for 3 h. The reaction was monitored by thin-layer chromatography (TLC) and mass spectrometry. Upon completion, the solvent was evaporated under reduced pressure to give a white solid. The desired product was obtained as a light white solid by silica column chromatography with dichloromethane and methanol (9:1, v/v) as the eluent. Yield: 1.05 g (80%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 10.10 (s, 1 H), 8.17 (d, J = 8.5 Hz, 2H), 7.91 (d, J = 8.5 Hz, 2H), 7.82-7.77 (m, 6H), 7.75-7.70 (m, 4H). ESI-MS (CH_2Cl_2): m/z calcd for $[M-PF_6]^+$, 291.39; found: 290.95; $[M-PF_6+CH_3OH]^+$, 323.43; found:323.00.

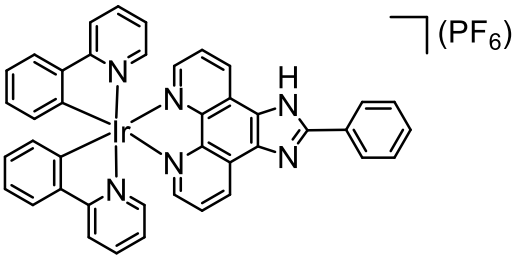


4-(1H-9,10-phenanthromidazolyl)triphenylsulfonium hexafluorophosphate. 1,10-phenanthroline-5,6-dione (400 mg, 0.917 mmol), (4-formylphenyl)diphenylphosphonium hexafluorophosphate (212 mg, 1.009 mmol) and ammonium acetate (1.925 mg, 25 mmol) were dissolved in 12 mL glacial acetic acid in a round bottom flask. The mixture was stirred well and heated to reflux for 3 h. After cooling to room temperature, 10 mL H_2O was added to the reaction solution, and then adjust the solution pH to about 7 and the solution becomes cloudy. The solvent was evaporated under reduced pressure, then a small amount of water is added to completely dissolve the product for lyophilization. The product was obtained in a yield of 62% as a white solid, which was used directly in the next step without further purification. ESI-MS (CH_2Cl_2): m/z calcd for $[M-PF_6]^+$, 481.60; found: 481.16.



[Ir(ppy)$_2$(4-(1H-9,10-phenanthromidazolyl)triphenylsulfonium)](PF$_6$)$_2$ (Ir-PAG).

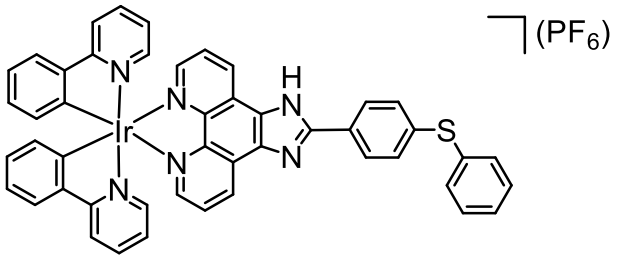
Complex **Ir-PAG** was synthesized according to a similar procedure reported previously.[2] Briefly, a mixture of 4-(1*H*-9,10-phenanthromidazolyl)triphenylsulfonium hexafluorophosphate (0.4 mmol, 2 equiv) and $[Ir(ppy)_2Cl]_2$ (0.2 mmol, 1 equiv) in CH_2Cl_2/CH_3OH (2:1, v/v) was heated to reflux under N_2 atmosphere for 4 h in the dark. After the end of the reaction, the solution was cooled to room temperature and a 6-fold excess of NH_4PF_6 was added, and the mixture was stirred for 1 h. The mixture was filtered to remove insoluble salts, and then the filtrate was evaporated to dryness under reduced pressure to get the crude product. The crude product was then dissolved with a small amount of CH_2Cl_2 and purified by column chromatography with CH_2Cl_2/CH_3OH (9:1, v/v) as the eluent. It was obtained as a yellow powder. Yield: 0.320g (63%). ^{1}H NMR (400MHz, DMSO-d_6): δ = 14.70 (s, 1H), 9.18 (t, J = 8.4 Hz, 2H), 8.63 (d, J = 8.7 Hz, 2H), 8.28 (d, J = 8.2 Hz, 2H), 8.20 (d, J = 4.2 Hz, 2H), 8.14 (d, J = 8.5 Hz, 4H), 7.99-7.89 (m, 10H), 7.85 (q, J = 7.2, 6.1 Hz, 4H), 7.52 (s, 2H), 7.08 (t, J = 7.5 Hz, 2H), 6.98 (dt, J = 11.0, 7.3 Hz, 4H), 6.30 (d, J = 7.5 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6): δ = 168.72, 152.12, 152.03, 151.02, 150.69, 146.46, 145.85, 140.53, 136.36, 134.22, 134.19, 133.29, 133.04, 132.11, 130.59, 127.05, 127.01, 126.91, 125.65, 124.23, 121.82. ESI-MS (CH_2Cl_2): m/z calcd for $[M-2PF_6-H]^+$, 981.19; found: 981.56. Elemental analysis: calcd (%) for $C_{53}H_{37}N_6SP_2F_{12}Ir$: C, 50.04; H, 2.93; N, 6.61; found: C, 49.87;H, 2.98; N, 6.55.



[Ir(ppy)$_2$(2-phenyl-1H-9,10-phenanthroimidazole)](PF$_6$) (Ir-PH).

Complex **Ir-PH** was synthesized in a manner identical to that described for **Ir-PAG**. It was obtained

as a yellow powder. Yield: 0.305g (78%). ^{1}H NMR (400MHz, DMSO-d_6): δ = 14.36 (s, 1H), 9.21 (t, J = 7.1 Hz, 2H), 8.33 (d, J = 7.4 Hz, 2H), 8.28 (d, J = 8.2 Hz, 2H), 8.20-8.07 (m, 4H), 7.97 (d, J = 7.6 Hz, 2H), 7.89 (t, J = 7.8 Hz, 2H), 7.68 (t, J = 7.5 Hz, 2H), 7.61 (t, J = 7.3 Hz, 1H), 7.55-7.49 (m, 2H), 7.08 (t, J = 7.5 Hz, 2H), 6.98 (dt, J = 14.5, 7.2 Hz, 4H), 6.30 (d, J = 7.4 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6): δ = 168.75, 152.19, 150.98, 150.21, 145.98, 145.87, 140.51, 134.06, 133.06, 132.09, 131.08, 128.91, 128.45, 126.90, 125.66, 124.20, 121.80. ESI-MS (CH_2Cl_2): m/z calcd for $[M-PF_6]^+$, 796.93; found: 797.48. Elemental analysis: calcd (%) for $C_{41}H_{28}N_6PF_6Ir \cdot 2H_2O$: C, 50.36; H, 3.3 N, 8.59 ; found: C, 50.22; H, 3.38; N, 8.73.



[Ir(ppy)$_2$(4-(1H-9,10-phenanthromidazolyl)diphenylsulfide)](PF$_6$) (Ir-SPH).

Complex **Ir-SPH** was synthesized in a manner identical to that described for **Ir-PAG**. It was obtained as a yellow powder. Yield: 0.300g (68%). ^{1}H NMR (400MHz, DMSO-d_6): δ = 14.36 (s, 1H), 9.17 (d, J = 7.8 Hz, 2H), 8.28 (d, J = 7.9 Hz, 4H), 8.21-8.14 (m, 2H), 8.14-8.05 (m, 2H), 7.97 (d, J = 7.8 Hz, 2H), 7.89 (t, J = 7.7 Hz, 2H), 7.65-7.38 (m, 9H), 7.08 (t, J = 7.5 Hz, 2H), 6.98 (dt, J = 14.0, 7.1 Hz, 4H), 6.30 (d, J = 7.5 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-d_6): δ = 168.73, 153.94, 152.14, 150.98, 145.85, 140.51, 134.06, 133.84, 133.05, 132.08, 131.84, 131.72, 130.17, 129.33, 126.89, 125.65, 124.20, 121.79. ESI-MS (CH_2Cl_2): m/z calcd for $[M-PF_6]^+$, 905.09; found: 905.54. Elemental analysis: calcd (%) for $C_{47}H_{32}N_6SPF_6Ir \cdot 3H_2O$: C, 51.13; H, 3.47; N, 7.61; found: C, 52.96; H, 3.34; N, 7.43.

<u>Photolysis of Ir(III) complexes detected by MS and ^{1}H NMR spectra</u>

The solutions of Ir(III) complexes (500 μM) were prepared in DMSO-d_6 and analyzed by MS and ^{1}H NMR just before and after being irradiated by an LED lamp with a wavelength of 425 nm (40 mW cm^{-2}) for 30 min.

Photolysis of Ir-PAG detected by HPLC

Four copys of **Ir-PAG** solutions (500 μM) were prepared in DMSO/H_2O (4:1, v/v), which was subjected to light irradiation (425 nm, 40 mW cm^{-2}) for 0, 5, 10, 30 min. The photolysis was monitored by HPLC (Thermo) with a Hypersil God Dim reverse phase column (100 mm × 2.1 mm, Thermo). Peaks were detected at 254 nm. We used H_2O with 0.1% trifluoroacetic acid (TFA) as mobile phase A and MeCN with 0.1% TFA as mobile phase B. Gradient elution was employed in the separation using a MeCN (0.1% TFA)/H_2O (0.1% TFA) mixture starting at 70/30 and changing linearly to 88/12 over 10 min at 2 mL/min flow rate.

Photoacid quantum yield measurement

Photoacid quantum yield was measured by using rhodamine B base as a sensor for photoacid.[1] The sample solutions were irradiated by an LED lamp with a wavelength of 425 nm, and the UV-Vis absorbance of rhodamine B at 555 nm was recorded every 2 s. The photoacid quantum yield of the tested sample is calculated according to the following equation:

$$\Phi_{H^+} = \frac{\Delta OD_{555} \cdot N_A}{10^3 \cdot \varepsilon_{555}^{RhB} \cdot I_0 \cdot \left[1 - 10^{-\bar{D}}\right] \cdot \Delta t}$$

Where ΔOD_{555} is the change in the optical density measured at 555 nm during the production of rhodamine B, N_A is the Avogadro number, ε_{555}^{RhB} is the extinction coefficient of rhodamine B at 555 nm, $\bar{D}$ is the average absorbance, I_0 is the light intensity in photon/(cm^2 s), Δt is the irradiation time in s.

Singlet oxygen quantum ($^1O_2$) yield measurement

The $^1O_2$ quantum yields (Φ_Δ) for the Ir(III) complexes were evaluated in methanol using a steady-state method with 1,3-diphenylisobenzofuran (DPBF) as the $^1O_2$ indicator and $[Ru(bpy)_3]Cl_2$ as the standard (Φ_Δ = 0.81).[3] Briefly, air-equilibrated methanol solutions containing the tested complexes and DPBF (100 μM) were prepared in the dark, and then the LED lamp with the wavelength of 425 nm was used for light irradiation. The absorbance of DPBF at 418 nm was recorded every 2 s of irradiation. The absorbance of the Ir(III) complexes and $[Ru(bpy)_3]Cl_2$ at the wavelength of 425 nm was adjusted at about 0.15. The Φ_Δ of the Ir(III) complexes were calculated

according to the following equation.

$$\Phi_{\Delta(x)} = \Phi_{\Delta(std)} \times (\frac{S_x}{S_{std}}) \times (\frac{F_{std}}{F_x})$$

where subscripts x and std represent the sample to be tested and the standard reference $[Ru(bpy)_3]Cl_2$, respectively, *S* is the slope of the absorption curve of DPBF at the wavelength of 418 nm over time. *F* is the absorption correction factor, which is given by $F = 1–10^{-OD}$ (OD represents the optical density of sample and $[Ru(bpy)_3]Cl_2$ at 425 nm).

Cell lines and culture conditions

The cells used in this paper were all obtained from Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). A549 and A549R cells were cultured in RPMI 1640 media, and HLF cells were cultured in DMEM media. The media contain 10% fetal bovine serum, 100 μg/mL streptomycin and 100 U/mL penicillin. The cells were cultured using tissue culture flasks and placed in a humidified incubator at 37 ℃ with 5% CO_2.

Cytotoxicity in the dark and phototoxicity test

Cytotoxicity in the dark: The cytotoxicity was determined by MTT method. The cells were digested with 0.25% trypsin to form a single cell suspension, and the viable cells were counted with a hemocytometer. Adjust the viable cell concentration to 5×10^4/mL and inoculate the cells into 96-well culture plates (160 μL/well). After incubation for 24 h, aspirate the old medium. The tested compounds were first dissolved in DMSO (1%, v/v) and then diluted with fresh cell media. The cells were incubated different concentrations of the drug diluted in the media at 37 ℃ for 44 h. 20 μL of MTT (5 mg/mL) was added to each well and incubate for another 4 h. The media was carefully removed and each well was added with 150 μL of DMSO, the plate was then shaken on a shaker for about 10 minutes, and the OD value at 595 nm was measured using a microplate reader.

Phototoxicity: Cells were treated with different concentrations of drug and incubated for 12 h in the dark, the drug-containing media was removed, and 200 μL of fresh medium was added. The cells was then irradiated with an LED light source (40 mW cm^{-2}) with a wavelength of 425 nm for 15 min. MTT was added after 32 h incubation in the dark. Subsequent treatment was the same as the dark

cytotoxicity test.

For **hypoxic conditions**, cells were first incubated with drug at 37 ℃ for 8 h under normoxic conditions and further incubated for 4 h under hypoxic conditions, and then sujected to PDT treatment immediately.

Colocalization assay

A549 cells cultured in a 35 mm Corning dish were incubated with 10 μM of Ir(III) complexes at 37 ℃ for 1 h. The cells were further co-incubated with 150 nM of LTDR or 150 nM of MTDR for another 0.5 h. The cells were washed three times with PBS and immediately observed with a laser confocal microscope. The excitation wavelength of Ir(III) complexes is 405 nm, the excitation wavelength of LTDR and MTDR is 633 nm. The fluorescence/phosphorescence collection wavelength is 600 ± 20 nm (Ir) and 670 ± 20 nm (LTDR and MTDR).

Measurement of mitochondrial membrane potential

A549 cells seeded in a 35 mm Corning cell culture dishes were incubated with different concerntrations of **Ir-PAG** at 37 ℃ for 12 h in the dark, followed by irradiation with an LED light source (40 mW cm^{-2}) with a wavelength of 425 nm for 15 min. The cells were washed twice with serum-free medium and then incubated with rhodamine 123 (5 μg/ml) in serum-free medium at 37 ℃ for 30 min. The cells were washed twice with serum-free medium, and immediately observed with a laser confocal microscope. The excitation wavelength of rhodamine 123 is 488 nm and the fluorescence collection wavelength is 520 ± 20 nm.

Intracellular ATP detection

A549 cells seeded into 96-well culture plates were incubated with different concerntrations of **Ir-PAG** at 37 ℃ for 12 h in the dark, followed by irradiation with an LED light source (40 mW cm^{-2}) with a wavelength of 425 nm for 15 min. Immediately afterwards, the cellular ATP levels were measured by using a commercial kit (CellTiter-Glo® Luminescent Cell Viability Assay kit; G7570, Promega, USA).

Cellular ROS detection

A549 cells seeded in a 35 mm Corning cell culture dish were incubated with **Ir-PAG** (1 μM) at 37 ℃ for 6 h in the dark. The cells were further incubated with serum-free medium containing 10 μM H_2DCF-DA for 20 min in the dark, followed by irradiation with an LED light source (40 mW cm^{-2}) at a wavelength of 425 nm for 30 s. The cells were washed twice with serum-free medium, and immediately observed with a laser confocal microscope. The excitation wavelength of DCF is 488 nm and the fluorescence collection wavelength is 520 ± 20 nm.

Annexin V/propidium iodide double staining assay

The assays were performed according to similar procedures previously reported.[4]

Supporting figures and tables

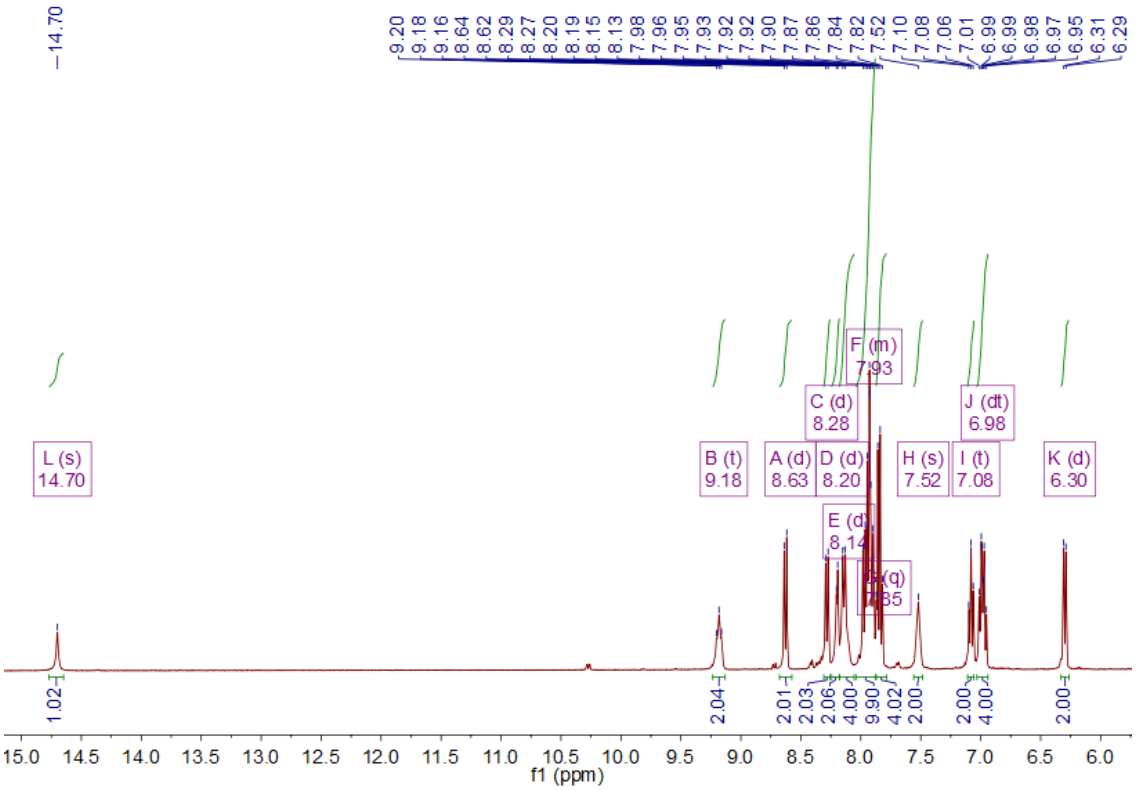


Fig. S1 ^{1}H NMR spectrum of **Ir-PAG** in DMSO-d_6 at 298 K.

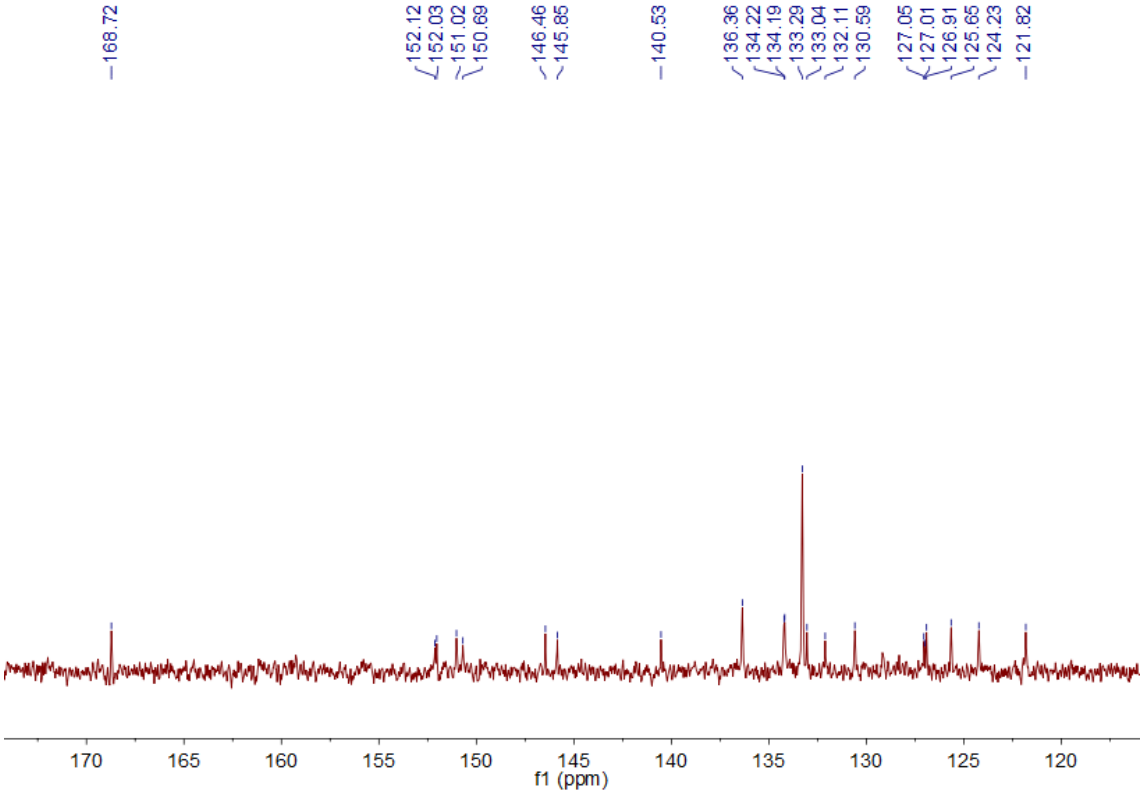


Fig. S2 ^{13}C NMR spectrum of **Ir-PAG** in DMSO-d_6 at 298 K.

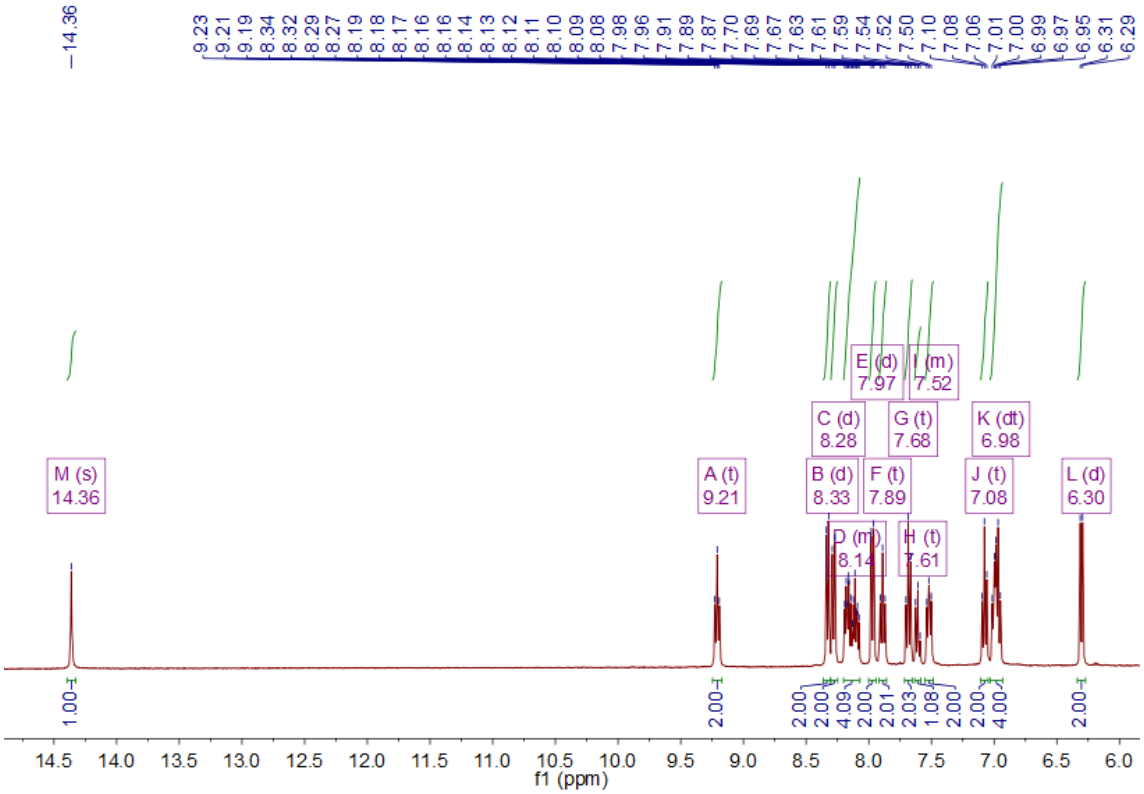


Fig. S3 ^{1}H NMR spectrum of **Ir-PH** in DMSO-d_6 at 298 K.

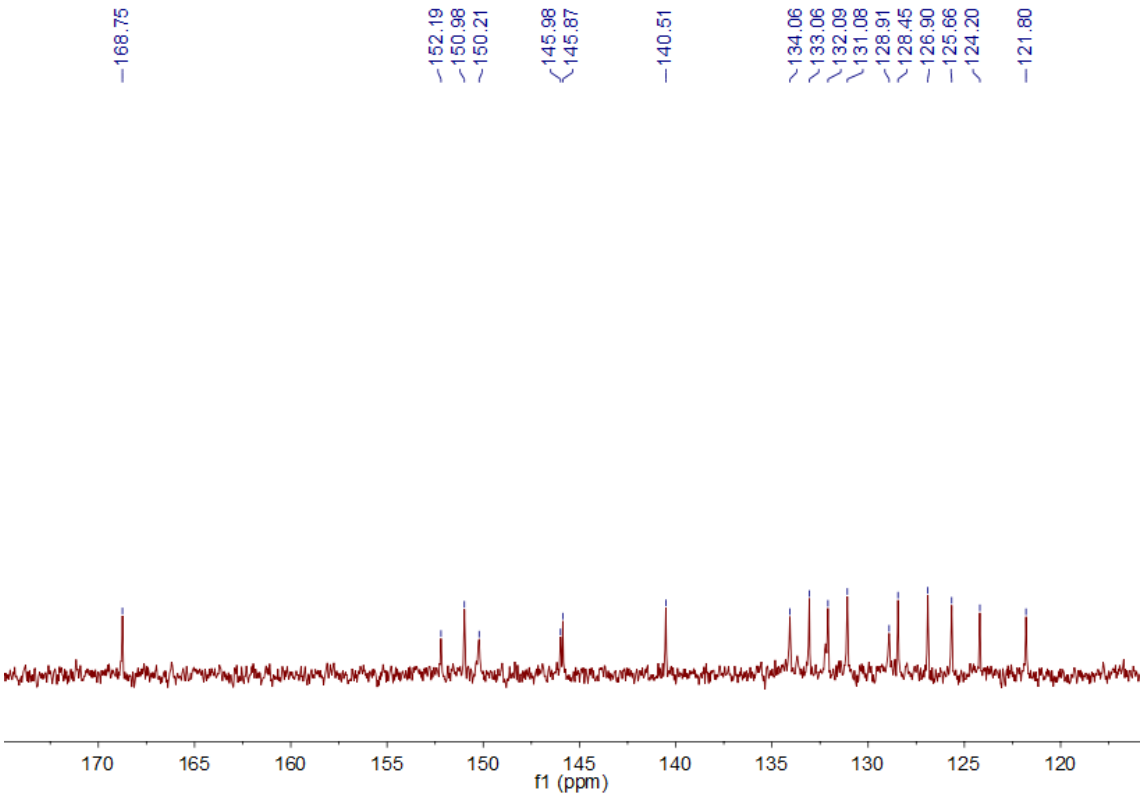


Fig. S4 ^{13}C NMR spectrum of **Ir-PH** in DMSO-d_6 at 298 K.

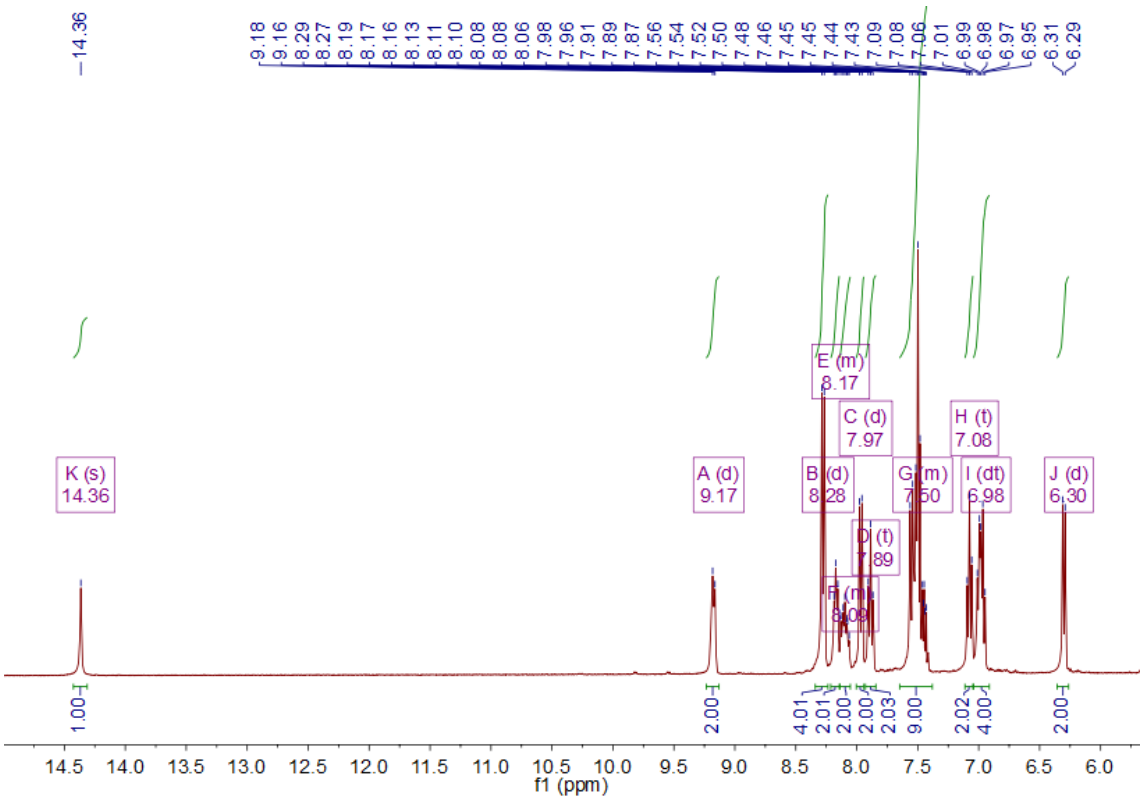


Fig. S5 ^{1}H NMR spectrum of **Ir-SPH** in DMSO-d_6 at 298 K.

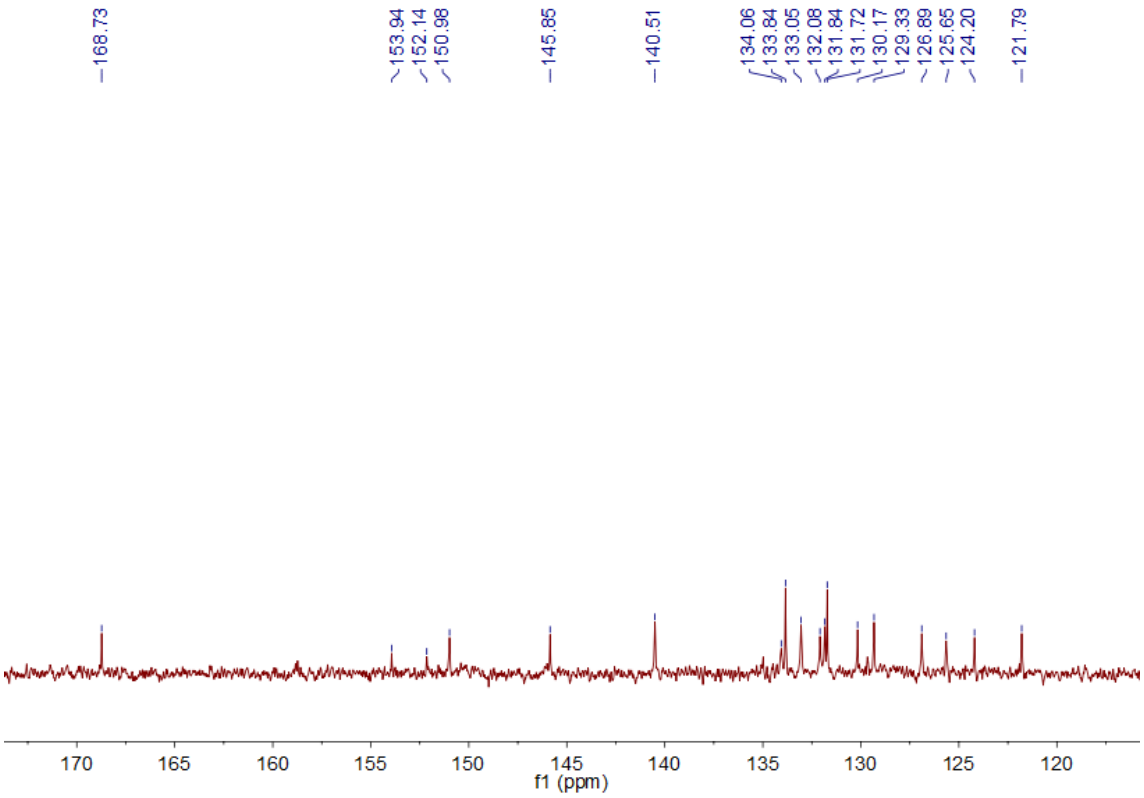


Fig. S6 ^{13}C NMR spectrum of **Ir-SPH** in DMSO-d_6 at 298 K.

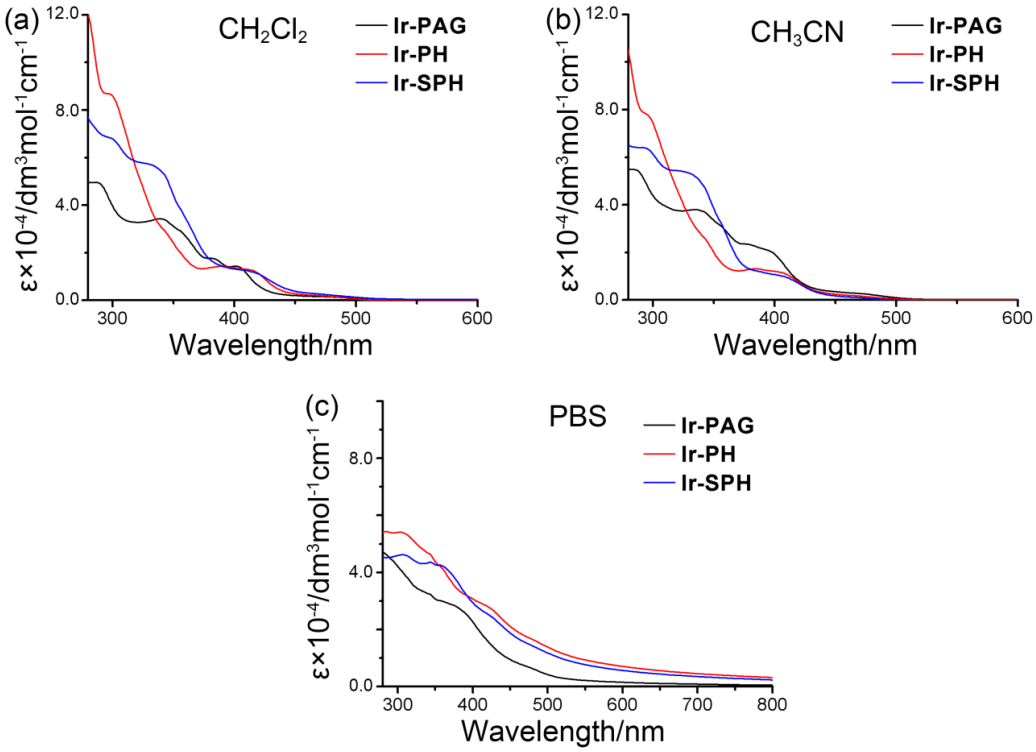


Fig. S7 UV/Vis absorption spectra of **Ir-PAG**, **Ir-PH** and **Ir-SPH** (10 μM) measured in (a) CH_2Cl_2, (b) CH_3CN and (c) PBS at 298 K.

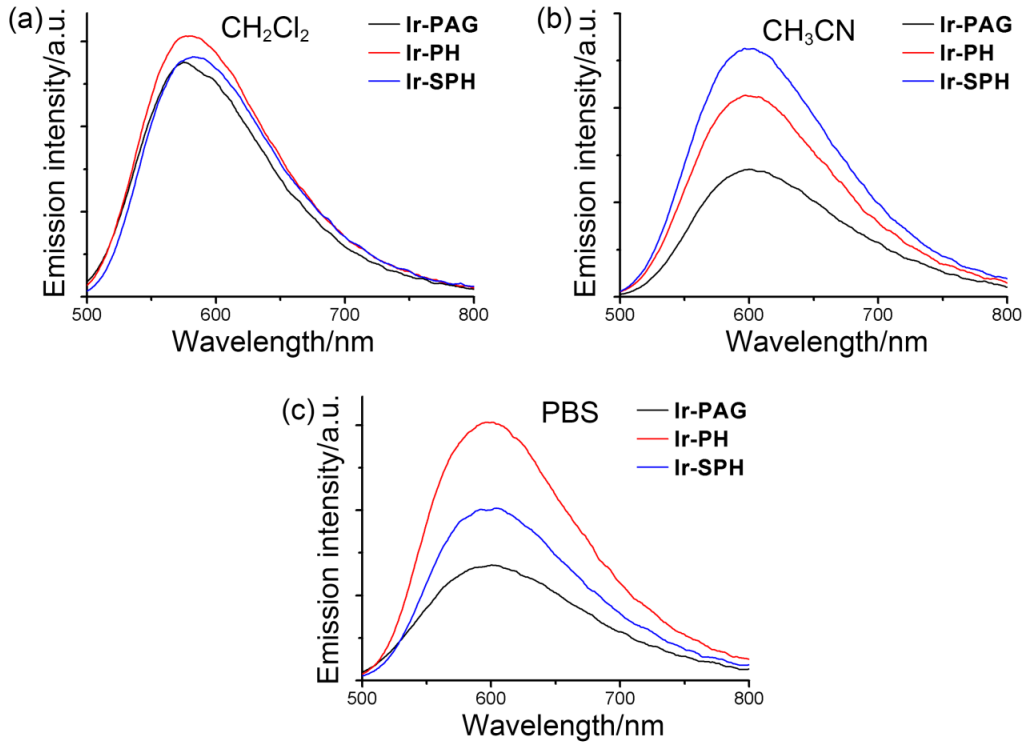


Fig. S8 Emission spectra of **Ir-PAG**, **Ir-PH** and **Ir-SPH** (10 μM) measured in (a) CH_2Cl_2, (b) CH_3CN and (c) PBS at 298 K.

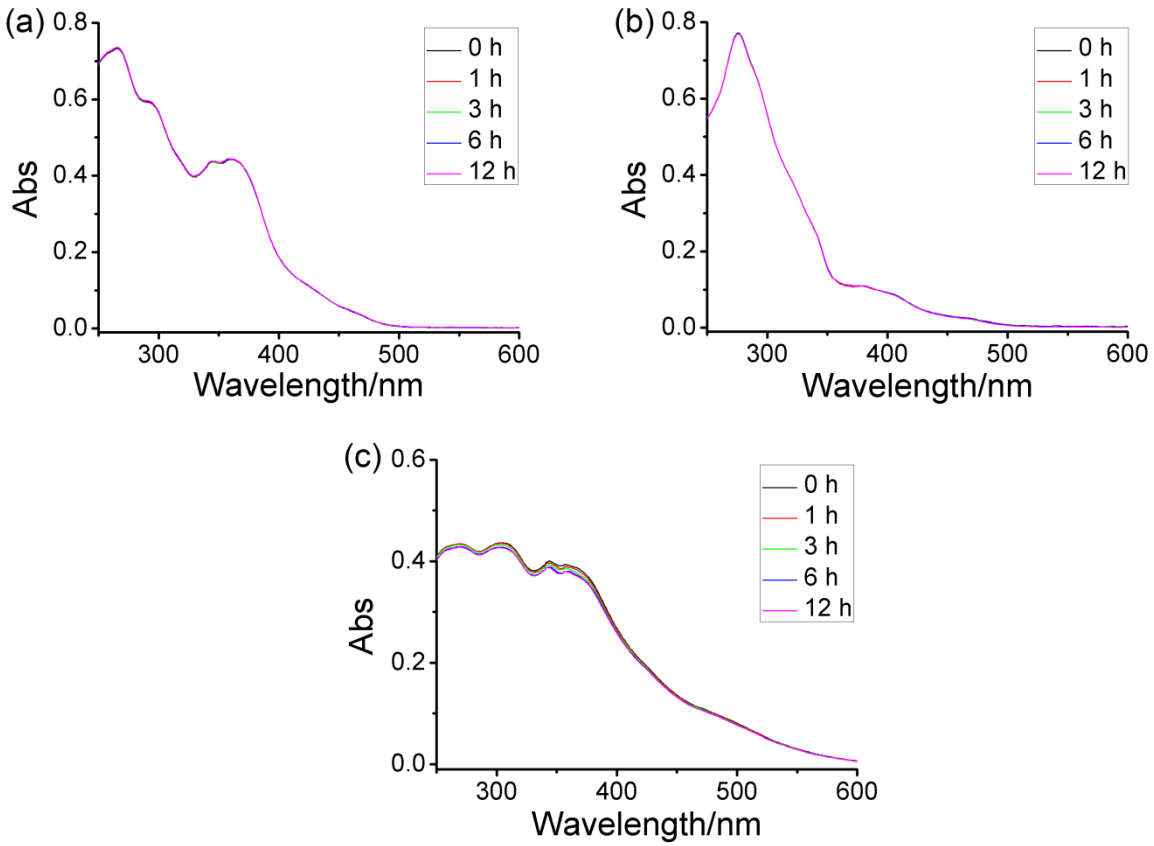


Fig. S9 Time-dependent UV/vis absorption spectra of (a) **Ir-PAG**, (b) **Ir-PH** and (c) **Ir-SPH** (10 μM) measured in PBS/DMSO (4:1, v/v) at 298 K.

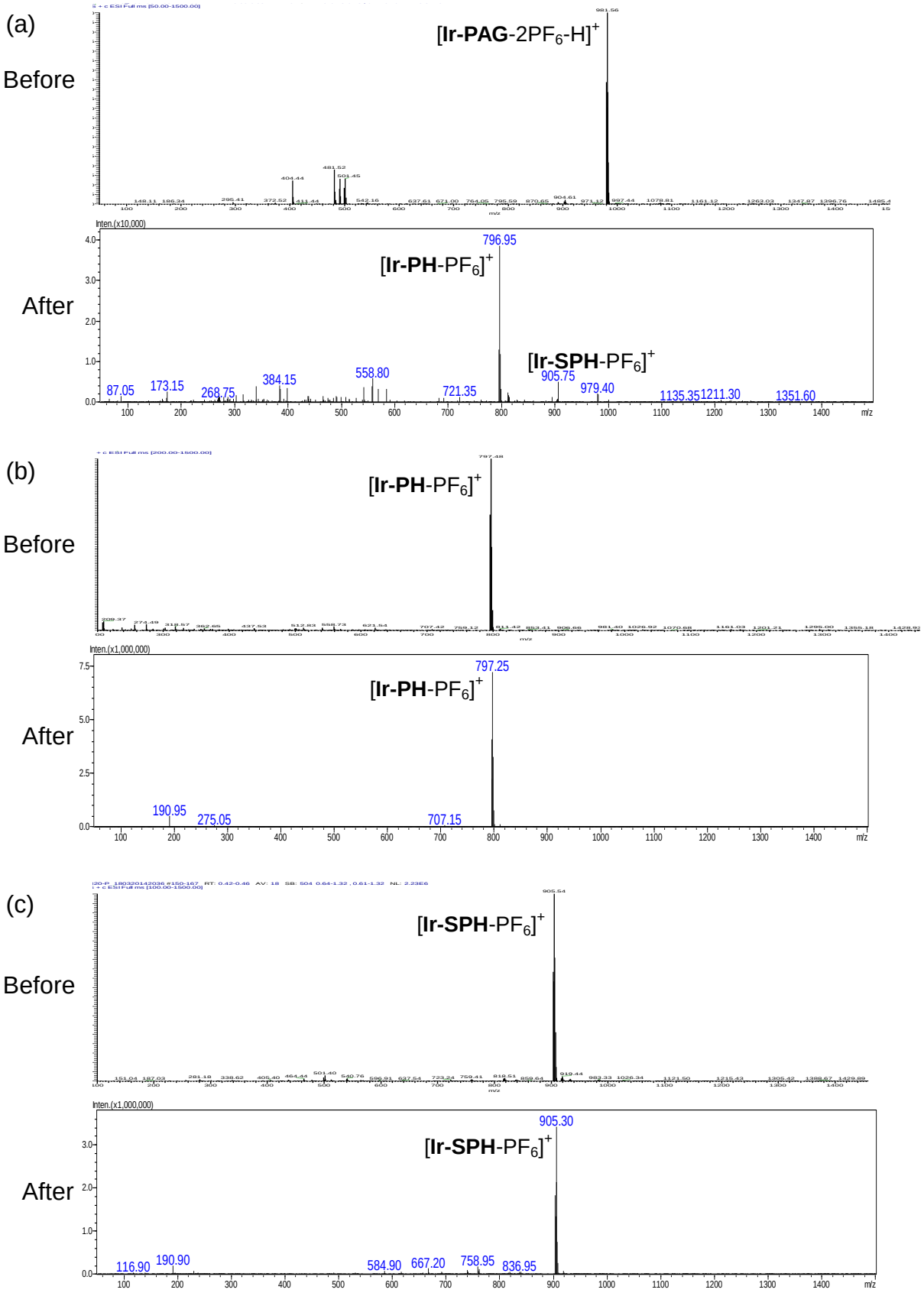


Fig. S10 ESI-MS spectra of (a) **Ir-PAG**, (b) **Ir-PH** and (c) **Ir-SPH** (500 μM) in DMSO-d_6 before and after light irradiation (425 nm, 40 mW·cm^{-2}) for 30 min.

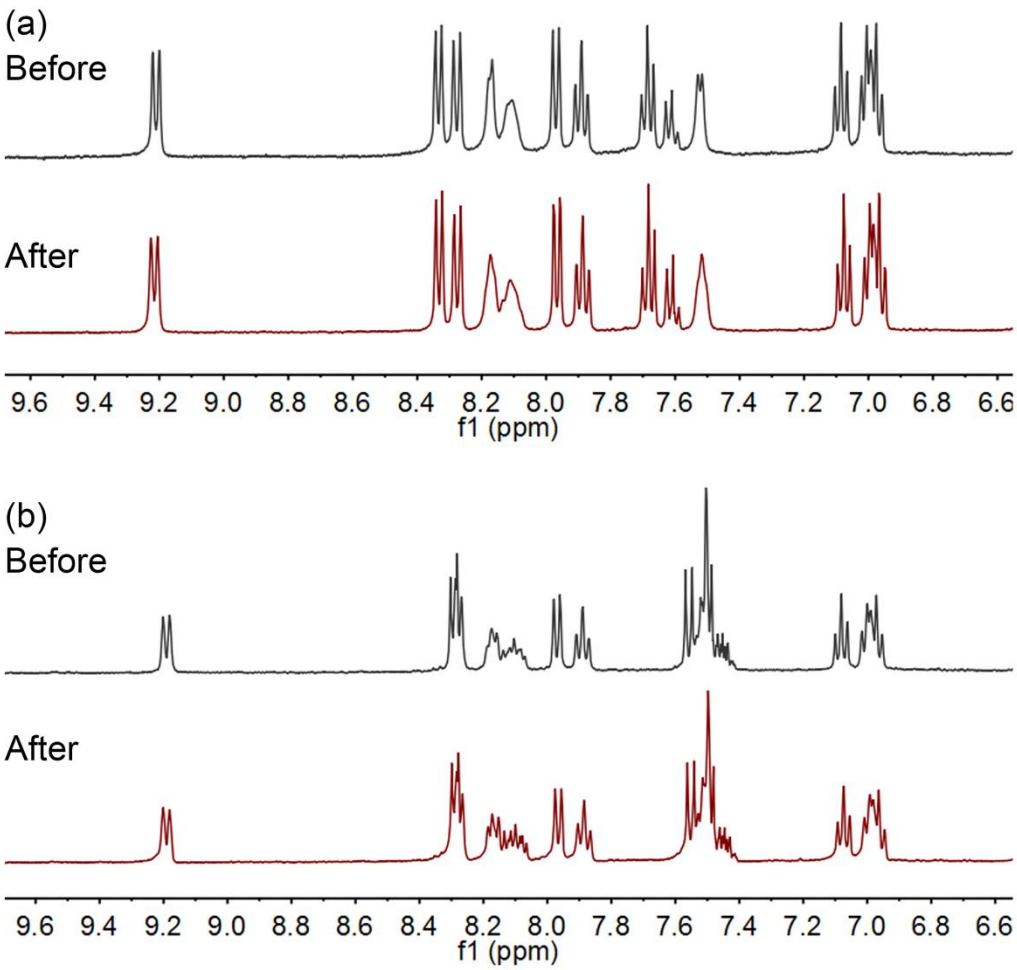


Fig. S11 ^{1}H NMR spectra of (a) **Ir-PH** and (b) **Ir-SPH** (500 μM) in DMSO-d_6 before and after light irradiation (425 nm, 40 mW·cm^{-2}) for 30 min.

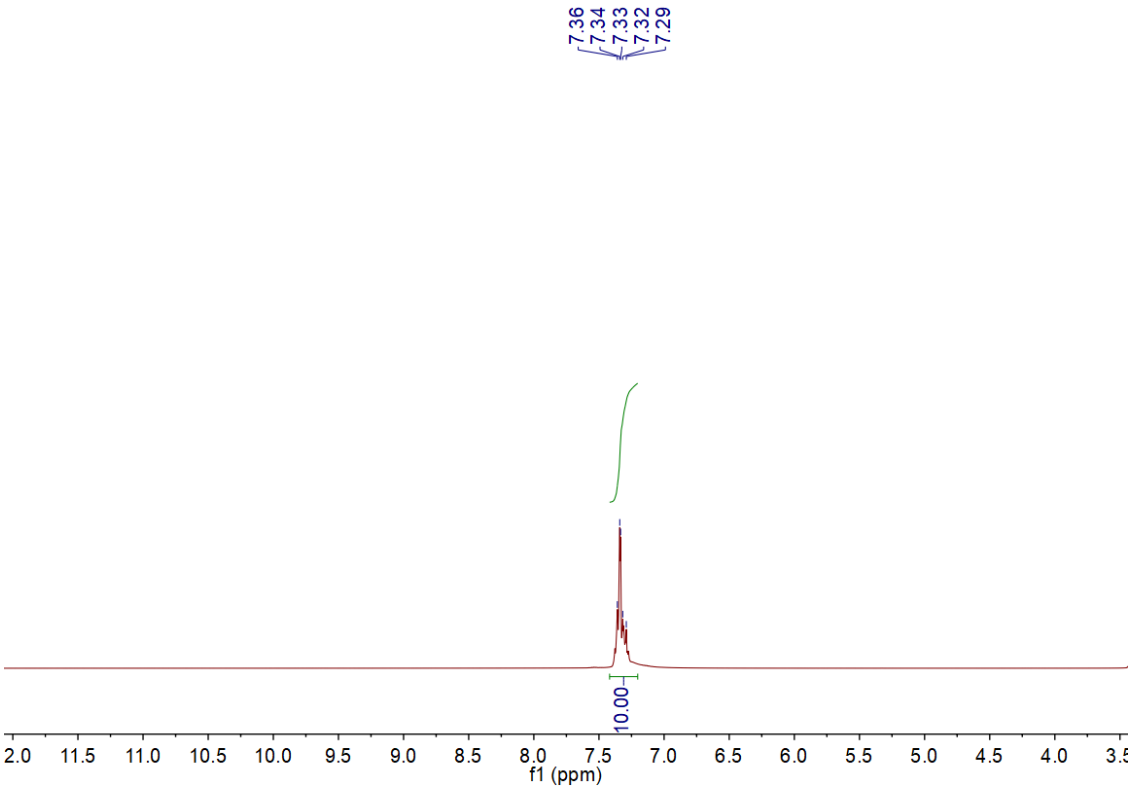


Fig. S12 ^{1}H NMR spectrum of diphenyl sulfide in DMSO-d_6 at 298 K.

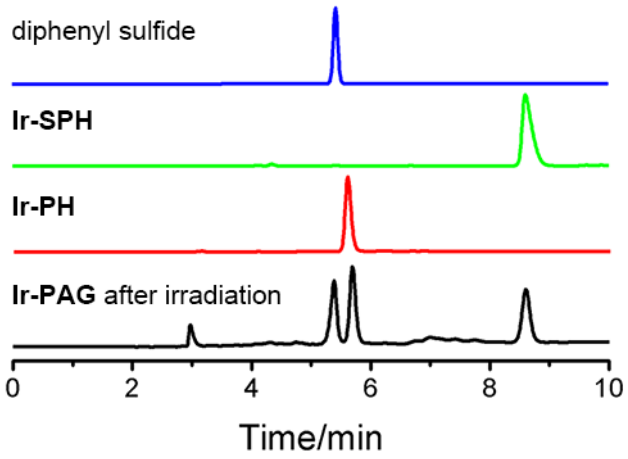


Fig. 13 Comparison of the HPLC retention times of **Ir-PH**, **Ir-SPH** and diphenyl sulfide with the photolysis products of **Ir-PAG**.

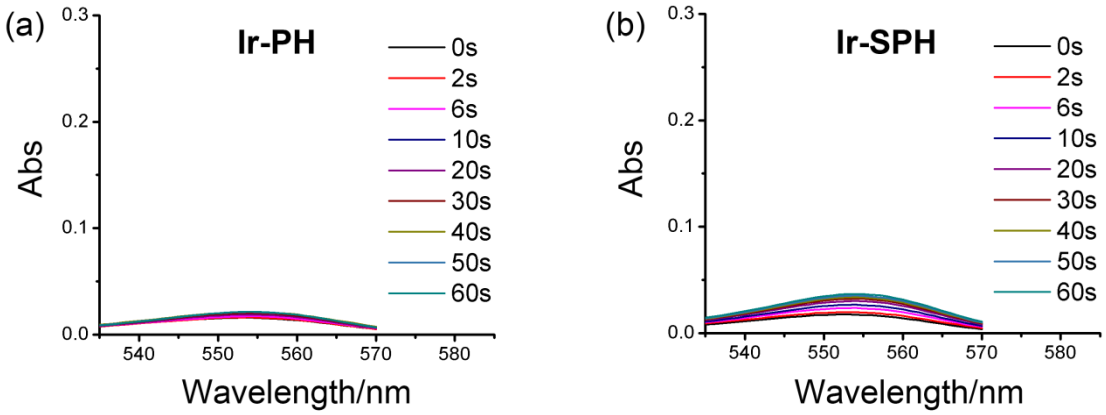


Fig. S14 Absorption spectra of rhodamine B base (10 μM) in CH_2Cl_2 mixed with (a) **Ir-PH** and (b) **Ir-SPH** (1 μM) upon light irradiation (425 nm, 2 mW·cm^{-2}) for different time intervals.

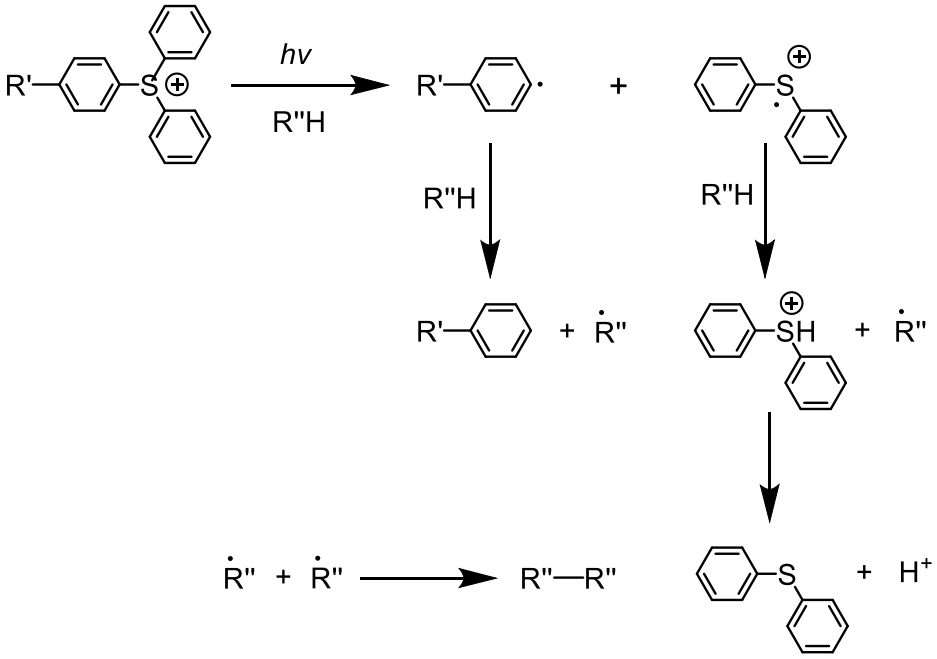


Fig. S15 The proposed mechanism for **Ir-PAG** to generate photoacid.[5] R′ represents the Ir(III)-containing fragment of **Ir-PAG**, and R″ represents solvent.

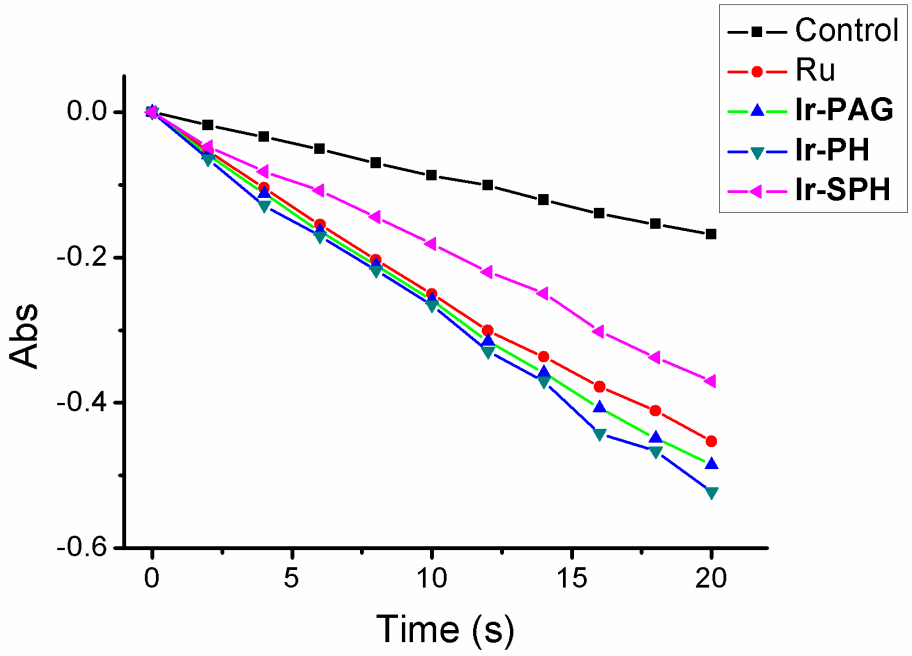


Fig. S16 Detection of $^1O_2$ production via changes in the absorbance of DPBF at 418 nm versus irradiation time in the presence of the tested complexes in aerated methanol. $[Ru(bpy)_3]Cl_2$ (Ru) was used as the reference in the Φ_Δ measurements (0.81 in aerated methanol)[3].

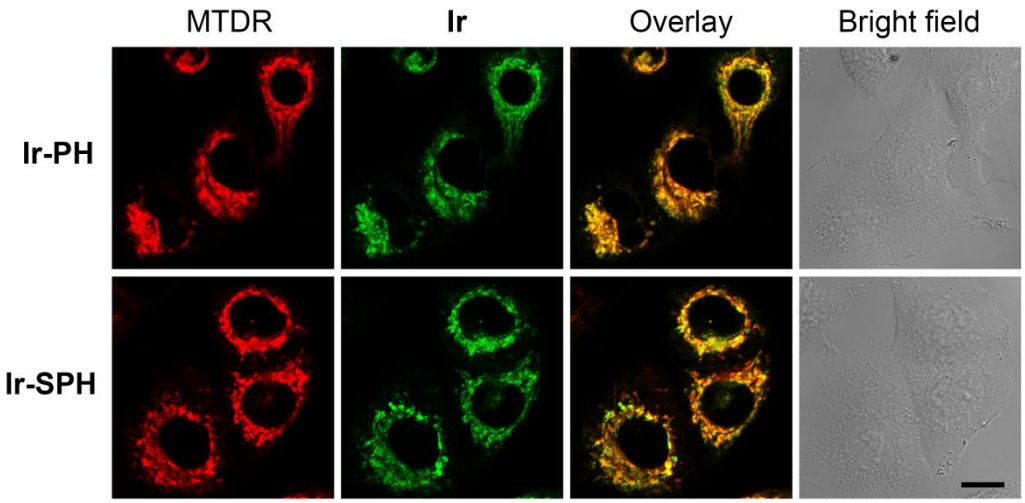


Fig. S17 CLSM co-localization images of **Ir-PH** and **Ir-SPH** (10 μM, 1 h) with MitoTracker Deep Red (MTDR; 150 nM, 30 min). λ_{ex} = 633 nm (MTDR) and 405 nm (**Ir-PH** and **Ir-SPH**); λ_{em} = 600 ± 20 nm (**Ir-PH** and **Ir-SPH**) and 670 ± 20 nm (MTDR). All images share the same scale bar, 20 μm.

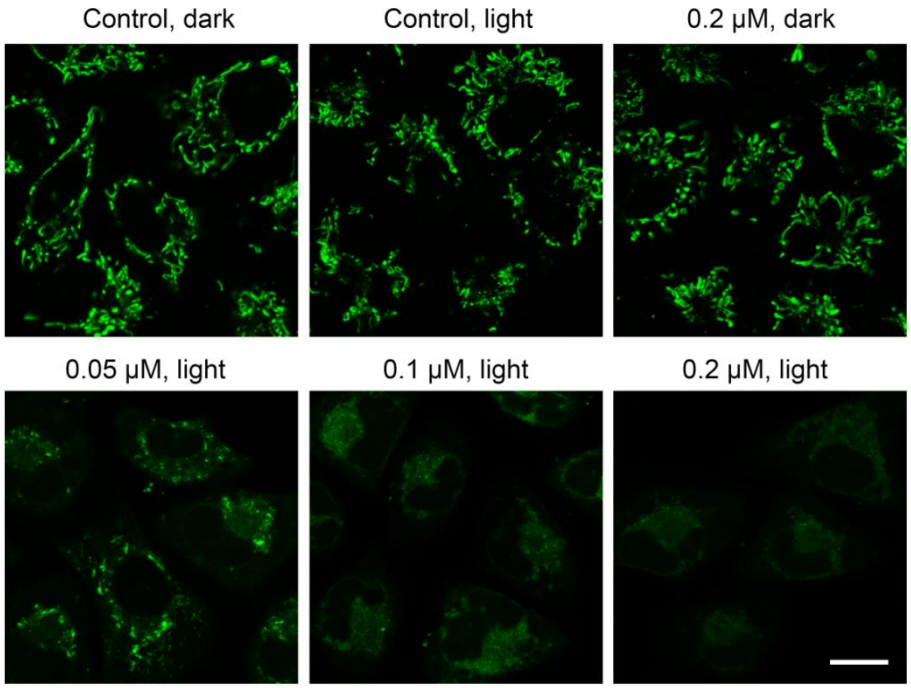


Fig. S18 Effects of **Ir-PAG**-mediated PDT on MMP analyzed by confocal microscopy. A549 cells were irradiated with an LED light source (40 mW·cm^{-2}) at a wavelength of 425 nm for 15 min, and then stained with rhodamine 123. All images share the same scale bar, 20 μm.

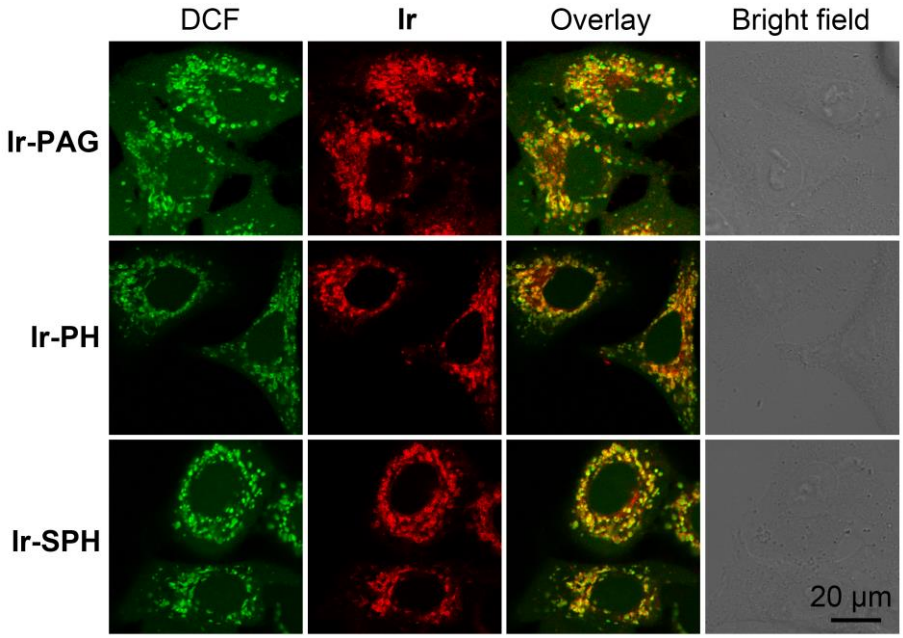


Fig. S19 Cellular ROS detection after A549 cells were incubated with 1 μM **Ir-PAG**, **Ir-PH** and **Ir-SPH** for 6 h and labeled with H_2DCF-DA for 20 min. Cells were then irradiated with light (425 nm, 40 mW·cm^{-2}) for 30 s, and subjected to CLSM immediately. λ_{ex} = 488 nm (DCF) and 405 nm (**Ir**); λ_{em} = 520 ± 20 nm (DCF) and 600 ± 20 nm (**Ir**). Scale bars, 20 μm.

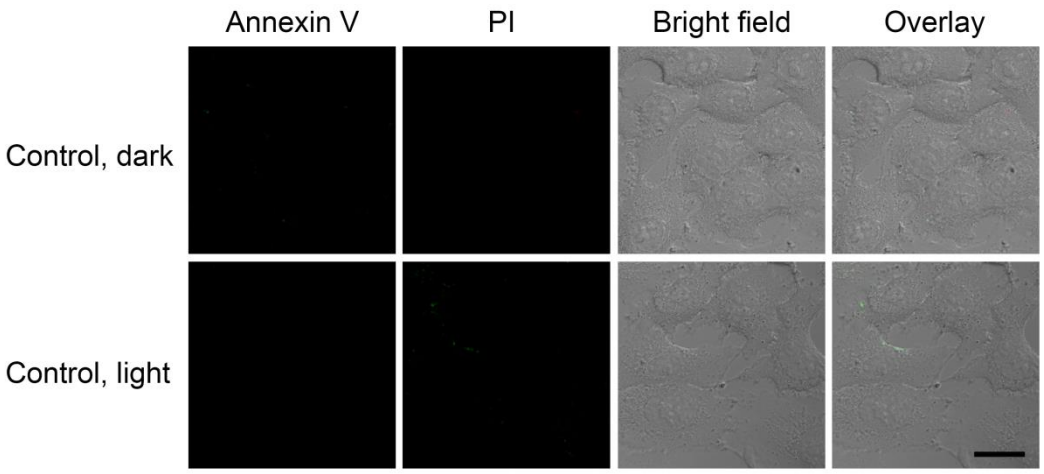


Fig. S20 Detection of cell death in A549 cells co-stained with annexin V-FITC and PI by confocal microscopy. Control cells were incubated in the dark or irradiated with light (425 nm, 40 mW·cm^{-2}, 15 min). λ_{ex} = 488 nm (annexin V-FITC and PI); λ_{em} = 520 ± 20 nm (annexin V-FITC) and 620 ± 20 nm (PI). All images share the same scale bar, 20 μm.

Table S1 Photophysical data of **Ir-PAG**, **Ir-PH** and **Ir-SPH**

Complex	Medium	λ_{em}[a]/nm	Φ[b]	τ_0[c]/ns
	CH_2Cl_2	578	0.132	237
Ir-PAG	CH_3CN	600	0.065	90
	PBS	600	0.018	386
	CH_2Cl_2	579	0.193	212
Ir-PH	CH_3CN	596	0.154	79
	PBS	601	0.011	309
	CH_2Cl_2	582	0.118	247
Ir-SPH	CH_3CN	597	0.158	82
	PBS	604	0.014	362

[a] Emission maximum, λ_{ex} = 405 nm. [b] The emission quantum yields were determined using $[Ru(bpy)_3]Cl_2$ in N_2-saturated CH_2Cl_2 (Φ = 0.059),[6] CH_3CN (Φ = 0.062)[7] and PBS (Φ = 0.042)[8] as the references. [c] The lifetimes were measured at the emission maxima.

Table S2 (Photo)cytotoxicity of the tested complexes toward A549R and HLF cells under normoxic conditions.

Complex	IC_{50} (μM)			
	A549R		HLF	
	Dark[a] (Light)[b]	PI[c]	Dark[a] (Light)[b]	PI[c]
Ir-PAG	17.5±1.3 (0.030±0.004)	583	50.1±3.5 (0.26±0.06)	193
Ir-PH	8.9±0.6 (0.036±0.003)	247	10.8±1.4 (0.19±0.05)	56.8
Ir-SPH	7.6±0.7 (0.018±0.003)	422	17.8±2.8 (0.18±0.03)	98.9

[a] Cells were treated with the tested complexes for 48 h. [b] Cells were treated with the tested complexes for 12 h before irradiation. [c] PI = IC_{50}(dark)/IC_{50}(light).

Supporting references

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