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Supporting Information

Ruthenium(II) complexes as bioorthogonal two-photon photosensitizers for tumour-specific photodynamic therapy against triple-negative breast cancer cells

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Experimental Section

Supporting figures and Tables

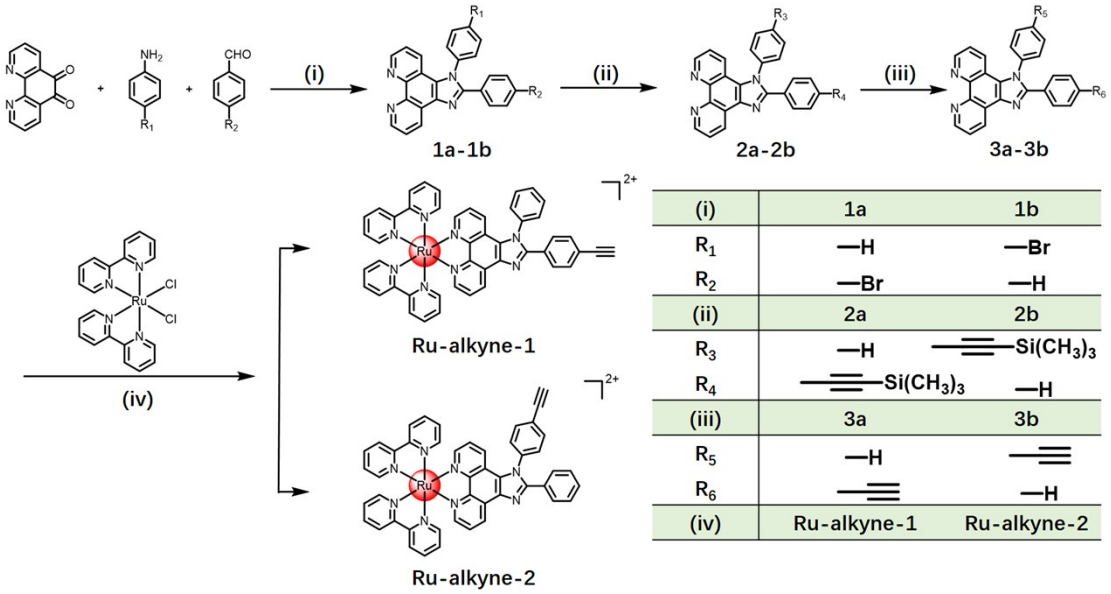
Materials and instruments

Unless otherwise noted, all chemical reagents and solvents were commercially available and used without further purification. Double distilled (DD) water was used throughout all of the experiments. 1,10-Phenanthroline-5,6-dione were purchased from Energy Chemical. 1,3-diphenyliso-benzofuran (DPBF), PBS, sodium pyruvate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Rhodamine B, agarose, Ac_4ManNAz, tris(3-hydroxypropyltriazolylmethyl) amine (THPTA), 2',7'-dichlorofluorescin diacetate (DCFH-DA) and Lactate Colorimetric Assay Kit were purchased from Sigma-Aldrich and used without further purification. Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, DiO (membrane dye), Hoechst 33342 (nuclear dye) were purchased from Invitrogen. Electrospray ionization mass spectra (ES-MS) were recorded on a LCQ system (Finnigan MAT, USA). Microanalysis (C, H, and N) was carried out using a Perkin-Elmer 240Q elemental analyzer. The ^{1}H NMR spectra were recorded on a Bruker advance III (500 MHz) Nuclear Magnetic Resonance Spectrometry. High-resolution electrospray ionization mass spectra (HR-MS) were recorded by Orbitrap LC/MS (Q Exactive, Thermo, German). The UV-Vis spectra were recorded on a Varian Cary 300 spectrophotometer. LC-MS chromatography was measured on LC-MS 2020 (Shimadzu, Japan) with C18 column (Shim-pack GIST C18, 5um, 4.6×250). Emission spectra were recorded on a PerkinElmer LS 55 fluorescence spectrometer at room temperature. Two-photon absorption cross-section measurements were performed by a modelocked Ti: Sapphire laser (Coherent, USA) with a repetition rate of 80 MHz, and a femtosecond optical parametric amplifier (spectral tuning range 720–840 nm). Luminescence lifetime studies were performed with an Edinburgh FLSP-920 photon-counting system using a pulse laser (450 nm) as the excitation source. Luminescence quantum yields of iridium complexes in MeOH solution were measured with reference to $[Ru(bpy)_3]^{2+}$ (Φ_{PL} = 0.042, aerated MeOH, 25 °C). Cell imaging was conducted on a LSM 880 (Carl Zeiss, Germany) Laser Scanning Confocal Microscope. Visible one-photon irradiation (λ_{irr} = 450 nm) in PDT was provided by a commercially available LED visible area light source (Height LED Instruments, China).

Synthesis and characterization

$Ru(bpy)_2Cl_2$[1] and 2-(4-bromophenyl)-1-phenyl-1H-imdazo[4,5-f][1,10]phenanthroline (1a)[2]

were prepared according to previous methods. Synthetic routes of the main ligands, **Ru-alkyne-1** and **Ru-alkyne-2** were described in **Scheme S1**.



(i)	1a	1b
R_1	—H	—Br
R_2	—Br	—H
(ii)	2a	2b
R_3	—H	—Si(CH$_3$)$_3$
R_4	—Si(CH$_3$)$_3$	—H
(iii)	3a	3b
R_5	—H	
R_6		—H
(iv)	Ru-alkyne-1	Ru-alkyne-2

Scheme S1 Synthetic route of **Ru-alkyne-1** and **Ru-alkyne-2**. (i) CH_3COOH, reflux overnight. (ii) TMSA, DMF/N(Et)$_3$ (5:1), CuI, Pd(Ph$_3$P)$_4$, Ar, 100 °C, overnight. (iii) MeOH, K_2CO_3, r.t., 1 hr. (iv) EtOH/H_2O (5:1), Ar, 85 °C, 8 hrs.

Synthesis of 1-phenyl-2-(4-((trimethylsilyl)ethynyl)phenyl)-1H-imidazo[4,5-f][1,10]phenanthroline (2a)

The compound was synthesized according to the modified literature[3]. A mixture of 2-(4-bromophenyl)-1-phenyl-1H-imdazo[4,5-*f*][1,10]phenanthroline (450 mg, 1 mmol), excess trimethylsilylacetylene (1.5 mL) in trimethylamine and dimethylformamide mixture (v/v, 1/4, 25 mL) was degassed by three freeze-pump-thaw cycles. Tetrakis(triphenylphosphine)palladium (58 mg, 5 % mmol) and copper iodide (20 mg, 10 % mmol) were then added. The reaction mixture was stirred overnight at 100 °C under an argon atmosphere. After the reaction, the mixture was cooled to room temperature, filtered, and evaporated. The product was extracted with dichloromethane (DCM, 3 × 75 mL). The organic extract was washed with aqueous ammonium chloride (2 × 75 mL), water (3 × 100 mL), and dried over anhydrous sodium sulfate. After evaporating the solvent, the dark residue was purified by flash column chromatography (100 - 200 mesh of silica gel, eluent solution from 100:1 to 50:1 of DCM and MeOH mixture). The blue fluorescent band was collected and evaporated to obtain a yellow powder, 350 mg. Yield: 75%. Anal. Calcd for $C_{30}H_{24}N_4Si$(%): C,

76.89; H, 5.16; N, 11.96. Found: C,76.85; H, 5.19; N, 11.94. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.21 (dd, *J* = 4.4, 1.7 Hz, 1H), 9.14 (dd, *J* = 8.1, 1.8 Hz, 1H), 9.06 (dd, *J* = 4.3, 1.6 Hz, 1H), 7.77 (dd, *J* = 8.1, 4.4 Hz, 1H), 7.69 – 7.63 (m, 3H), 7.55 – 7.48 (m, 4H), 7.45 (dd, *J* = 8.6, 1.9 Hz, 1H), 7.41 (d, *J* = 8.4 Hz, 2H), 7.30 (dd, *J* = 8.4, 4.3 Hz, 1H), 0.24 (s, 9H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 151.44, 148.93, 148.04, 144.68, 144.09, 137.81, 136.20, 132.13, 132.06, 131.91, 130.79, 130.63, 130.44, 129.61, 128.98, 128.70, 128.55, 128.45, 128.10, 127.08, 124.04, 123.96, 123.60, 122.26, 119.80, 104.31, 96.47, 29.70.HR-ESI-MS (CH_3OH), Calcd: m/z =469.18430 $[M + H^+]^+$, Found:469.18188.

Synthesis of 2-(4-ethynylphenyl-1H- imidazo[4,5-f][1,10] phenanthroline (3a)

1-phenyl-2-(4-((trimethylsilyl)phenyl)-1H-imidazo[4,5-f][1,10]phenanthroline (200 mg) and potassium carbonate (100 mg) were added to 10 mL of methanol and then were stirred at room temperature for 1 hour. The reaction mixture was filtered and washed with DCM twice. The filtrate was evaporated under reduced pressure and purified by flash column chromatography (100 - 200 mesh of silica gel, eluent solution is 50:1 of DCM and MeOH mixture) to afford yellow solid, 152 mg. Yield: 90%. Anal. Calcd for $C_{27}H_{16}N_4$(%): C, 81.80; H, 4.07; N, 14.13. Found: C,81.76; H, 4.12; N, 14.12.^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.21 (dd, *J* = 4.4, 1.8 Hz, 1H), 9.14 (dd, *J* = 8.1, 1.8 Hz, 1H), 9.06 (dd, *J* = 4.3, 1.7 Hz, 1H), 7.77 (dd, *J* = 8.1, 4.4 Hz, 1H), 7.72 – 7.64 (m, 3H), 7.59 – 7.51 (m, 4H), 7.43 (dt, *J* = 6.4, 2.1 Hz, 3H), 7.30 (dd, *J* = 8.5, 4.4 Hz, 1H), 3.15 (s, 1H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 151.22, 148.98, 148.07, 144.74, 144.14, 137.79, 136.22, 132.10, 130.75, 130.68, 130.51, 130.06, 129.03, 128.69, 128.08, 127.12, 123.95, 123.60, 123.03, 122.26, 119.80, 82.99, 79.02. HR-ESI-MS (CH_3OH), Calcd: m/z =397.14477 $[M + H^+]^+$, Found:397.14242.

Synthesis of Ru-alkyne-1

Ligand 1 (50 mg) and $Ru(bpy)_2Cl_2$ (51 mg) were mixed in 20 mL ethanol and water (v/v = 5:1) solution. The mixture was refluxed at 85 °C under argon atmosphere shielding light for 8 hours to give rise to an orange solution and then was evaporated under reduced pressure to dryness. The crude product was purified by flash column chromatography (100 - 200 mesh of neural Al_2O_3) using methanol and acetonitrile mixture (v/v = 1:10) as eluent solution to collect orange band, which was evaporated to obtain the orange powder, 94 mg. Yield: 85%. Anal. Calcd for $C_{47}H_{32}N_8Cl_2Ru$ (%): C, 64.09; H, 3.76; N, 12.62. Found: C, 64.06; H, 3.40; N, 12.61.^{1}H NMR (500 MHz, DMSO-d_6) δ 9.22

(dd, *J* = 8.3, 1.3 Hz, 1H), 8.98 - 8.91 (m, 4H), 8.26 – 8.18 (m, 2H), 8.17 – 8.09 (m, 3H), 8.06 – 7.98 (m, 2H), 7.85 (d, *J* = 5.6 Hz, 1H), 7.84 – 7.70 (m, 6H), 7.68 – 7.57 (m, 7H), 7.53 (d, *J* = 8.2 Hz, 2H), 7.46 – 7.42 (m, 1H), 7.41 – 7.36 (m, 2H), 4.44 – 4.37 (m, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 157.20, 157.13, 157.03, 153.24, 151.84, 151.22, 150.52, 146.02, 145.83, 138.38, 136.86, 136.65, 132.27, 131.73, 131.36, 131.05, 129.83, 129.75, 129.19, 128.41, 128.28, 127.51, 126.31, 125.92, 125.05, 123.69, 121.88, 118.55, 83.58, 83.15.HR-ESI-MS (CH_3OH), Calcd: m/z =405.08912 [M - $2Cl^-$]$^{2+}$, Found:405.08714.

Synthesis of 1-(4-bromophenyl)-2-phenyl-1H-imidazo[4,5-f][1,10]phenanthroline (1b)

This compound was synthesized using the same procedure as 2-(4-bromophenyl)-1-phenyl-1H-imdazo[4,5-f][1,10]phenanthroline, using benzaldehyde and 4-bromoaniline instead of 4-bromobenzaldehyde and aniline, respectively. A pink powder was obtained as the product, 1674 mg. Yield: 93%. Anal. Calcd for $C_{25}H_{15}BrN_4$(%): C, 66.53; H, 3.35; N, 12.41. Found: C, 66.51; H, 3.38; N, 12.39 ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.21 (dd, *J* = 4.4, 1.8 Hz, 1H), 9.14 (dd, *J* = 8.1, 1.8 Hz, 1H), 9.08 (dd, *J* = 4.3, 1.7 Hz, 1H), 7.77 (dd, *J* = 8.3, 4.6 Hz, 3H), 7.58 – 7.53 (m, 2H), 7.50 (dd, *J* = 8.4, 1.7 Hz, 1H), 7.44 – 7.41 (m, 2H), 7.40 – 7.34 (m, 4H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 152.31, 148.96, 148.06, 144.57, 143.99, 136.88, 136.19, 133.79, 130.87, 130.39, 129.57, 129.46, 129.39, 128.56, 127.88, 126.63, 124.41, 123.91, 123.66, 122.34, 119.63. HR-ESI-MS (CH_3OH), Calcd: m/z =451.05529 $[M + H]^+$, Found:451.05013.

Synthesis of 2-phenyl-1-(4-((trimethylsiyl)ethynyl)phenyl)-1H-imidazo[4,5-f][1,10]phenanthroline (2b)

This compound was synthesized using the same procedure as **1b**, using 1-(4-bromophenyl)-2-phenyl-1H-imidazo[4,5-f][1,10]phenanthroline instead of 1-phenyl-2-(4-((trimethylsilyl)phenyl)-1H-imidazo[4,5-f][1,10]phenanthroline. A grey powder was collected and purified by column chromatography, 328 mg. Yield: 70%. Anal. Calcd for $C_{30}H_{24}N_4Si$ (%): C, 76.89; H, 5.16; N, 11.96. Found: C, 76.85; H, 5.18; N, 11.99. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.26 (dd, *J* = 4.5, 1.7 Hz, 1H), 9.20 (dd, *J* = 8.1, 1.7 Hz, 1H), 9.10 (dd, *J* = 4.4, 1.6 Hz, 1H), 7.82 (dd, *J* = 8.1, 4.4 Hz, 1H), 7.76 – 7.71 (m, 2H), 7.58 – 7.55 (m, 2H), 7.53 (dd, *J* = 8.4, 1.6 Hz, 1H), 7.50 – 7.46 (m, 2H), 7.38 – 7.33 (m, 4H), 0.32 (s, 9H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 152.42, 148.51, 148.00, 143.86, 143.24,

137.46, 136.11, 134.03, 131.48, 129.56, 129.46, 129.33, 128.67, 128.53, 128.26, 126.82, 125.44, 124.08, 123.72, 122.60, 119.84, 103.13, 97.76, 46.13, 8.66.HR-ESI-MS (CH_3OH), Calcd: m/z =469.18430 $[M + H]^+$, Found:.469.18192.

Synthesis of 1-(4-ethynlphenyl)-2-phenyl-1H-imidazo[4,5-f][1,10]phenanthroline (3b)

This compound was synthesized similar to that of **3a**, using 2-phenyl-1-(4-((trimethylsiyl)ethynyl)phenyl)-1H-imidazo[4,5-f][1,10]phenanthroline instead of 1-phenyl-2-(4-((trimethylsilyl)phenyl)-1H-imidazo[4,5-f][1,10]phenanthroline. A pink solid was obtained, 149 mg. Yield: 88%. Anal. Calcd for $C_{27}H_{16}N_4$ (%):C, 81.80; H, 4.07; N, 14.13. Found: C, 81.78; H, 4.09; N, 14.13. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.20 (dd, *J* = 4.4, 1.8 Hz, 1H), 9.14 (dd, *J* = 8.1, 1.8 Hz, 1H), 9.07 (dd, *J* = 4.3, 1.7 Hz, 1H), 7.78 – 7.72 (m, 3H), 7.57 – 7.54 (m, 2H), 7.52 – 7.47 (m, 3H), 7.38 – 7.31 (m, 4H), 3.30 (s, 1H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 152.22, 148.99, 148.06, 144.68, 144.13, 138.02, 136.27, 134.16, 130.77, 129.57, 129.50, 129.35, 128.87, 128.52, 127.94, 126.69, 124.38, 123.95, 123.60, 122.28, 119.66, 82.06, 80.03.HR-ESI-MS (CH_3OH), Calcd: m/z =397.14477 $[M + H^+]^+$, Found:397.14250.

Synthesis of Ru-alkyne-2

Ru-alkyne-2 was synthesized using the same procedure of **Ru-alkyne-1,** except that **3b** was used instead of **3a**, 91 mg. Yield: 82%. Anal. Calcd for $C_{47}H_{32}N_8Cl_2Ru$(%): C, 64.09; H, 3.76; N, 12.62.Found: C, 64.06; H, 3.78; N, 12.60.^{1}H NMR (500 MHz, DMSO-d_6) δ 9.20 (dd, *J* = 8.3, 1.4 Hz, 1H), 8.96 – 8.85 (m, 4H), 8.21 (dt, *J* = 16.1, 8.0 Hz, 2H), 8.16 – 8.08 (m, 3H), 8.06 – 8.03 (m, 1H), 7.99 – 7.97 (m, 1H), 7.86 – 7.77 (m, 6H), 7.71 – 7.66 (m, 1H), 7.63 – 7.53 (m, 7H), 7.47 -7.43 (m, 3H), 7.39 – 7.35 (m, 2H), 4.60 – 4.43 (m, 1H).^{13}C NMR (126 MHz, DMSO-d_6) δ 157.21, 157.12, 157.05, 154.25, 151.82, 151.18, 150.52, 145.87, 145.77, 138.38, 137.17, 136.65, 134.44, 131.04, 130.55, 129.81, 129.39, 129.06, 128.62, 128.40, 128.27, 128.14, 127.48, 126.48, 125.91, 125.05, 124.77, 121.83, 84.05, 82.82.HR-ESI-MS (CH_3OH), Calcd: m/z = 405.08912 $[M - 2Cl^-]^{2+}$, Found:405.08710.

Synthesis of Ru-triazole-2

Ru-alkyne-2 (44 mg, 0.05 mmol) and (azidomethyl)benzene (20 mg, 0.15 mmol) were dissolved in

5 mL ethanol and water mixture (1:1, v/v), followed by 5% $CuSO_4$ and ascorbate sodium (1:2) under argon. After stirred at 37 °C for 2 h, the reaction mixture was evaporated under reduced pressure and allowed to be purified by flash column chromatography (100-200 mesh of neural Al_2O_3) using methanol and acetonitrile mixture (v/v = 1:10) as the eluent solution. The orange band was collected. After evaporating, an orange powder was obtained, 46 mg. Yield: 90%. Anal. Calcd for $C_{54}H_{39}Cl_2N_{11}Ru$ (%): C, 63.97; H, 3.88; N, 15.20. Found: C, 63.93; H, 3.93; N, 15.17. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.21 (dd, *J* = 8.3, 1.4 Hz, 1H), 8.97 – 8.83 (m, 5H), 8.25 – 8.09 (m, 7H), 8.04 – 7.97 (m, 2H), 7.87 – 7.82 (m, 3H), 7.80 (dt, *J* = 5.5, 1.1 Hz, 1H), 7.68 – 7.62 (m, 3H), 7.62 – 7.54 (m, 5H), 7.46 – 7.33 (m, 10H), 5.70 (s, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 171.91, 157.21, 157.12, 157.03, 154.25, 151.84, 151.12, 150.46, 145.89, 145.78, 138.37, 136.60, 136.38, 136.18, 133.46, 131.05, 130.49, 129.86, 129.77, 129.51, 129.32, 129.05, 128.74, 128.59, 128.48, 128.39, 128.25, 127.54, 126.40, 125.94, 125.05, 123.30, 121.93, 53.66, 22.98.HR-ESI-MS (CH_3OH), Calcd: 471.62112 [M - $2Cl^-$]$^{2+}$, Found:471.61824.

Kinetic study on copper-catalyzed alkyne-azide cycloaddition (CuAAC) reaction

The reaction rate constants of cycloaddition of ruthenium(II) complexes containing the alkyne group and Ac_4ManNAz were calculated based on HPLC-MS analysis results according to the previous literature[4, 5]. Ruthenium-alkyne (50 μM) was reacted in the presence of 25 μM $CuSO_4$, 2.5 mM freshly prepared sodium ascorbate and 125 μM (THPTA) and various concentrations of Ac_4ManNAz(150, 200, 250 μM) at 37 °C for different time scale. The reaction was quenched with 10 mM ethylenediaminetetraacetic acid (EDTA). Detailed conditions of HPLC-MS are shown in **Table. S2.** Pseudo-first order rate constant kobs, was calculated for each concentration of Ac_4ManNAz by fitting the alkyne areas versus time using the following equation,

$$Y = a \times (1 - e^{kobs \times t})$$

Where Y is the alkyne area monitored by absorbance at 254 nm, and t is time. The pseudo-first-order rate constant, kobs, was then plotted against the concentration of Ac_4ManNAz to yield the second-order rate constant using the following equation,

$$kobs = k_2 \times c(Ac_4ManNAz).$$

Single oxygen generation in solution

The $^1O_2$ quantum yields[6] were measured by monitoring the photooxidation of DPBF (60 μM) in methanol in the presence of **Ru-triazole-2**. The $OD_{450\ nm}$ values of **Ru-triazole-2** and $[Ru(bpy)_3]^{2+}$ solutions were adjusted to ca. 0.10, respectively. The methanol solution with 1/$[Ru(bpy)_3]^{2+}$/methanol (control) and DPBF were fully aerated and were subjected LED irradiation (450 ± 10 nm, 10.9 mW cm^{-2}). The absorbance of the mixture at 411 nm was recorded with an interval of 2 seconds. $[Ru(bpy)_3]^{2+}$ was used as the reference for $^1O_2$ sensitization. The quantum yield of **Ru-triazole-2** was calculated by the following equations,

$$\Phi_\Delta^s = \Phi_\Delta^r \times (s^s \times F^r)/(s^r \times F^s)$$

wheres denotes the calibrated slope of a linear fit of the cumulative changes of absorbance at 411 nm vs. cumulative irradiation time. F represents the absorption correction factor. Superscript "s" stands for the sample, and "r" stands for the reference, i.e. $[Ru(bpy)_3]^{2+}$.

Electron spin resonance (ESR) assay

The ESR spectra were recorded at room temperature (25 °C) on a Bruker Model A300 spectrometer at 1 G field modulation and 100 G scan range with 20 mW microwave power. An area light source was used for irradiation for 240 s (450 ± 10 nm, 10.9 mW cm^{-2}). **Ru-triazole-2** (OD_{450} = 0.50) was dissolved in aerated methanol containing 100 mM TEMP as a $^1O_2$ spin trap. The mixed solution was raised into capillary tubes by siphon effect in the dark. The loaded capillary tubes were sintered at one terminal and subjected to irradiation. Then the measurement was carried out to plot the ESR spectra of the reaction product. $Ru(bpy)_3^{2+}$ (OD_{450} = 0.50) was used as standard.

Two-photon absorption cross-sections determination

The two-photon absorption cross-sections were determined according to an established method developed by Webb and Xu[7]. The two-photon-induced luminescence of **Ru-alkyne-2** and **Ru-triazole-2** were measured using quartz cuvettes in methanol solution. The samples were excited with laser pulses of 35 fs produced by the mode-locked Ti:Sapphire laser with a repetition rate of 1 kHz, and a femtosecond optical parametric amplifier was utilized. The emission from the methanol solution of 1 was collected at a 90-degree angle by a high numerical aperture lens and directed to an entrance slit of the spectrometer. Rhodamine B was employed as the reference. The quadratic dependence of the two-photon induced luminescence intensity on the excitation

power was verified at the corresponding wavelength of their respective maximal two-photon absorption cross-sections. The two-photon excitation ratios of the reference and sample can be expressed by the following formula:

$$\sigma_s = \sigma_r \frac{\Phi_r c_r I_s n_s}{\Phi_s c_s I_r n_r}$$

in which σ represents the two-photon absorption cross-sections, Φ stands for the quantum yield, c is the concentration, I is the integrated luminescence intensity, and n is the refractive index. Subscript "s" stands for sample, and "r" stands for reference, i.e. Rhodamine B.

Cell culture.

Human breast cancer MDA-MB-231 cells and normal breast cells MCF-10A were provided by American Type Culture Collection (ATCC). The cells were cultured in DMEM medium containing 10% heat-inactivated FBS and maintained in a humidified incubator with 5% CO_2 at 37 °C.

A general protocol for cell-surface labeling of azido glycan on live MDA-MB-231 cells and imaging by confocal microscopy.

MDA-MB-231 cells were seeded at 1×10^5 cells/mL confocal dishes and grown at 37 °C and 5% CO_2 in DMEM medium with 50 μM Ac_4ManNAz for 3 days. The medium was then gently aspirated, and the cells were washed twice with 1× PBS buffer. $CuSO_4$ (25 μM) and THPTA (125 μM) were added to the dishes in 1X PBS buffer containing **Ru-alkyne-2** (10 μM). A freshly prepared sodium ascorbate (100 mM) was added to establish a final concentration of 2.5 mM with gentle shaking. After incubation at room temperature for 1 hour, the cells were stained with Hoechst 33342 for 30 minutes or DiO for 20 minutes, following the standard protocols of the manufacturer. The cells were then washed twice with 1× PBS to be imaged by a confocal microscope. For **Ru-alkyne-2** detection, the excitation wavelength was 810 nm, and the emission filter was 590-630 nm, for Hoechst detection, the excitation wavelength was 405 nm and the emission filter was 430-470 nm and for DiO detection, the excitation wavelength was 488 nm and the emission was collected from 510 nm to 540 nm.

Flow Cytometry Study.

MDA-MB-231 cells in a 6-well plate were precultured overnight and incubated with 50 μM Ac_4ManNAz for 3 days. After the click reaction described above, the cells were washed with 1× PBS and treated with trypsin, washed with medium twice, and subjected to flow cytometry analysis using Cyan-LX (DakoCytomation). The mean fluorescence was determined by counting 10,000 events.

Cytotoxicity Studies.

MTT assays[8, 9] were used to assess the cell viability of MDA-MB-231 or MCF-10A cells after the click reaction with **Ru-alkyne-2** and further exposed with irradiation (λ = 810 nm). The cells in 96-well plates were first incubated with a medium containing 50 μM Ac_4ManNAz for 3 days. After the click reaction described above, then the cells were washed with 1× PBS twice and exposed to light irradiation (6 J cm^{-2}, 5 min). The cells were further incubated in a fresh medium for 48 h and washed with 1× PBS. Then MTT in 1× PBS solution (100 μL, 0.5 mg mL^{-1}) was added into each well. After incubation for 3 h, the supernatant was discarded and the precipitate was dissolved in DMSO (150 μL) with gentle shaking. The absorbance of MTT at 595 nm was monitored by the microplate reader. The cells without any treatment were used as control.

Detection of intracellular singlet oxygen

The intracellular singlet oxygen generation under two-photon laser excitation was measured by DCFH-DA as the singlet oxygen probe. The cells in confocal dishes were first incubated with a medium containing 50 μM Ac_4ManNAz for 3 days. After the click reaction described above, then the cells were washed with 1× PBS twice The cells were then incubated with PBS containing 10 μM DCFHDA at room temperature for 20 min. The culture medium was refreshed with fresh PBS and subjected to two-photon laser irradiation (810 nm, 6 J cm^{-2}). The fluorescent images were taken by an LSM 880 Carl Zeiss Laser Scanning Confocal Microscopy. For the DFH-DA channel, the excitation wavelength was 488 nm, and the emission filter was between 510-550 nm.

Membrane integrity assay

The fluorescent measure of the release of lactate dehydrogenase (LDH) from cells was used to assess the integrity of the damaged membrane resulting from the photodynamic therapy. The cells

in 96-well plates were first incubated with a medium containing 50 μM Ac_4ManNAz for 3 days. After the procedure of click reaction mentioned above, one of the assay plates was exposed to the irradiation of light source (810 nm, 6 J cm^{-2}) and another plate was kept in the dark. After that, both plates were removed from 37 °C incubator and equilibrate to 22 °C for 25 minutes. 100 μL CytoTox-ONE™ Reagent (Promega) was added to each well, shacked for 30 seconds, and then incubated at 22 °C for 10 minutes. The reaction was quenched by adding 50 μL Stop Solution (Promega). Fluorescence was measured by the microplate reader with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The cells without incubating with Ac_4ManNAz were used as control.

Live and dead cells staining assay

MDA-MB-231 cells were firstly seeded in 96 wells and then incubated with DMEM medium containing 50 μM Ac_4ManNAz for 3 days. After the click reaction described above, one of the assay plates was exposed to the irradiation of light source (810 nm, 6 J cm^{-2}) and another plate was kept in the dark. Calcein-AM (0.5 μM) and EthD-1 (2 μM) were added to each well for staining live and dead cells respectively.

Formation of 3D multicellular tumour spheroids (MCTSs)

The formation of MCTSs was conducted according to the previous protocols.[10] A suspension of 1.5 % agarose in PBS (w/v) was sterilized by a high-pressure steam sterilization pot for 20 min. The resulting gel was charged into 96-well micro-assay culture plates (50 μL/well) before cool down. The prepared plates were then exposed under UV irradiation for 3 h to ensure sterile. A suspension of MDA-MB-231 cells at 2×10^4 cells/mL was split charged into the prepared 96-well micro-assay culture plates with a volume of 200 μL/well. The culture medium was refreshed every two days. MDA-MB-231 MCTSs formed spontaneously in 3 days with a diameter of around 800 μm.

Z-stack imaging of 3D MCTSs

3D MCTSs pretreated with Ac_4ManNAz and conducted with click reaction, in the dark were carefully washed by PBS and subjected to CLSM for scanning. The one-/two-photon excited luminescence along the z-axis was collected and processed. The images were stacked in the z-stack

mode to give a final one-/two-photon z-axis stack imaging. For one-photon imaging, the excitation wavelength was 450 nm while the emission wavelength was 610 ± 20 nm. For two-photon imaging, the excitation wavelength was 810 nm and the emission filters were 610 ± 20 nm.

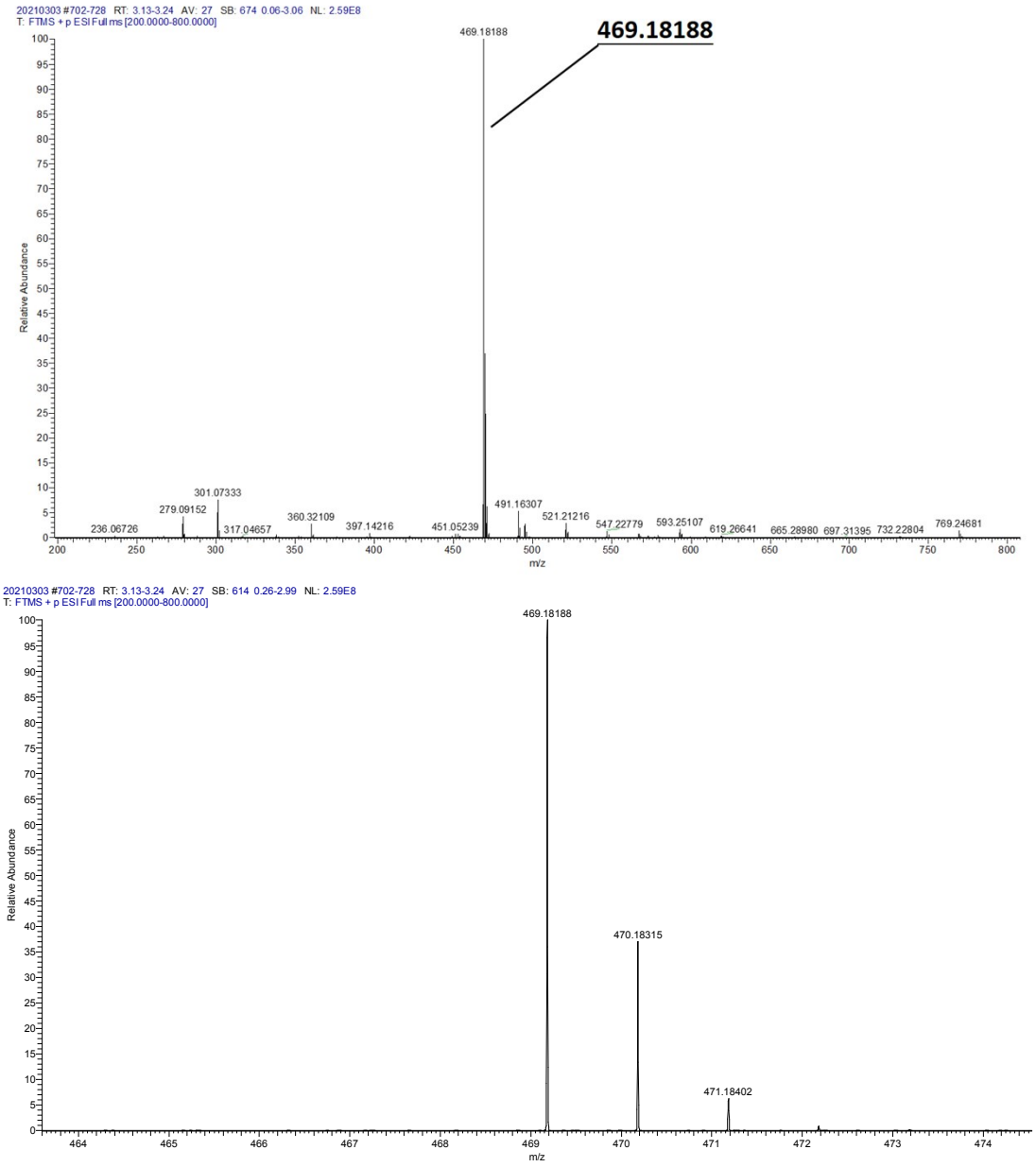


Fig. S1 HR-ESI-MS spectra of **1-phenyl-2-(4-((trimethylsilyl)ethynyl)phenyl)-1H-imidazo[4,5-f][1,10]phenanthroline (2a)**

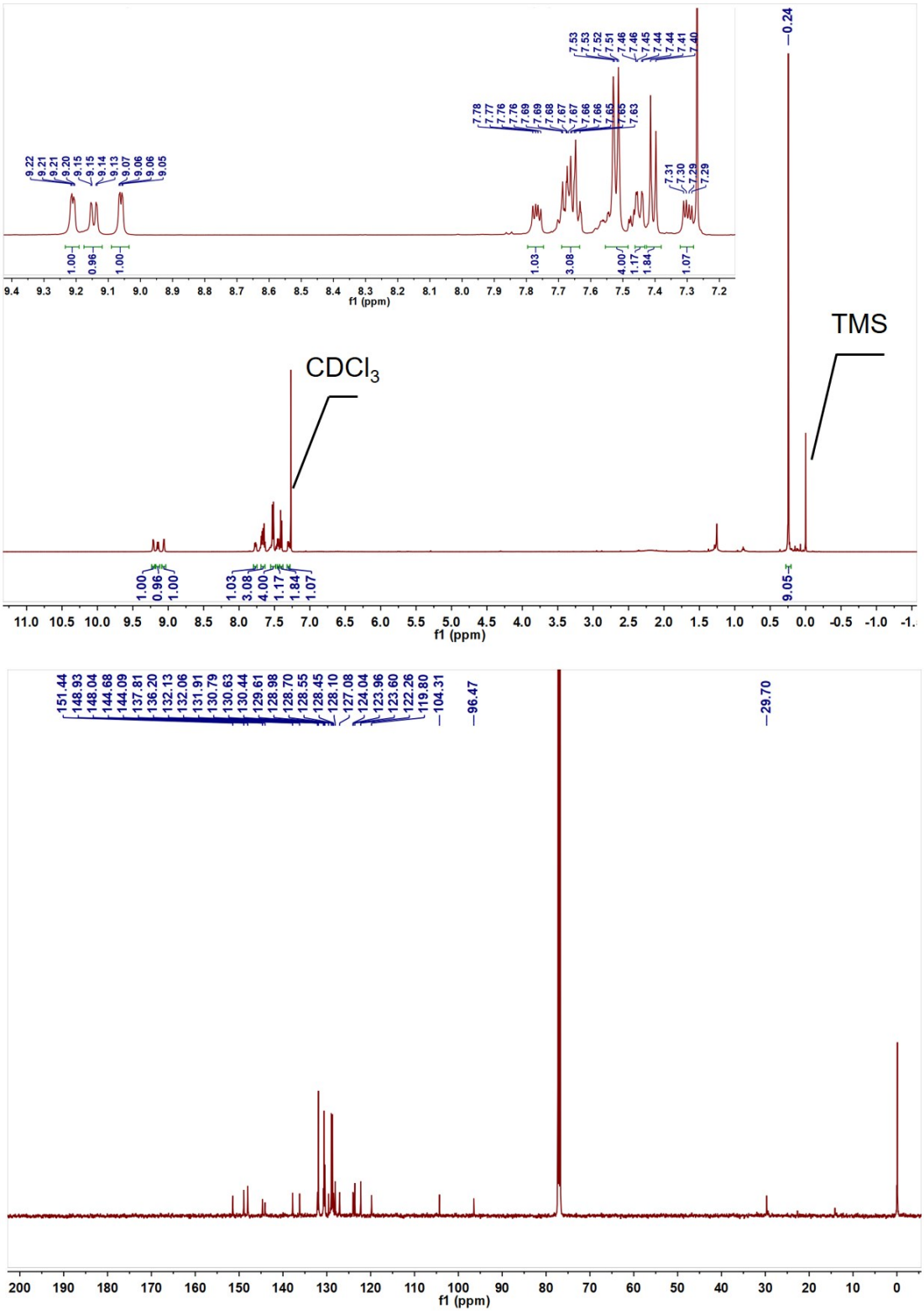


Fig. S2 ^{1}H NMR and ^{13}C NMR spectra of **1-phenyl-2-(4-((trimethylsilyl)ethynyl)phenyl)-1H-imidazo[4,5-f][1,10]phenanthroline (2a)**

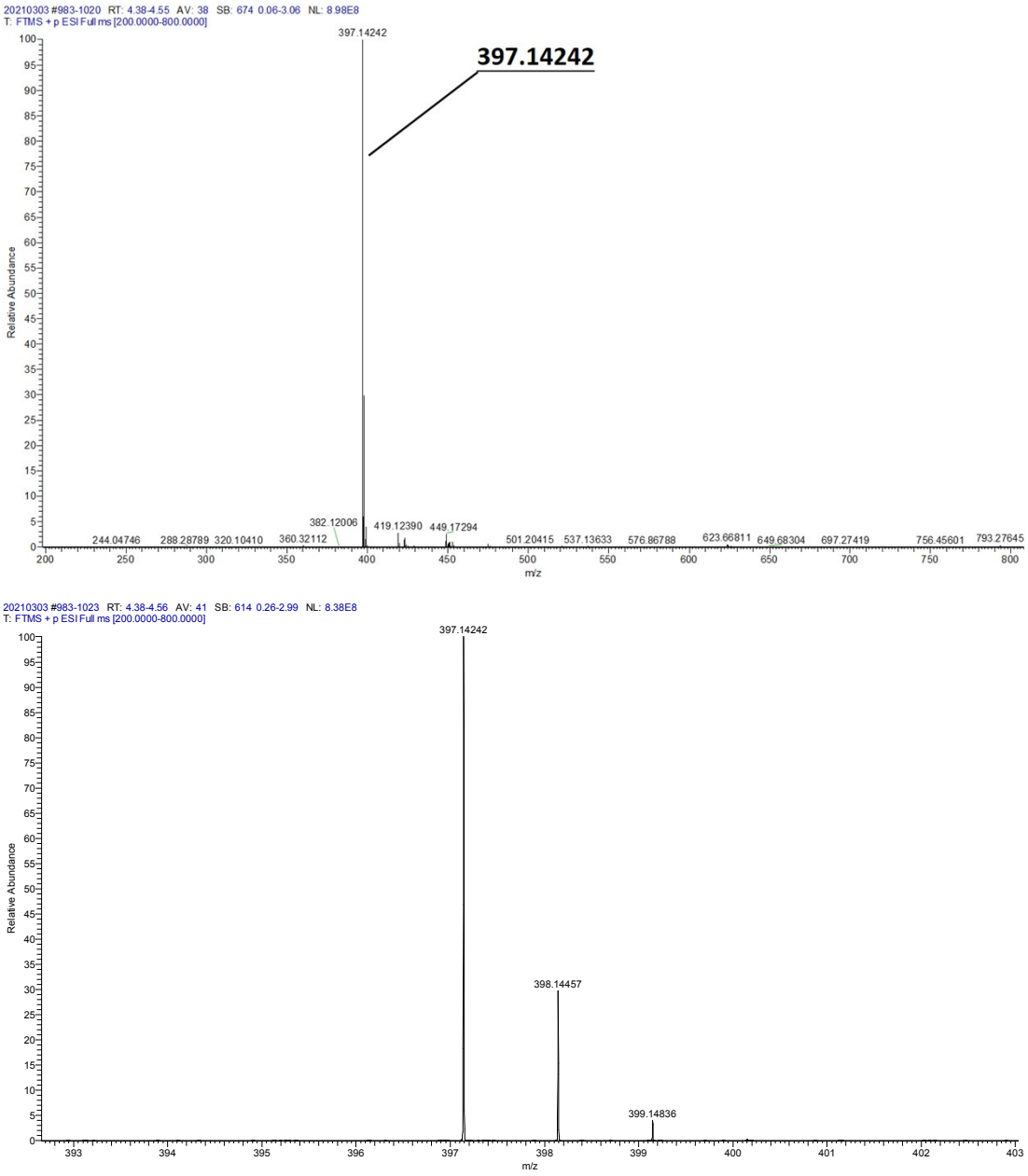


Fig. S3 HR-ESI-MS spectra of **2-(4-ethynylphenyl-1H- imidazo[4,5-f][1,10] phenanthroline (3a)**

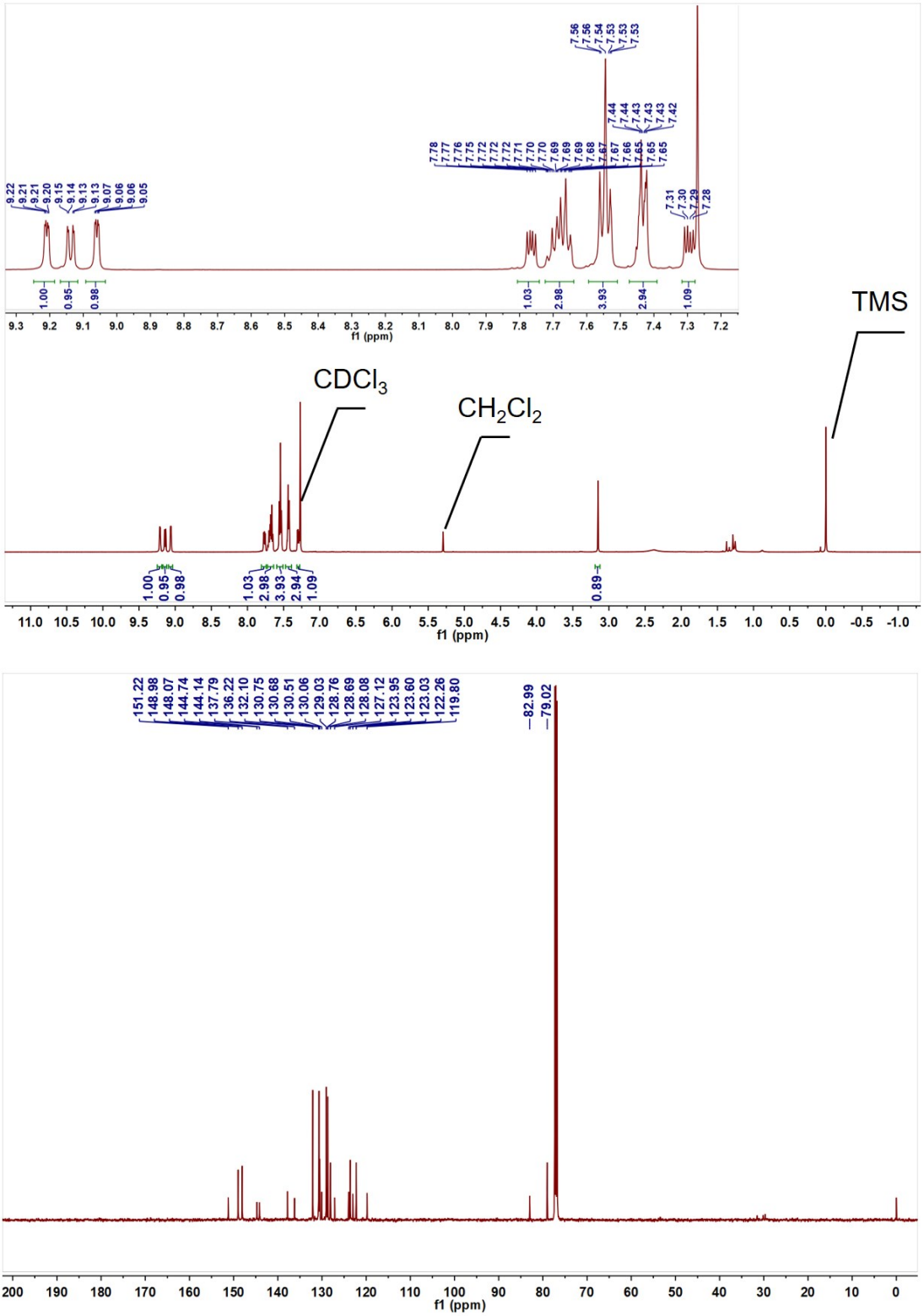


Fig. S4 ^{1}H NMR and ^{13}C NMR spectra of **2-(4-ethynylphenyl-1H- imidazo[4,5-f][1,10] phenanthroline (3a)**

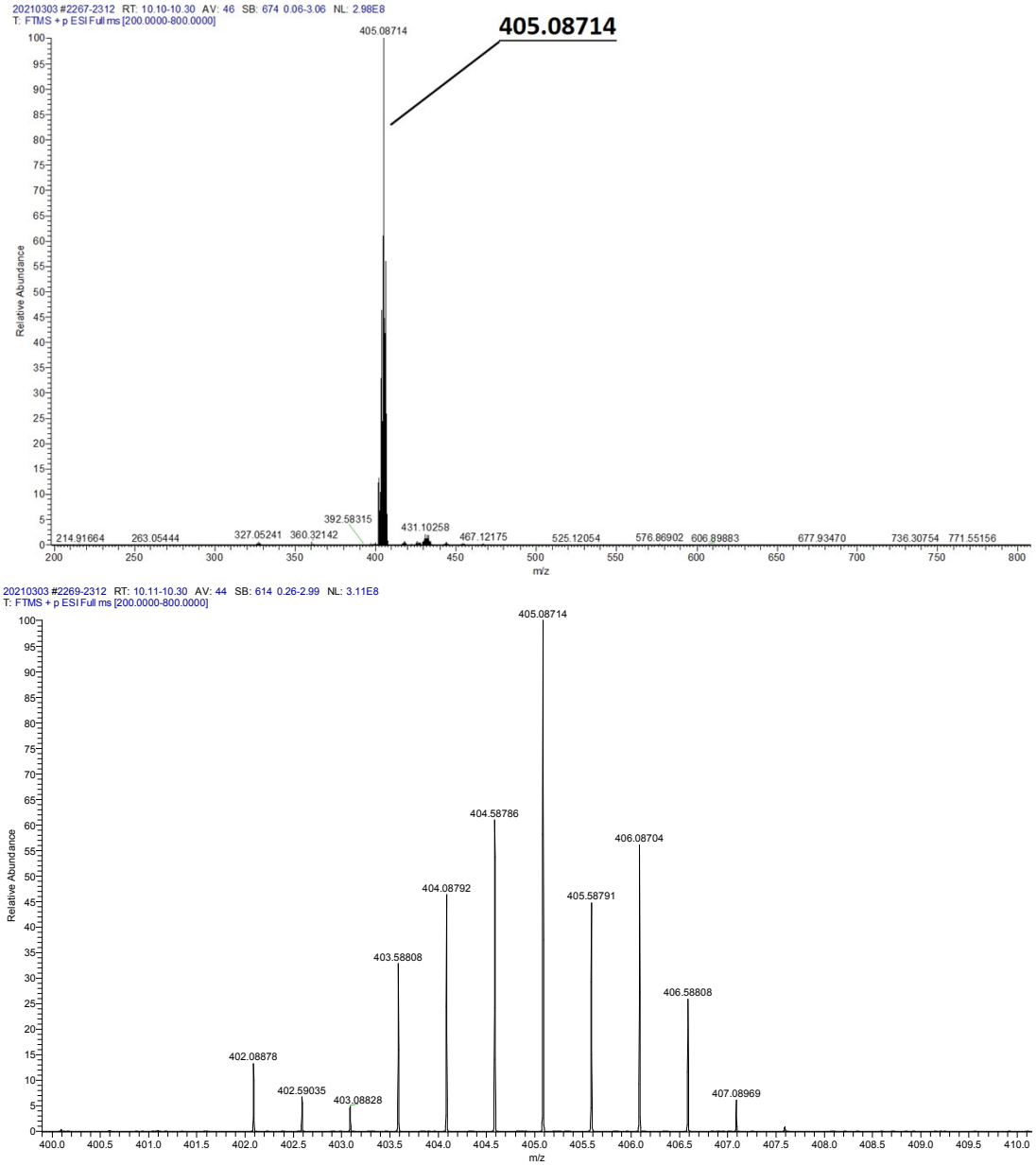


Fig. S5 HR-MS spectra of **Ru-alkyne-1**

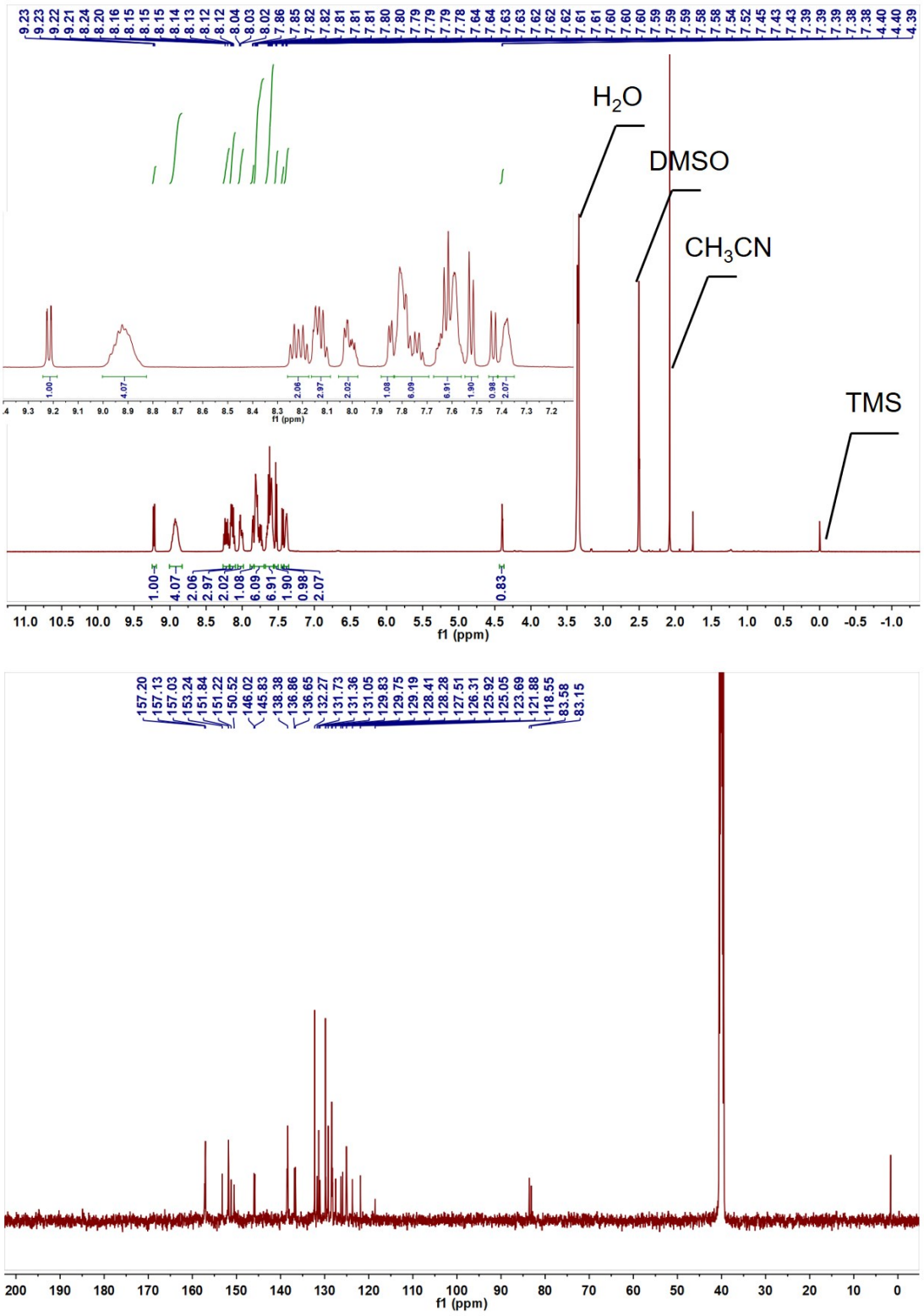


Fig. S6 ^{1}H NMR and ^{13}C NMR spectra of **Ru-alkyne-1**

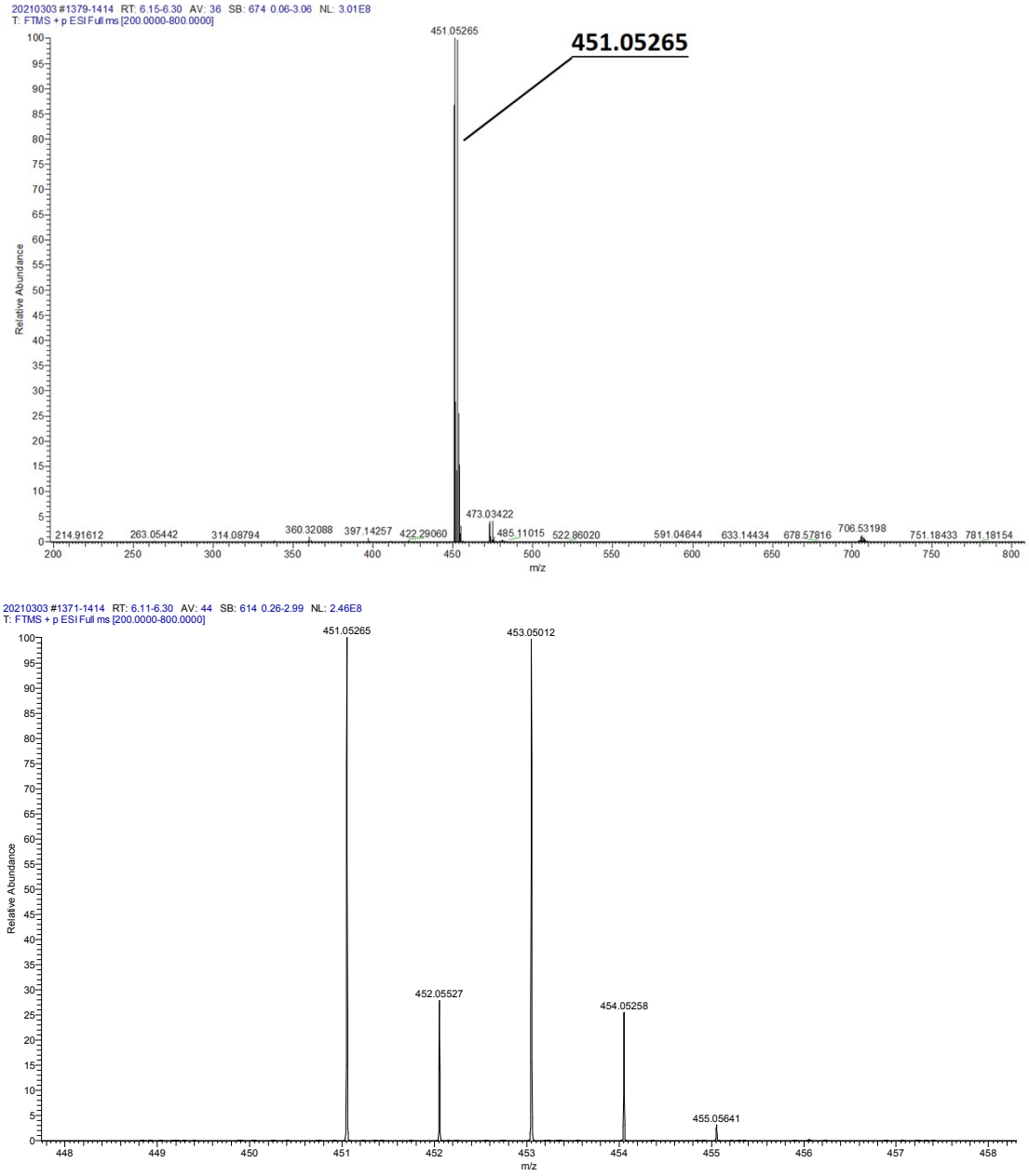


Fig. S7 HR-ESI-MS spectra of **1-(4-bromophenyl)-2-phenyl-1H-imidazo[4,5-f][1,10]phenanthroline (1b).**

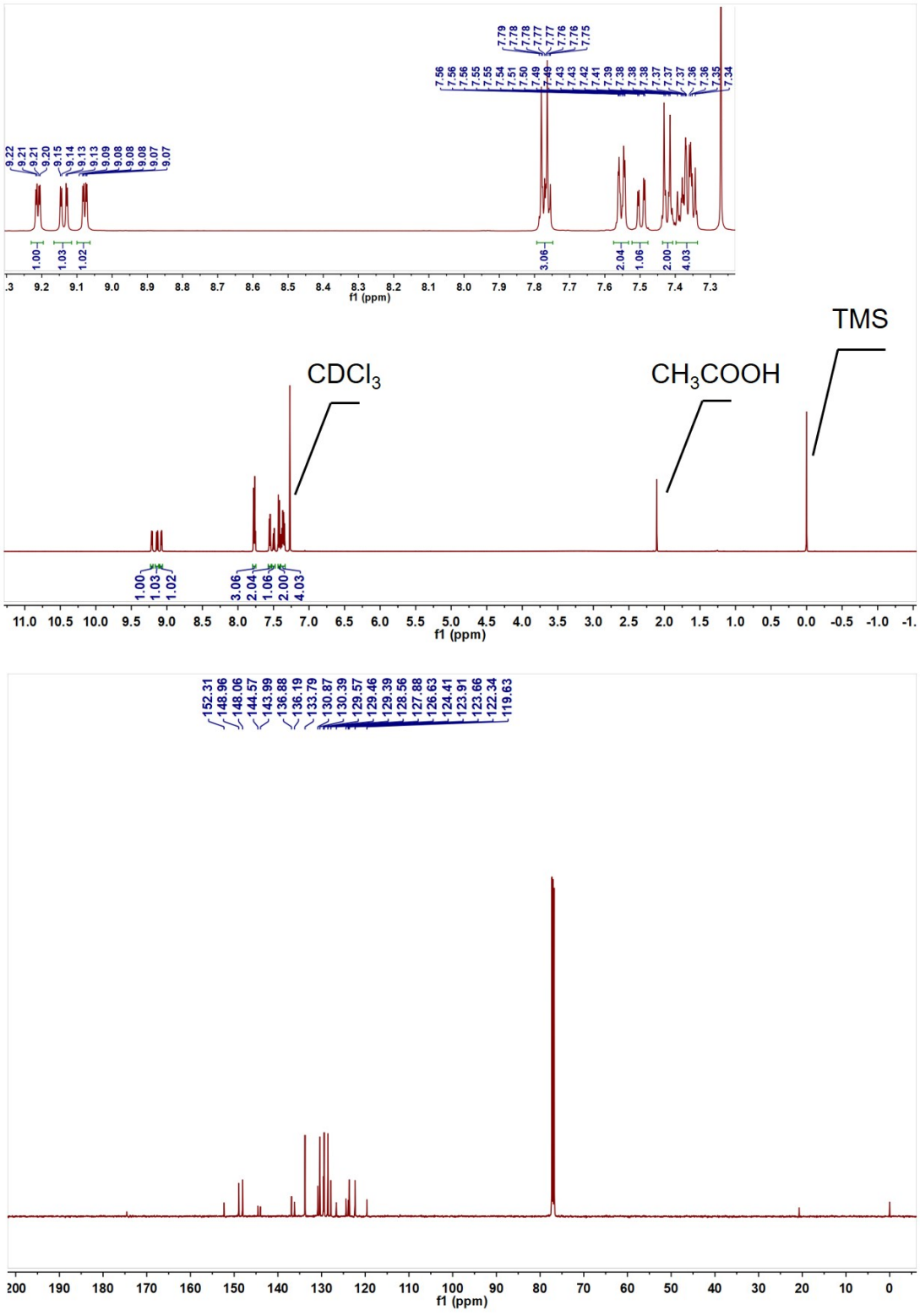


Fig. S8 ^{1}H NMR and ^{13}C NMR spectra of **1-(4-bromophenyl)-2-phenyl-1H-imidazo[4,5-f][1,10]phenanthroline (1b).**

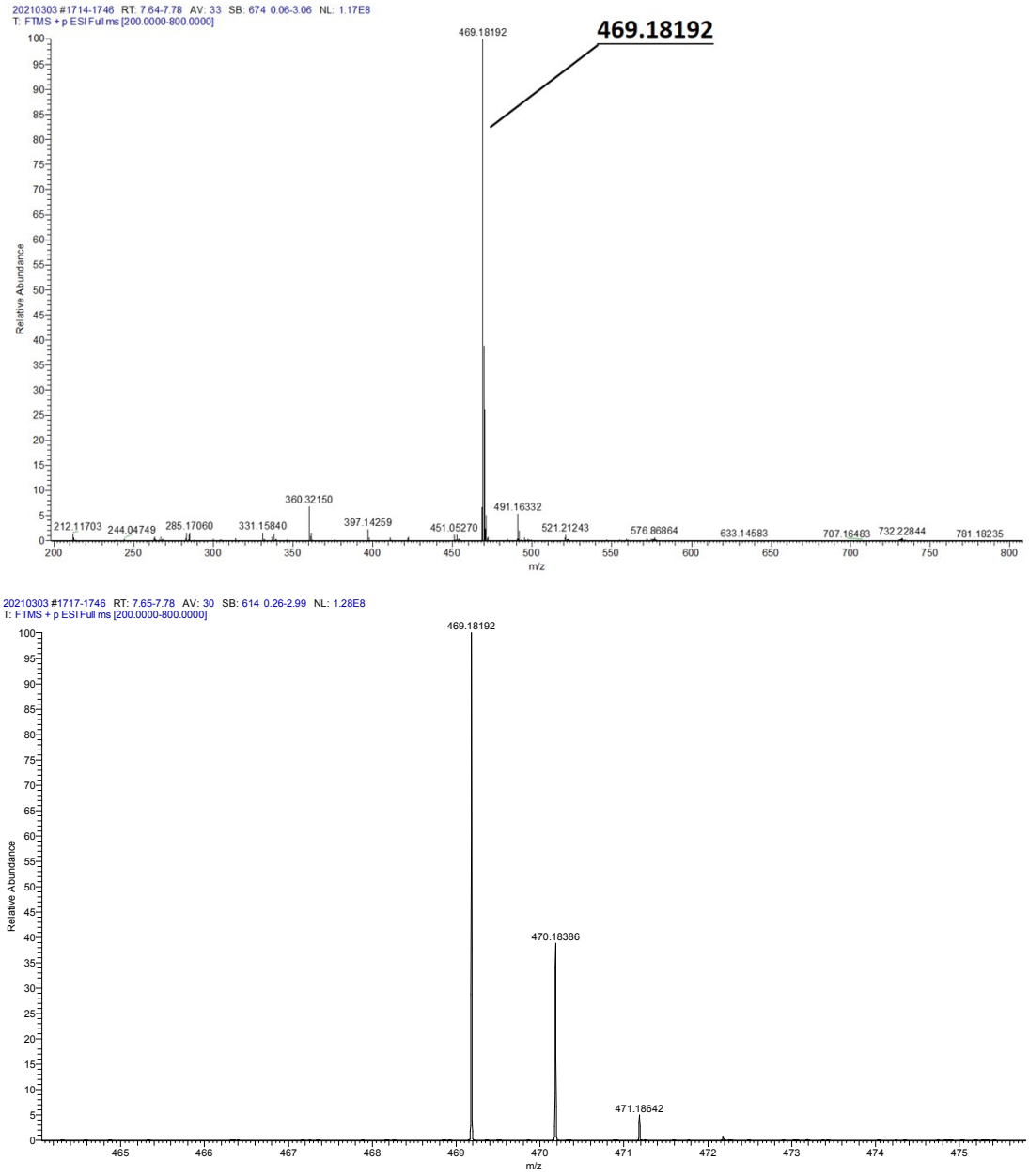


Fig. S9 HR-ESI-MS spectra of **2-phenyl-1-(4-((trimethylsiyl)ethynyl)phenyl)-1H-imidazo[4,5-f][1,10]phenanthroline (2b)**

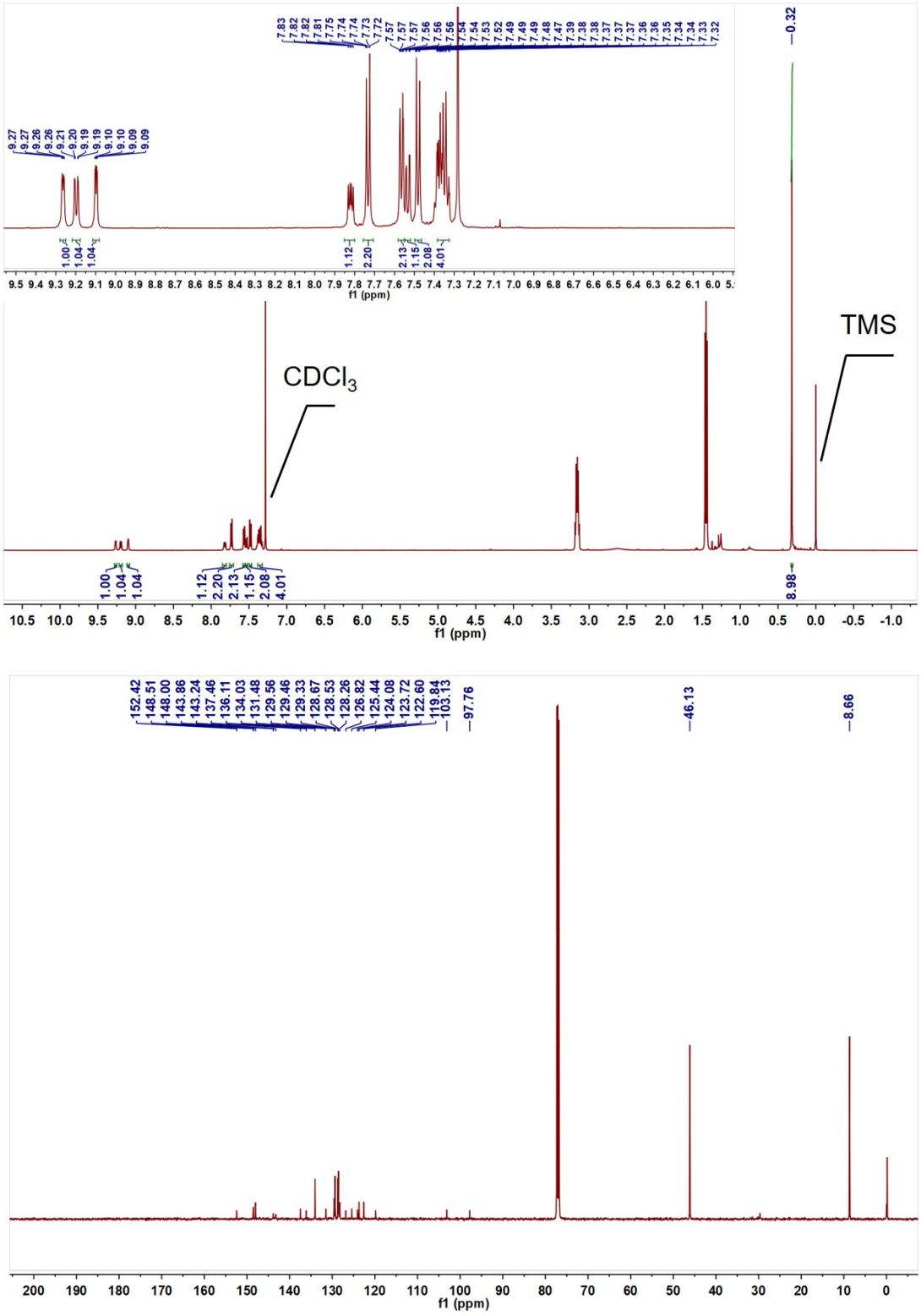


Fig. S10 ^{1}H NMR and ^{13}C NMR spectra of **2-phenyl-1-(4-((trimethylsiyl)ethynyl)phenyl)-1H-imidazo[4,5-f][1,10]phenanthroline (2b).**

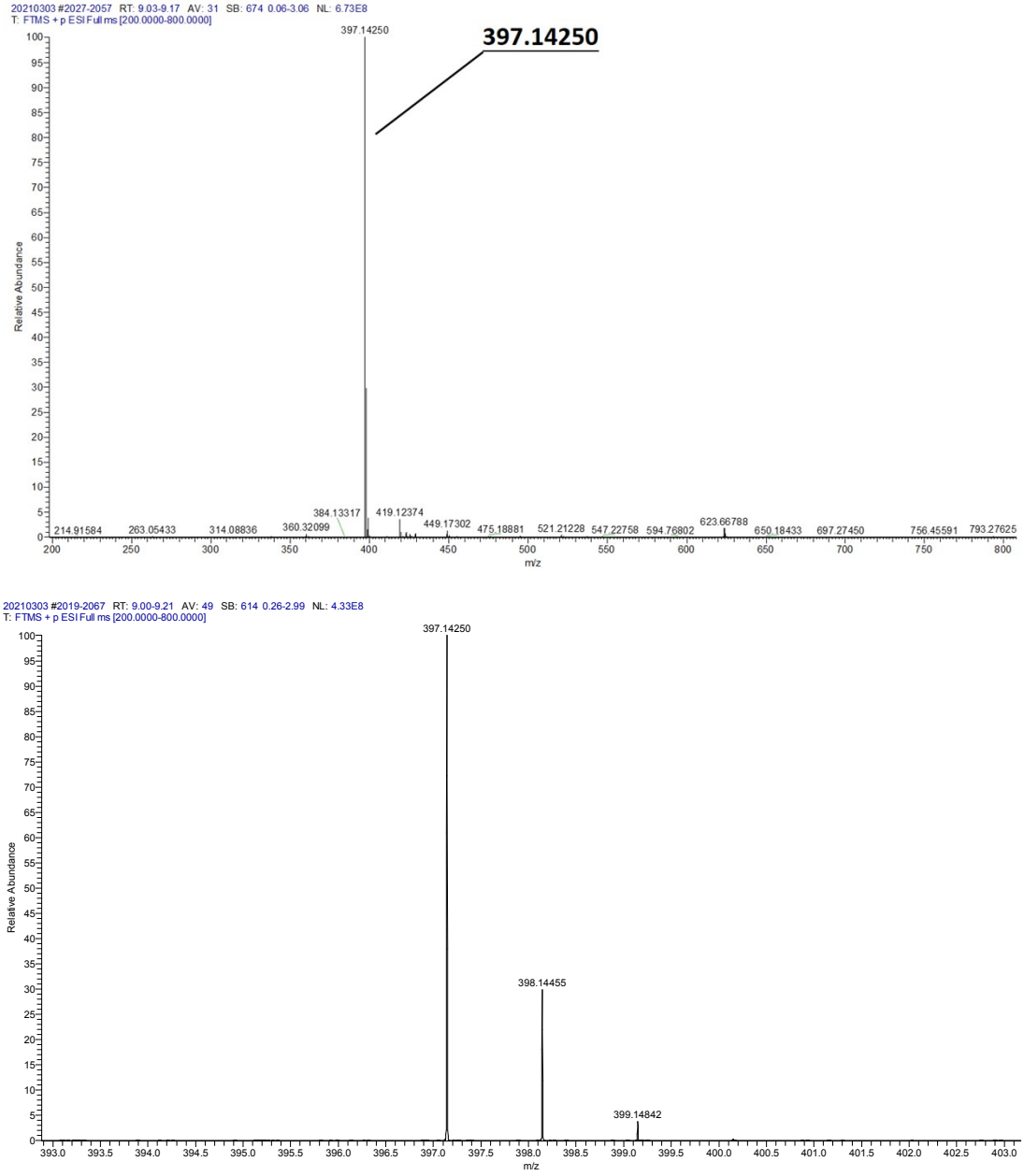


Fig. S11 HR-ESI-MS spectra of **1-(4-ethynlphenyl)-2-phenyl-1H-imidazo[4,5-f][1,10]phenanthroline (3b).**

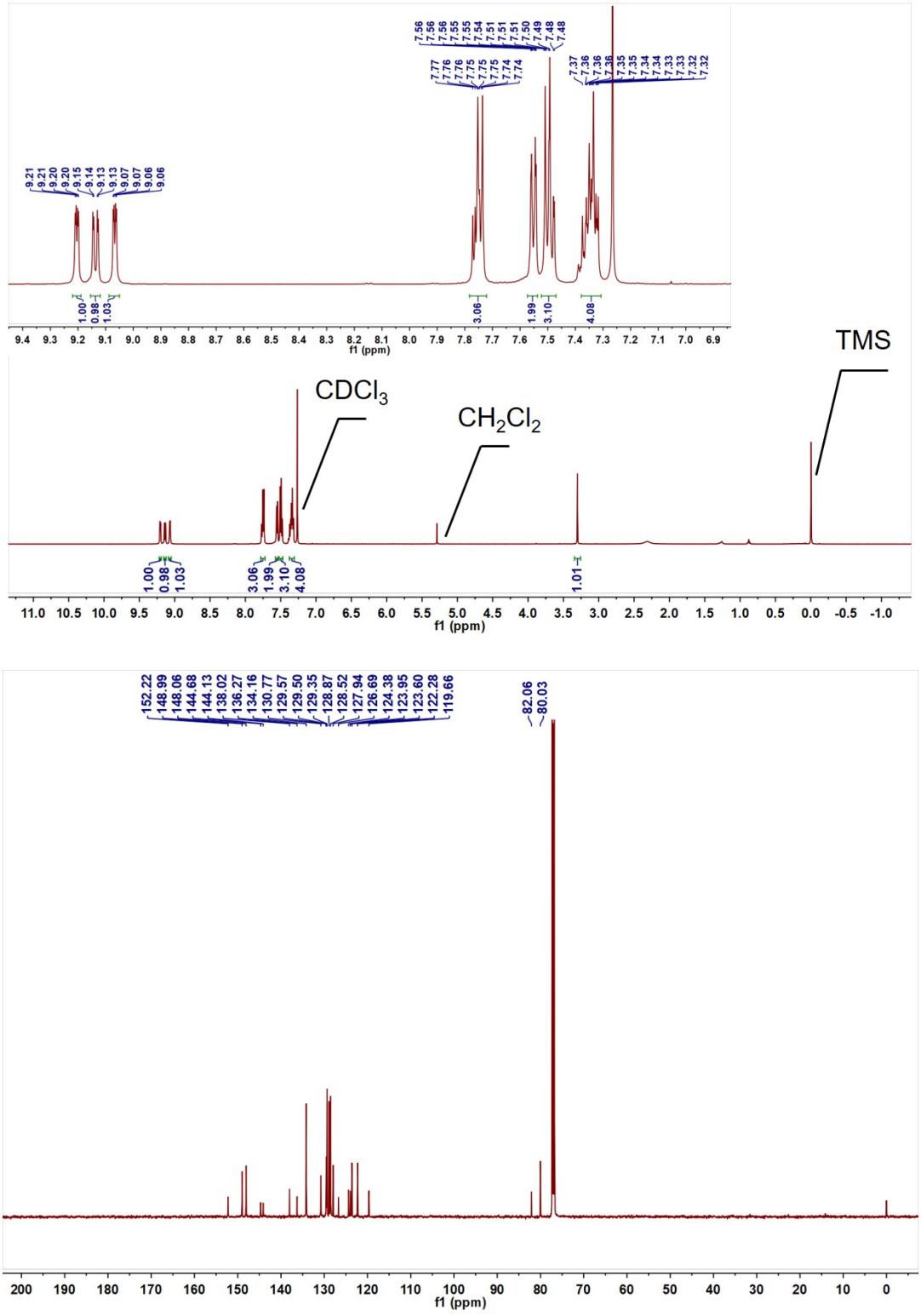


Fig. S12 ^{1}H NMR and ^{13}C NMR spectra of **1-(4-ethynlphenyl)-2-phenyl-1H-imidazo[4,5-f][1,10]phenanthroline (3b).**

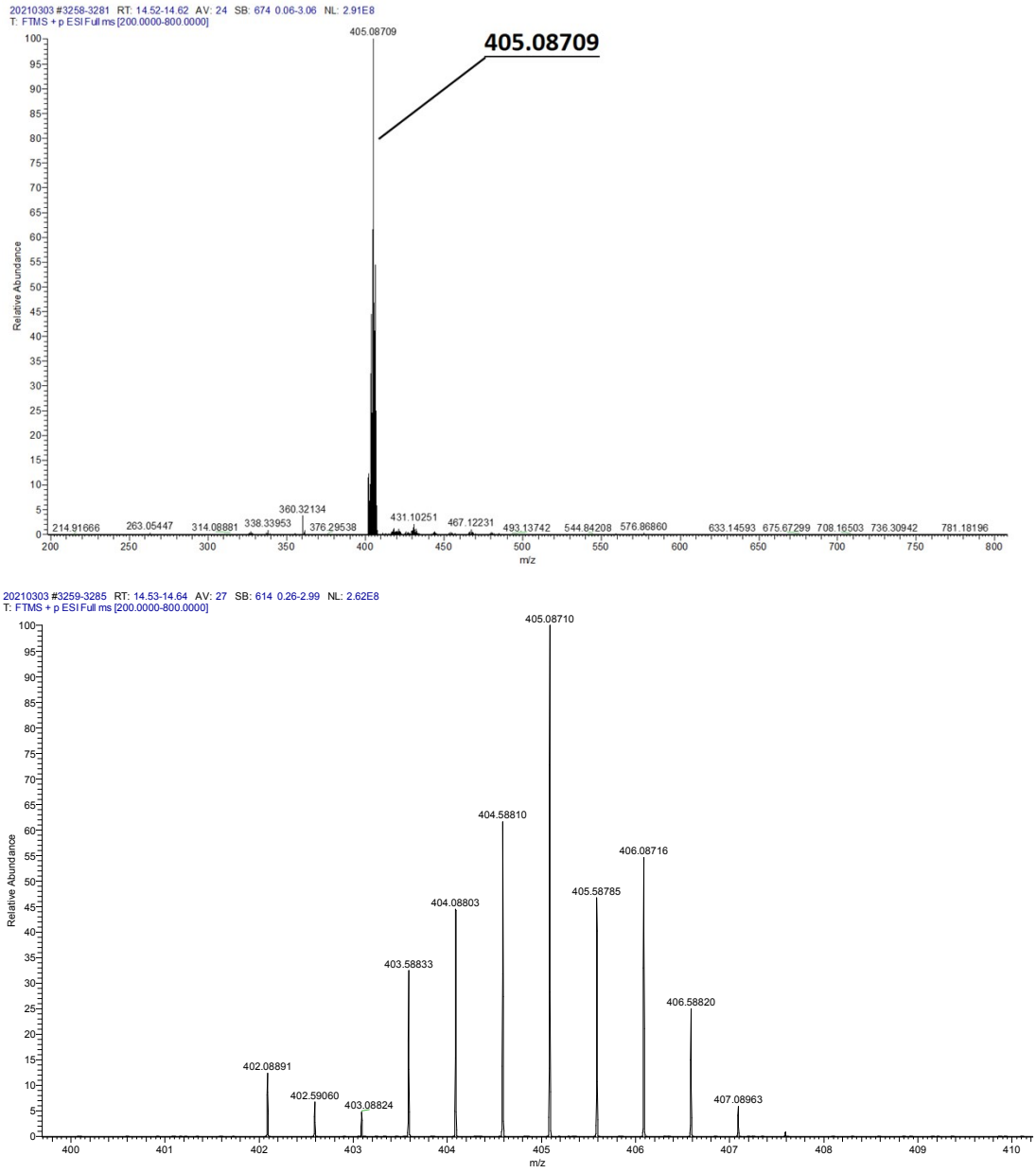


Fig. S13 HR-ESI-MS spectra of **Ru-alkyne-2**

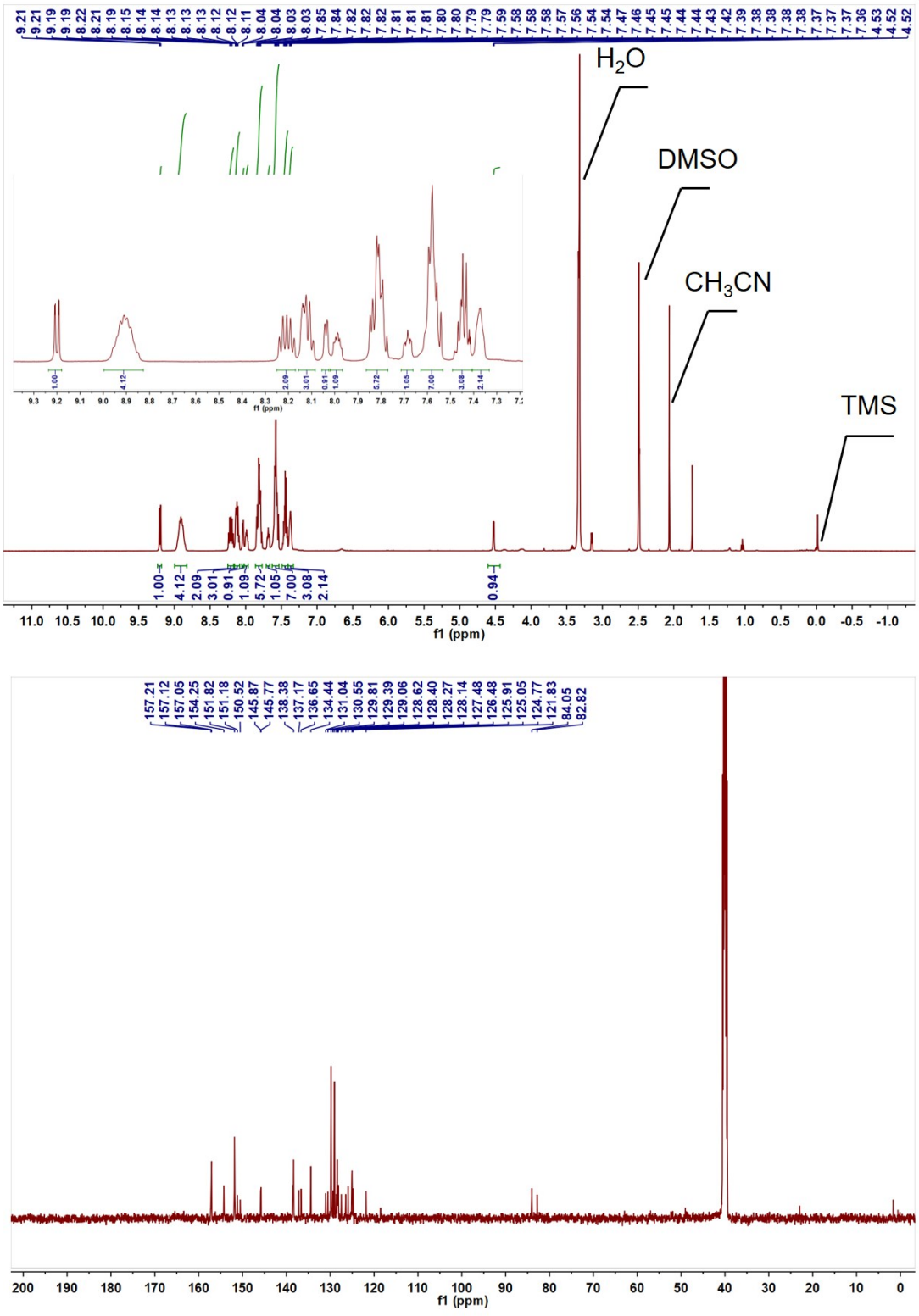


Fig. S14 ^{1}H NMR and ^{13}C NMR spectra of **Ru-alkyne-2**

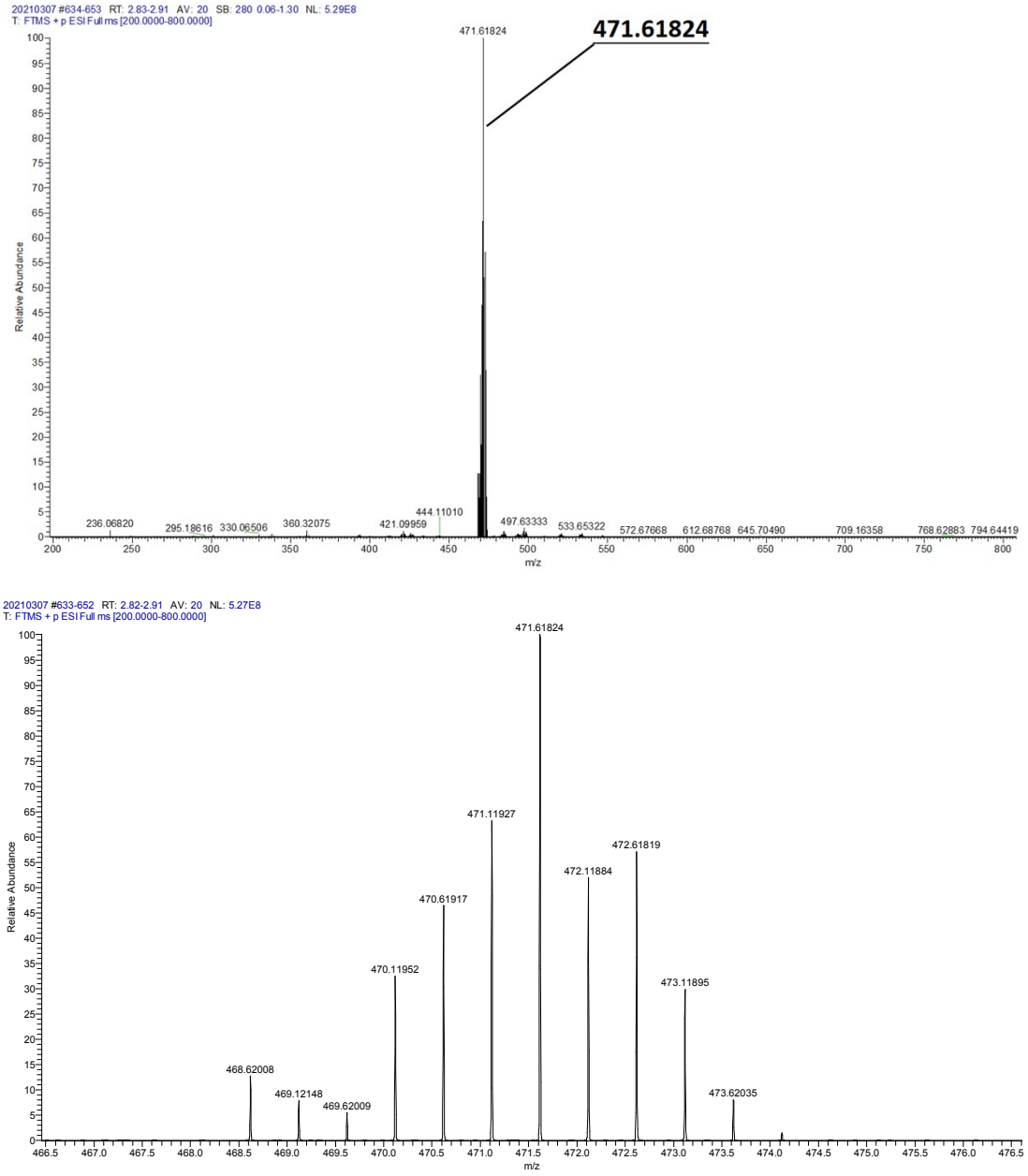


Fig. S15 HR-ESI-MS spectra of **Ru-triazole-2**

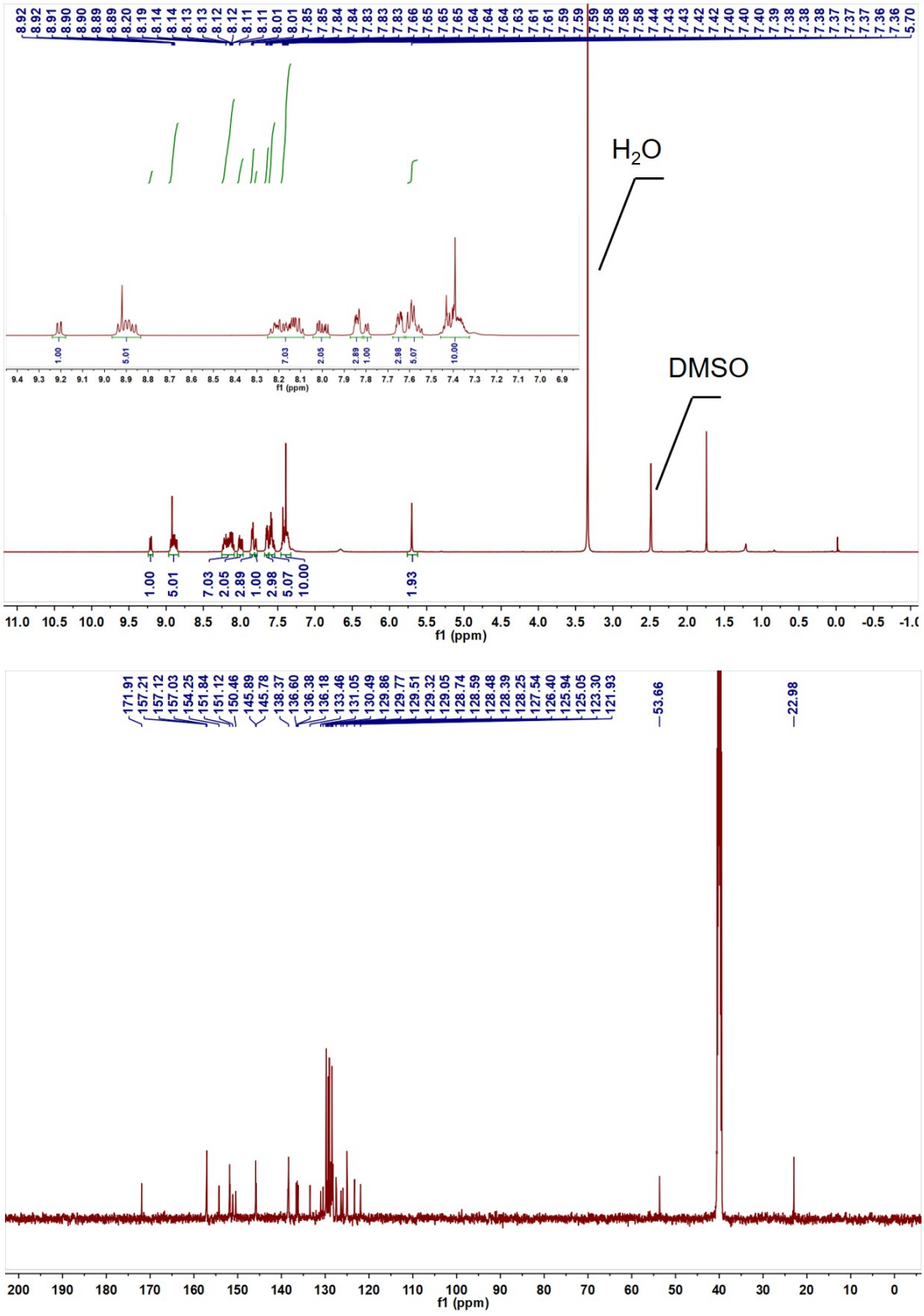


Fig. S16 ^{1}H NMR and ^{13}C NMR spectra of **Ru-triazole-2**

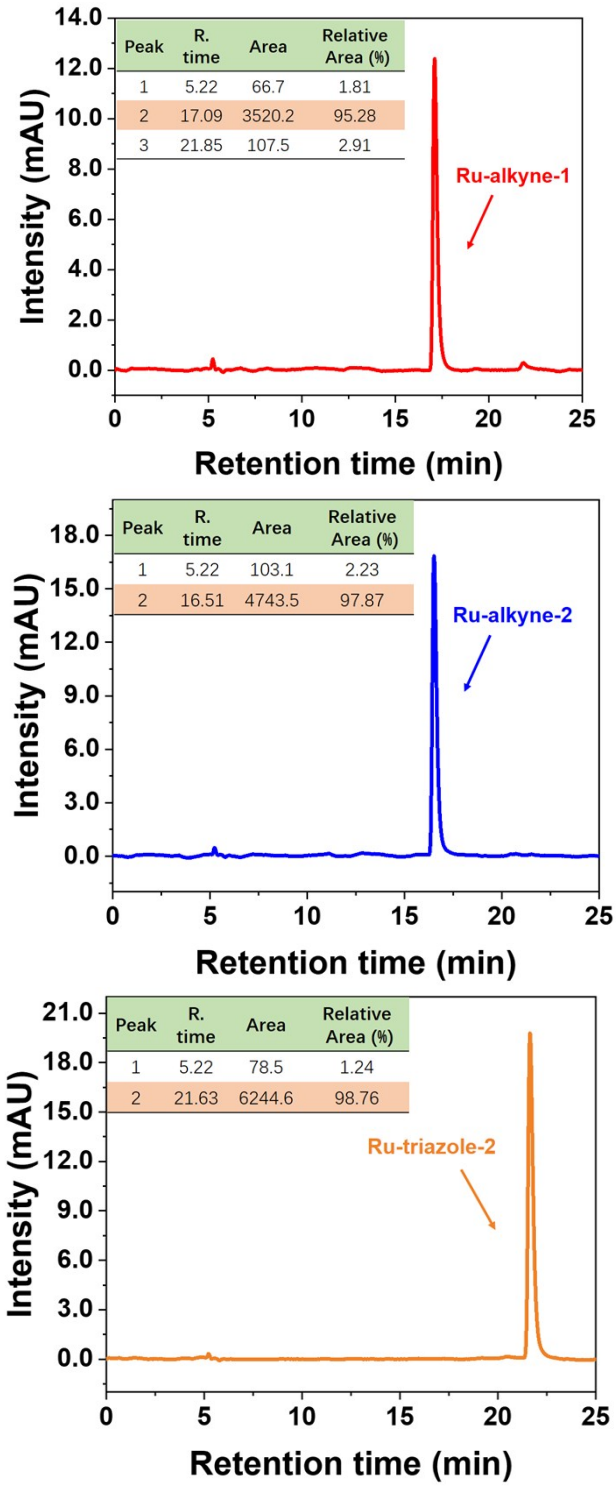


Fig. S17 HPLC trace of **Ru-alkyne-1**, **Ru-alkyne-2** and **Ru-triazole-2**.

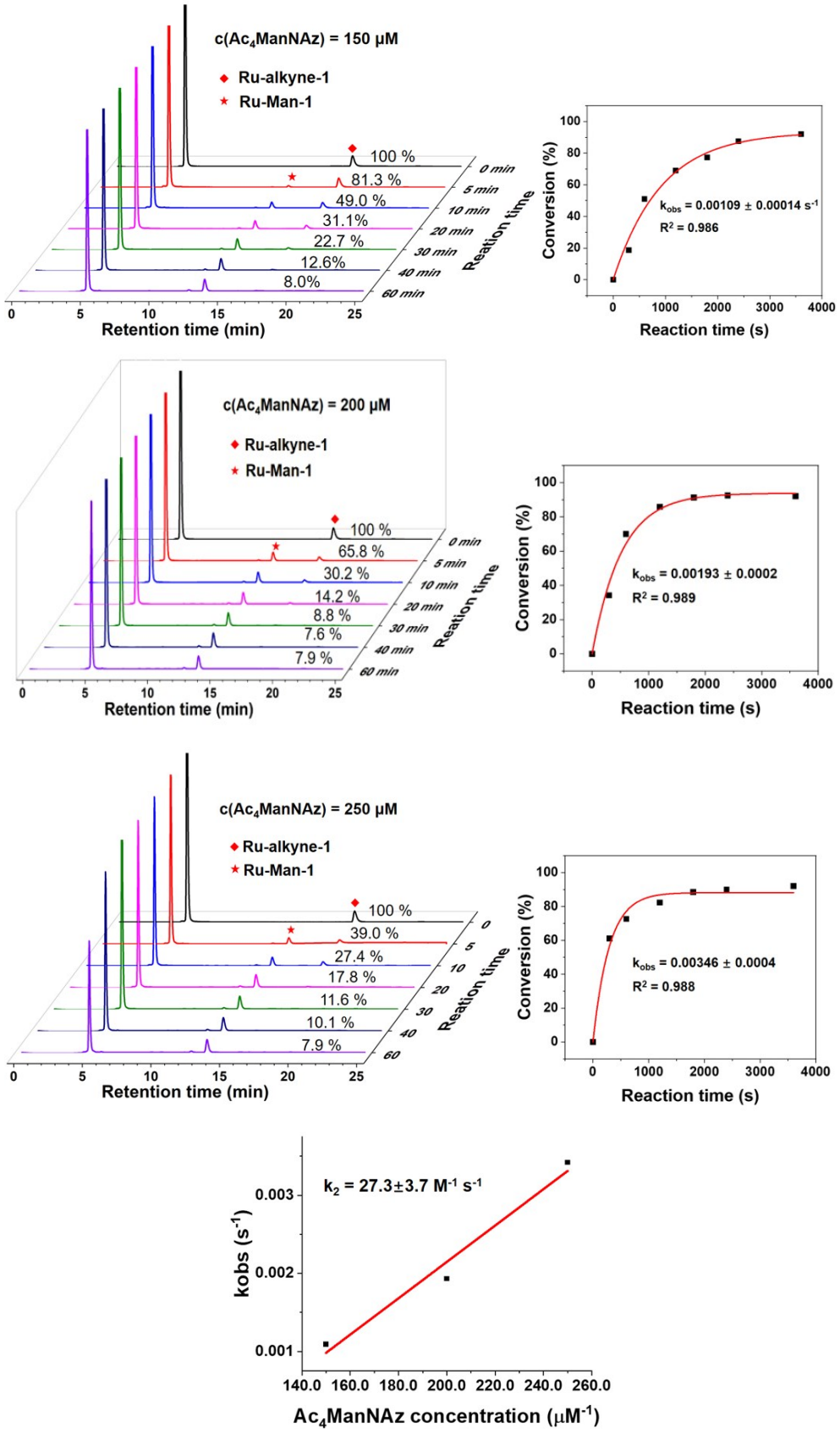


Fig. S18 HPLC-MS of CuAAC reaction of **Ru-alkyne-1** with different concentrations of Ac_4ManNAz.

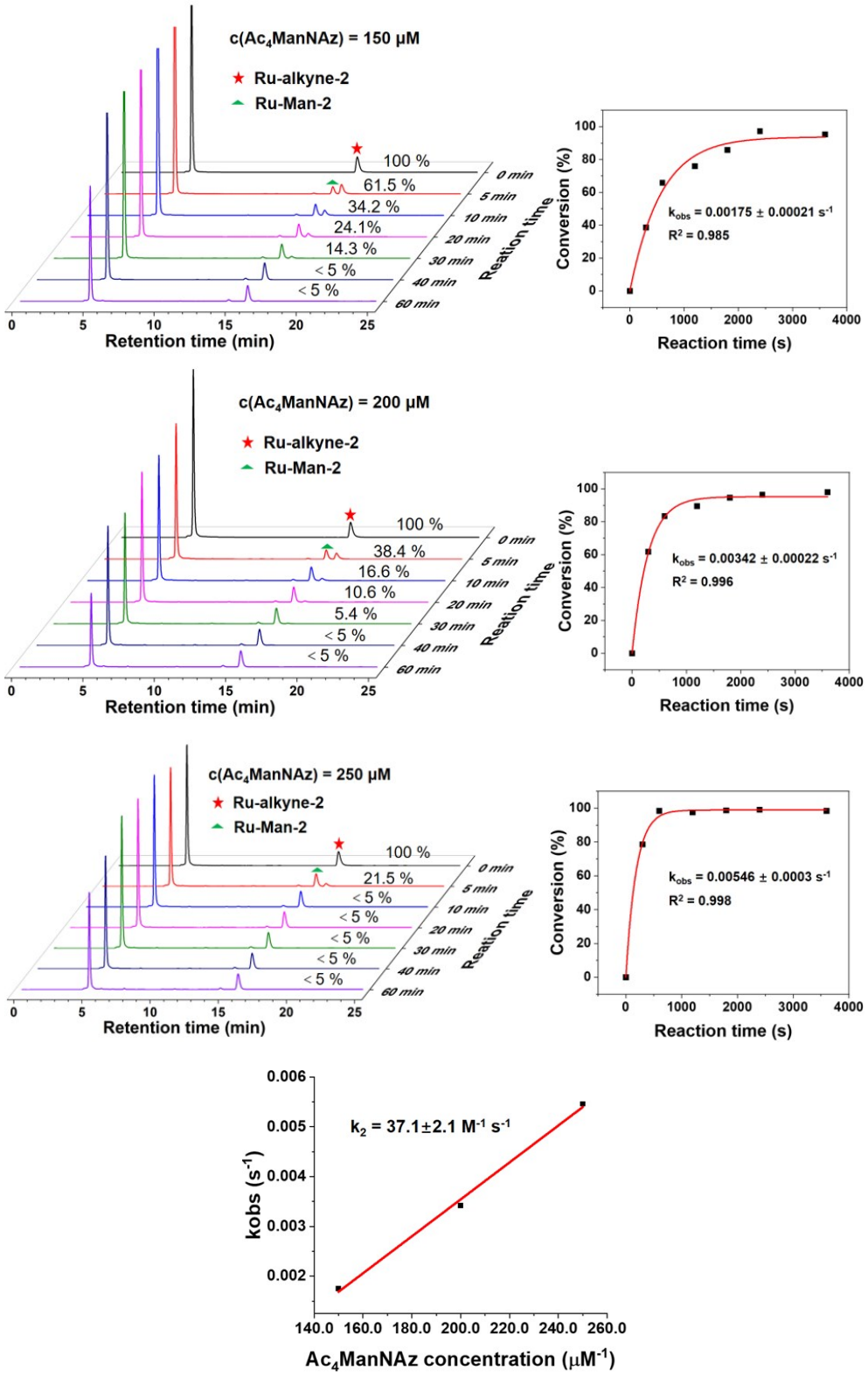


Fig. S19 HPLC-MS of CuAAC reaction of **Ru-alkyne-2** with different concentrations of Ac_4ManNAz.

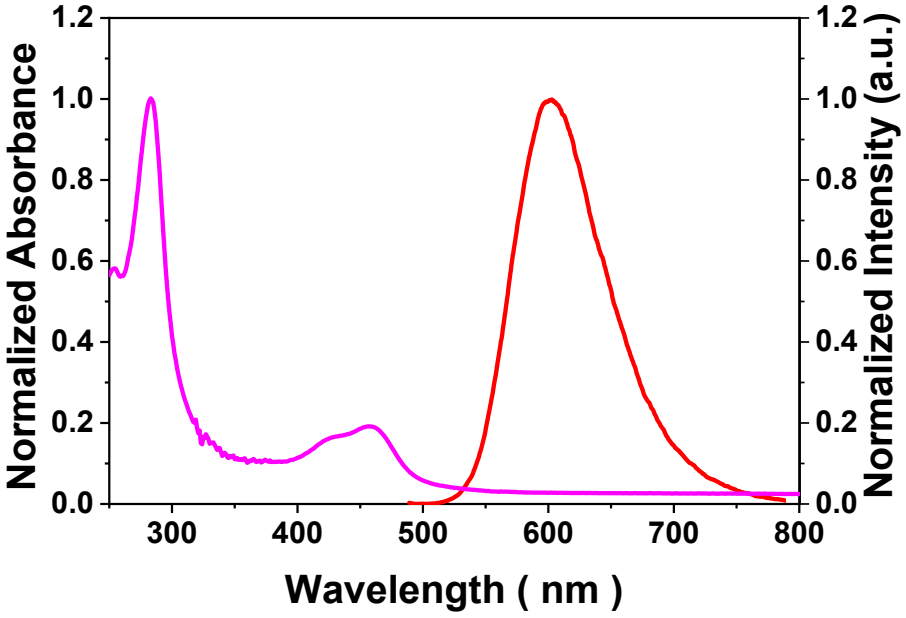


Fig. S20 Electronic absorption and emission spectra of **Ru-alkyne-2**

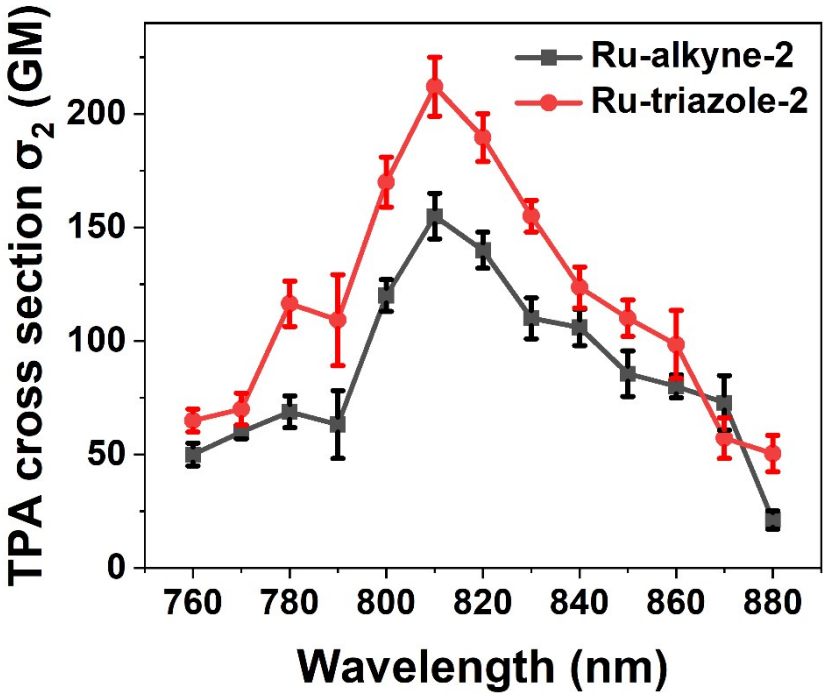


Fig. S21 Two-photon absorption cross sections of **Ru-alkyne-2** and **Ru-triazole-2** at different excitation wavelengths from 760 to 880 nm in methanol.

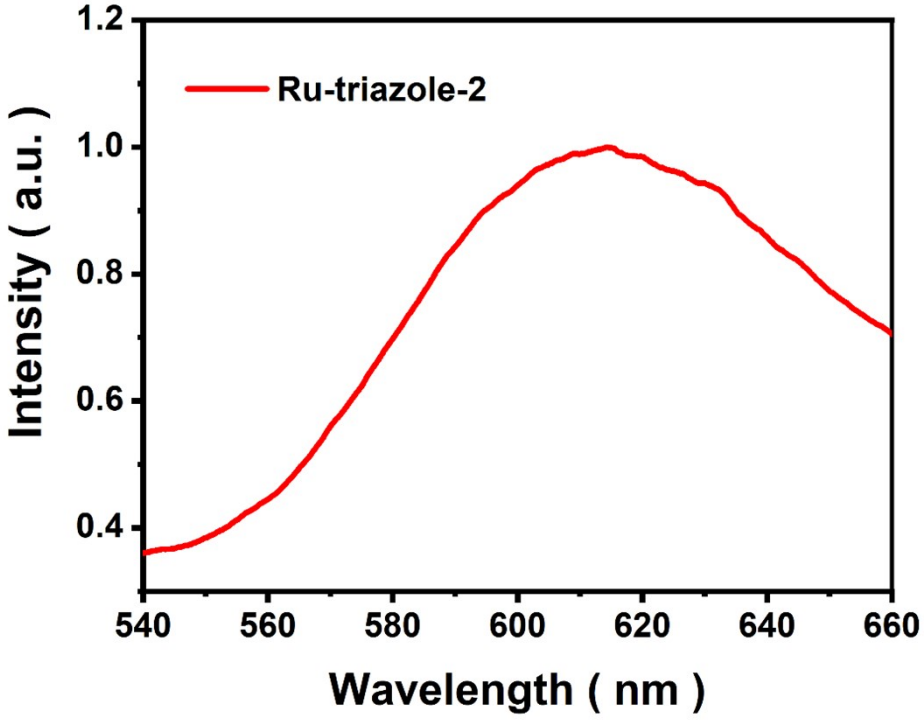


Fig. S22 Emission spectrum of **Ru-triazole-2** upon two-photon excitation (λ_{ex} = 810 nm)

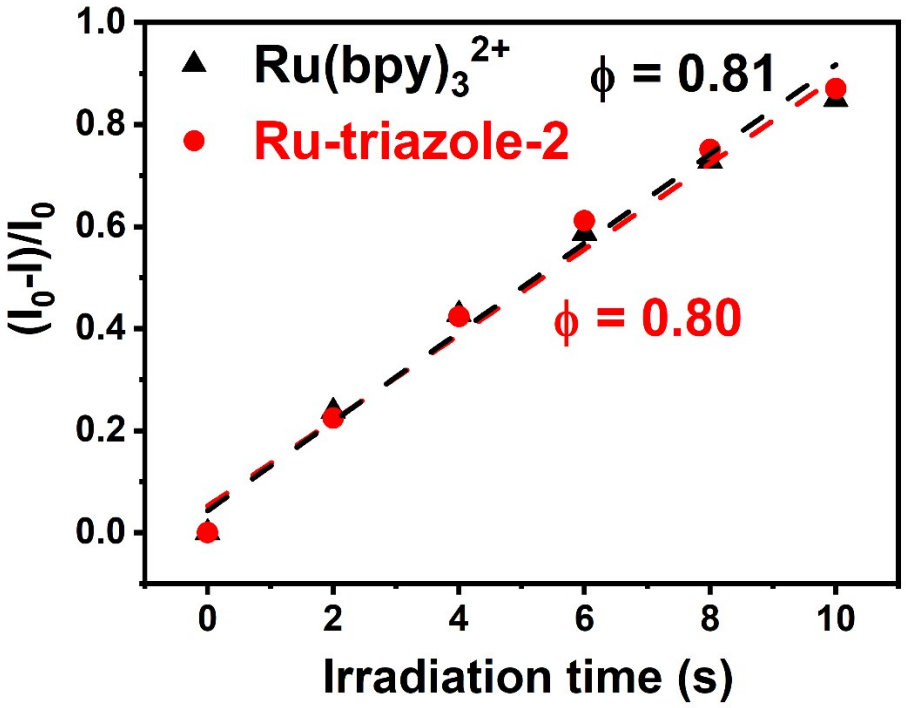


Fig. S23 Single oxygen quantum yields of **Ru-triazole-2** where $Ru(bpy)_3^{2+}$ was used as standard.

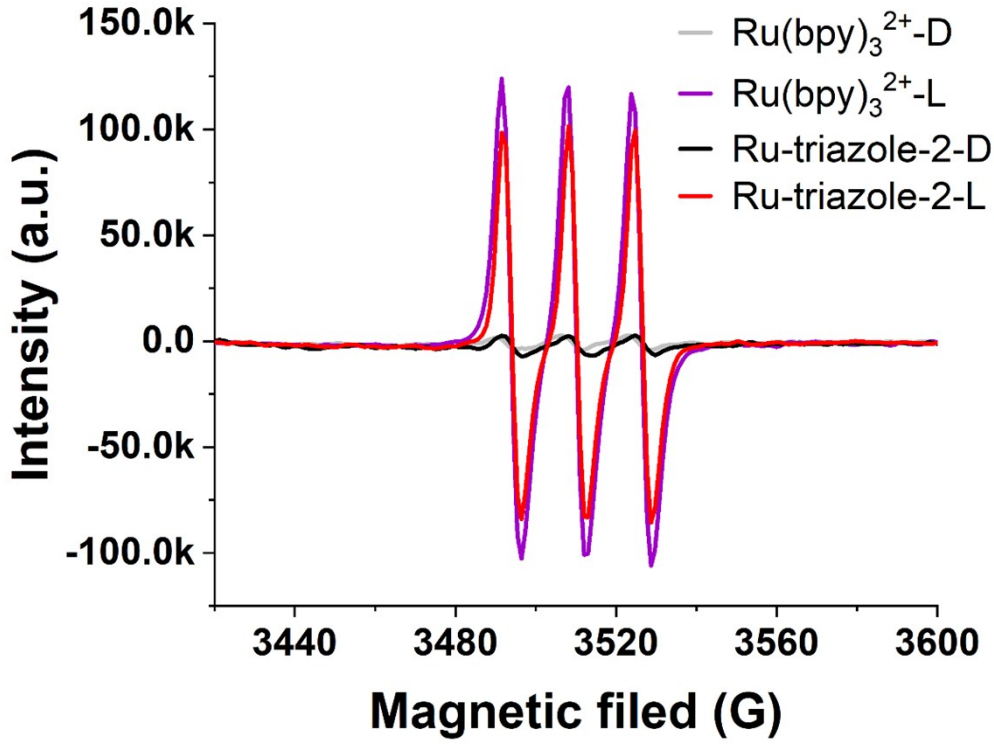


Fig. S24 ESR spectrum of **Ru-triazole-2** and $Ru(bpy)_3^{2+}$ in the presence of TEMP and irradiation.

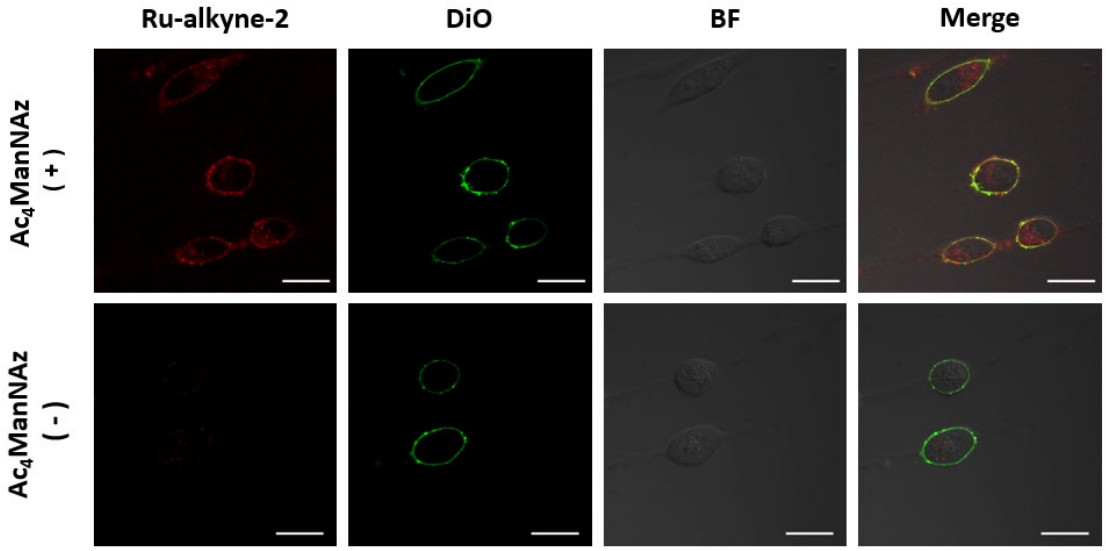


Fig.S25 MDA-MB-231 cells were seeded in confocal dishes and pre-incubated with or without 50 μM Ac_4ManNAz in DMEM medium for 3 days, and then removed the medium. The cells were washed by 1X PBS for twice, following the click chemistry procedure (10 μM **Ru-alkyne-2**, 25 μM $CuSO_4$, 125 μM THPTA, 2.5 mM sodium ascorbate in PBS) for an hour, and then stained by the DiO (10 μL, 1% DMSO) for another 30 min. Scale bar: 20 μm.

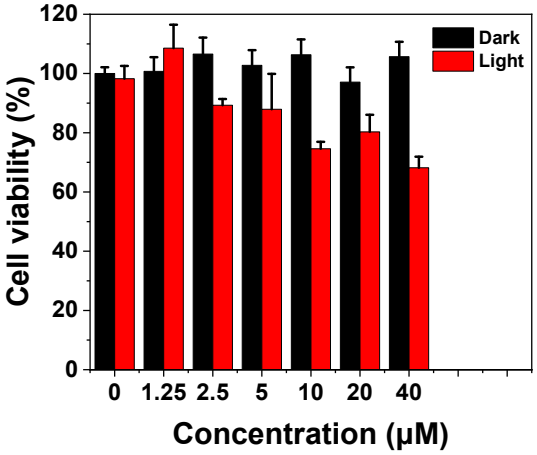


Fig. S26 (Photo)cytotoxicity of **Ru-alkyne-2** when being labelled to the MCF-10A cell line (pretreated with 50 μM Ac_4ManNAz for three days).

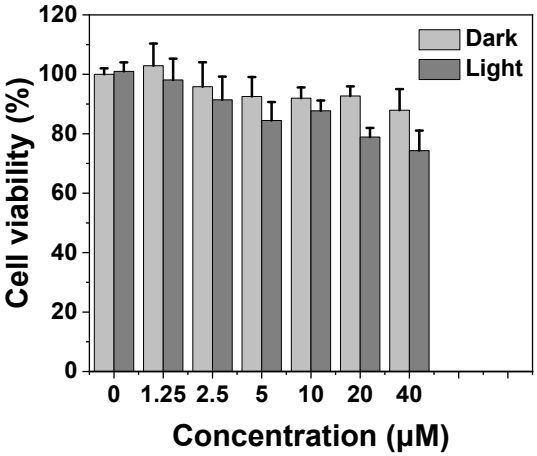


Fig. S27 (Photo)cytotoxicity of **Ru-alkyne-2** towards MCF-10A cell line that without Ac_4ManNAz pretreatment.

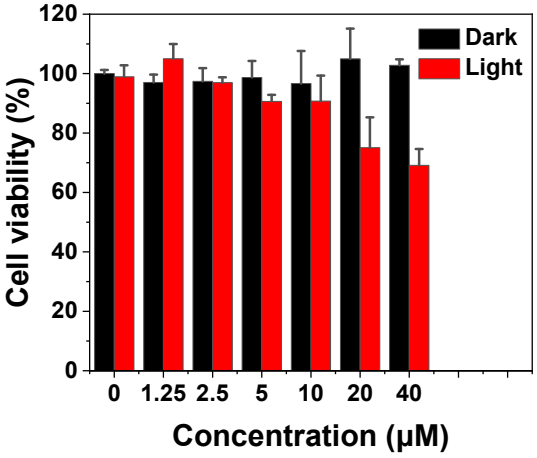


Fig. S28 (Photo)cytotoxicity of **Ru-triazole-2** towards MDA-MB-231 cell line

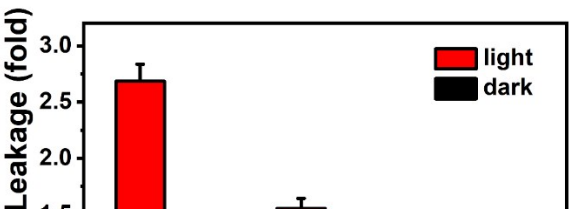


Fig. S29 Cell membrane integrity assay through LDH leakage

Table S1 photo-physical properties of **Ru-alkyne-2** and **Ru-triazole-2**

Complexes	λ_{abs} /nm	λ_{em} /nm	Φ_u	τ /ns	σ_2 /GM
Ru-alkyne-2	283, 455	609	0.053	354	155 (810 nm)
Ru-triazole-2	284, 455	609	0.039	342	212 (810 nm)

Table S2 detailed conditions of HPLC

Retention(min.)	A(%)	B(%)	Flow(mL/min.)
0.00	60	40	
15.00	55	45	
20.00	55	45	0.5
20.01	60	40	
25.00	60	40	

Chromatographic column: Shim-pack GIST C18; Dim.(mm):250*4.6; Particle Sz.(u): 5

Injection volume: 2 μL

Multi-Step Gradient: A(H_2O, 0.01 % TFA), B(MeCN)

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