Supporting Information

Synthesis and biological evaluation of ruthenium complexes bearing 1,2,4-triazole group as potential membrane-targeting antibacterial agents towards *Staphylococcus aureus*

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CONTENTS

1. General information

All reagents were purchased from commercial suppliers. 1,10-Phenanthroline, 1,2,4-triazole, 2,2'-bipyridine, 4,4'-dimethyl-2,2'-bipyridine, 4,4'-di-tert-butyl2,2'-bipyridine and 4,4'-dimethoxy-2,2'-bipyridine were purchased from Energy-chemical, while antibiotics and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sangon. 4',6'-diamidino-2'-phenylindole (DAPI) was purchased from Solarbio. Propidium iodide (PI) was purchased from 3A Materials. The *S. aureus* strains were obtained from China center of industrial culture collection (CICC). Enzyme-labeled instrument was purchased from BioTek Instruments. The bio-chemical incubator and constant temperature culture shaker were purchased from Yiheng Scientific Instruments. NMR spectra were recorded on a Bruker Avance 400MHz spectrometer. The HR-MS was carried out using a water G2-XS Q-TOF instrument. HPLC chromatogram were recorded on a 1260 Infinity from Agilent Technologies. Ru1-Ru4 was eluted by 0.1% formic acid solution and acetonitrile by gradient elution. Quantity of flow: 1 mL/min. Chromatographic column model: Supersil ODS 2.5 μm, Column specification: ID 4.6 mm × 250 mm. Cell fluorescence were recorded on a BIO-RAD fluorescent cell imager.

2. Synthesis and characterization of TPIP and Ruthenium Complexes

The 1,10-phenanthroline-5,6-dione was synthesized according to the literature procedures. To a 100mL round-bottom flask were added 830.4 mg of **compound 1** (4.8 mmol), 840 mg of 1,10-phenanthroline-5,6-dione (4 mmol) and 2.28 g of ammonium acetate (30 mmol) in 30 mL acetic acid. The reaction was heated to 120℃ under argon. After 6 hours, the reaction was cooled to 25℃ and was diluted with water to get a orange-red solution. A yellow precipitate was separated out when the PH of the solution was adjusted to near 7. The precipitate was washed with water for 3-4 times and dried in vacuum to acquire a yellow solid, 2-(4-(1H-1,2,4-triazol-1-yl)phenyl)-1H-imidazo[4,5-f][1,10]phenanthroline, called TPIP. ^{1}H-NMR (400 MHz, DMSO) δ: 13.87 (s, 1H), 9.44 (s, 1H), 9.05 (s, 2H), 8.93 (d, *J*=6.0 Hz, 2H), 8.45 (d, *J*=8.7 Hz, 2H), 8.32 (s, 1H), 8.14 (d, *J*=8.7 Hz, 2H), 7.90-7.81 (m, 2H); ^{13}C-NMR (101 MHz, DMSO) δ: 152.63 (s), 149.46 (s), 147.87 (s), 143.65 (s), 142.48 (s), 137.22 (s), 129.66 (s), 129.18 (s), 127.49 (s), 123.29 (s), 119.69 (s), 67.05 (s), 25.15 (s). HRMS (ESI) m/z: calcd. 364.1311 for $C_{21}H_{14}N_7$ $[M+H]^+$, found: 364.1329.

Synthesis and characterization of Ru1-Ru4.

The compounds $Ru(bpy)_2Cl_2$, $Ru(dmb)_2Cl_2$, $Ru(dtb)_2Cl_2$ and $Ru(dmob)_2Cl_2$ were prepared by previously described procedures.

Ru1 was prepared using the similar method to that as the literature with some modifications. A mixture of $Ru(bpy)_2Cl_2$ (242 mg, 0.5 mmol), TPIP (182.5 mg, 0.5 mmol) and ethylene glycol (10 mL) was refluxed under argon

for 8h to give a clear brown-red solution. The cooled reaction mixture was diluted with water and then potassium hexafluorophosphate was added. The obtained brown-red precipitate was purified by column chromatography on alumina with dimethylbenzene/acetonitrile (4:1, v/v) as an eluent. The solvent was removed under reduced pressure and orange-red solid was obtained. **Ru2** was prepared similar as **Ru1**, using $Ru(dmb)_2Cl_2$ (270 mg, 0.5 mmol) in place of $Ru(bpy)_2Cl_2$. **Ru3** was prepared similar as **Ru1**, using $Ru(dtb)_2Cl_2$ (354 mg, 0.5 mmol) in place of $Ru(bpy)_2Cl_2$. **Ru4** was prepared similar as **Ru1**, using $Ru(dmob)_2Cl_2$ (302 mg, 0.5 mmol) in place of $Ru(bpy)_2Cl_2$.

Ru1: ^{1}H-NMR (400 MHz, DMSO) δ: 14.73-14.28 (m, 1H), 9.43 (d, *J*=7.1 Hz, 1H), 9.09 (d, *J*=8.1 Hz, 2H), 8.87 (dd, *J*=15.5, 8.2 Hz, 4H), 8.51 (d, *J*=8.6 Hz, 2H), 8.33 (s, 1H), 8.25-7.81 (m, 13H), 7.60 (dd, *J*=11.1, 5.8 Hz, 4H), 7.39-7.31 (m, 2H); ^{13}C-NMR (101 MHz, DMSO) δ： 156.83 (s), 156.63 (s), 152.67 (s), 151.44 (s), 149.46 (s), 144.91 (s), 142.52 (s), 138.43-137.41 (m), 137.41-137.11 (m), 130.41 (s), 127.85 (d, *J*=12.8 Hz), 126.17 (s), 124.49 (s), 119.87 (s), 63.88 (s). HRMS (ESI) m/z: calcd. 388.5831 for $C_{41}H_{27}N_{11}Ru$ $[M-2H]^{2-}$, found: 388.5847.

Ru2: ^{1}H-NMR (400 MHz, DMSO) δ: 9.42 (s, 1H), 9.05 (d, *J*=8.0 Hz, 2H), 8.72 (d, *J*=17.1 Hz, 4H), 8.51 (d, *J*=8.6 Hz, 2H), 8.31 (s, 1H), 8.11 (d, *J*=8.2 Hz, 2H), 7.99 (s, 2H), 7.87 (d, *J*=6.0 Hz, 2H), 7.67 (d, *J*=5.7 Hz, 2H), 7.41 (t, *J*=6.2 Hz, 4H), 7.17 (d, *J*=5.7 Hz, 2H), 2.55 (s, 6H), 2.45 (s, 6H);^{13}C NMR (101 MHz, DMSO) δ: 156.29 (d, *J*=14.1 Hz), 152.64 (s), 150.48 (d, *J*=10.8 Hz), 149.46 (d, *J*=13.4 Hz), 144.99 (s), 142.48 (s), 137.30 (s), 129.95 (s), 129.39 (s), 128.45 (d, *J*=14.2 Hz), 127.81 (s), 125.98 (s), 125.84 (d, *J*=24.2 Hz), 125.01 (s), 119.83 (s), 20.74 (d, *J*=9.4 Hz). HRMS (ESI) m/z: calcd. 416.6144 for $C_{45}H_{35}N_{11}Ru$ $[M-2H]^{2-}$, found: 416.6163.

Ru3: ^{1}H-NMR (400 MHz, DMSO) δ: 9.46 (s, 1H), 9.09 (d, *J*=8.0 Hz, 2H), 8.89 (d, *J*=13.0 Hz, 4H), 8.50 (d, *J*=8.7 Hz, 2H), 8.33 (s, 1H), 8.17 (d, *J*=8.5 Hz, 2H), 8.02-7.92 (m, 4H), 7.64 (dd, *J*=20.2, 6.1 Hz, 4H), 7.48 (d, *J*=6.1 Hz, 2H), 7.34 (d, *J*=6.0 Hz, 2H), 1.43 (s, 18H), 1.34 (s, 18H); ^{13}C-NMR (101 MHz, DMSO) δ： 161.58 (d, *J*=17.7 Hz), 156.54 (d, *J*=18.3 Hz), 152.43 (s), 150.72 (s), 144.06 (s), 142.21 (s), 129.74 (s), 127.44 (s), 125.28 (s), 124.91-124.85 (m), 124.63 (d, *J*=23.7 Hz), 121.79 (s), 119.52 (s), 67.04 (s), 36.00-35.31 (m), 30.06 (d, *J*=11.9 Hz), 25.14 (s). HRMS (ESI) m/z: calcd. 500.7085 for $C_{57}H_{59}N_{11}Ru$ $[M-2H]^{2-}$, found: 500.7113.

Ru4:^{1}H-NMR (400 MHz, DMSO) δ: 14.45 (s, 1H), 9.46 (s, 1H), 9.06 (s, 2H), 8.54-8.43 (m, 6H), 8.34 (s, 1H), 8.22-8.13 (m, 4H), 7.93 (s, 2H), 7.64 (d, *J*=6.4 Hz, 2H), 7.34-7.19 (m, 4H), 6.93 (s, 2H), 4.03 (s, 6H), 3.93 (s, 6H);^{13}C-NMR (101 MHz, DMSO) δ: 166.53 (d, *J*=15.7 Hz), 157.94 (s), 152.78 (s), 128.04 (s), 120.01 (s), 114.01 (s), 111.28 (s), 99.57 (s), 63.89 (s), 56.76 (d, *J*=9.0 Hz). HRMS (ESI) m/z: calcd. 448.6043 for $C_{45}H_{35}N11O_4Ru$ $[M-2H]^{2-}$, found: 448.6065.

3. Antibacterial activity evaluation

Minimum inhibitory concentration (MIC) assay

The overnight cultured bacteria were diluted in MH medium to 1:1000 as the working suspension.50 μL of the compounds were diluted by two-fold serial dilution to the desired concentration (0.39-200 μg/mL) in a 96-well plate. Then, the bacterial suspension (200 μL) were equally added into each well. There were three groups parallel in each concentration. The plate was incubated at 37 ℃ for 18 h. The MH medium only with bacteria suspension was used as the positive control.

The growth curve assay

The compounds were diluted to the final concentration (0.195 μg/mL, 0.39 μg/mL, 0.78 μg/mL, 1.56 μg/mL, 3.125 μg/mL) by the 1:100 dilution bacteria suspension in a 24-well plate. The plate was cultured in a shaking table at 220 rpm, 37 ℃. Then, the OD was recorded at 600nm on a microplate reader every 30 minutes for 10 h. The bacteria suspension without treatment of compounds was used as the positive control.

Minimum bacterial concentration (MBC) and the time-killing curve assay

The ruthenium complexes and the bacteria suspension were added into the shaking bacterial tubes with the final concentration of 2 × MIC, 4 × MIC, 8 × MIC, 16 × MIC, 32 × MIC, 64 × MIC and 128 × MIC. After 24 h cultured in 37 ℃, the diluted bacteria suspension was spread onto LB agar plates. After 18 h, the MBC was determined by the plate with no visible the viability of the bacteria.

Like the MBC assay, the final concentration of the complexes were 2 × MIC, 4 × MIC, 8 ×MIC and 16 ×MIC. Then, the diluted bacteria suspension was spread onto LB agar plates at the time of 0, 30, 60, 90 and 120 minutes.

Fluorescence staining of bacteria

The logarithmic phase growing *S. aureus* were washed with PBS for 3 times, and diluted to OD_{600nm}=0.3 by PBS. The **Ru3** (4 × MIC) and bacterial suspension were shaken for 2 h in 37 ℃. Then, 30 μL of DAPI (10 μg/mL) and 30μL of PI (20 μg/mL) were added into 500 μL bacterial suspension, and then the bacterial suspension was cultured in the dark for 15 min per stain. The bacteria were observed by a fluorescent cell imager. The kanamycin (4×MIC) was used as a negative control.

Bacterial membrane depolarization assay

The logarithmic phase growing *S. aureus* were washed with PBS for 3 times, and diluted to OD_{600nm}=0.3 by PBS. The **Ru3** (1 × MIC, 4 × MIC) and bacterial suspension were shaken for 2 h in 37℃.Then, 30 μL of 3,3-Dipropylthiadicarbocyanine Iodide ($DiSC_3(5)$) was added into 500 μL bacterial suspension, and then the bacterial suspension was cultured in the dark for 1h. The bacteria were observed by a fluorescent cell imager.

Outer membrane permeabilization assay

The bacteria were cultured with MH medium to the logarithmic phase and then washed by PBS for three times. After that, the MH medium was changed into M9 lactose medium for overnight. Then, the M9 medium was wiped off and the bacteria was re-suspended by PBS to OD_{600nm}=0.4. The **Ru3** (2 × MIC), *S. aureus* suspension and 100 μL ONPG (10 mg/mL) were added into shaking bacterial tubes. The value was determined by ultraviolet spectrophotometer (excitation λ=415nm) every 30 min within 2h.

Membrane integrity study

The logarithmic phase growing *S. aureus* were washed with PBS for 3 times, and diluted to OD_{600nm}=0.3. The **Ru3** was added to obtain final concentration of 1 × MIC and 2 × MIC in a 24-well plate. After 2 h incubation at 37℃, the bacterial suspension was centrifuged (6000 rpm, 1 min) to harvest the supernatant. The value was determined by ultraviolet spectrophotometer (excitation λ=260nm). There were three groups parallel in each concentration. The negative control was untreated bacteria suspension.

Bacterial Morphology Assay (SEM)

The logarithmic phase growing *S. aureus* were washed with PBS for 3 times, and diluted to OD_{600nm}=0.3. The **Ru3** was added to obtain final concentration of 4×MIC and was cultured at 37℃ for 4h. After centrifugation, the *S. aureus* suspension was fixed overnight by a 2.5% glutaraldehyde in PBS at 4℃. Then, the stationary bacteria were washed with PBS for three times and trested with gradient ethanol (30, 50, 70, 80, 90 and 95%). After dehydrating, drying and coating, the samples were observed by a scanning electron microscopy.

Inhibition of bacterial toxin assay

The hemolytic rate was used to test the amount of bacterial toxin. The overnight cultured bacteria were diluted in MH medium to 1:100 as the working suspension. The **Ru3** was diluted to the desired concentrations (0.25×MIC, 0.5×MIC, 0.75×MIC) with the working suspension and was cultured for 14 h. Then, centrifuging the bacterial suspension to obtained the supernatant. The rabbits red blood cells (RBCs) were washed with PBS for three times. 1mL PBS, 40 μL supernatant and 30 μL RBCs were added to the sterilization 1.5 mL EP tubes and incubated at 37℃ for 30 minutes. After centrifugation, the supernatant (200 μL) was transferred to a 96-well plate and measured at 540nm on a microplate reader. The hemolytic rate was calculated by the follow:

$$\%hemolysis = \frac{OD^{sample} - OD^{nagetive\ control}}{OD^{positive\ control} - OD^{nagetive\ control}} \times 100$$

Inhibition the formation of *S. aureus* biofilm

S. aureus cultured overnight were diluted in MH medium to 1:100 as the working suspension. The **Ru3** was

diluted to the desired concentrations (0.25 × MIC, 0.5 × MIC, 0.75 × MIC, 1 × MIC, 2 × MIC, 4 × MIC) with the working suspension and was cultured for 48 h in the 24-well plates. There were three groups parallel in each concentration. Then, each well was washed with reverse osmosis (RO) water to remove the floating bacteria. After drying, the crystal violet solution was added to each well and shaken for 20 minutes. Washing and drying the floating stain, the crystal violet was dissolved by the 50% glacial acetic acid. The final value was determined at 595 nm on a microplate reader.

Activity of eliminate mature biofilm

The overnight cultured bacteria were diluted in MH medium to 1:100 as the working suspension. Bacterial suspension (200 μL) was added into each well and cultured for 24h at 37 ℃. The **Ru3** was diluted to five times of the desired concentration (0.39-200 μg/mL) by two-fold serial dilution. After washing the floating bacteria, 50 μL different complex solution and 200 μL fresh MH medium were added into each well and incubated for 24 h at 37℃. Then, the medium was slowly removed and washed for two times. The 100 μL 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (1%) was added into each well and incubated at 37℃ for 4h. 200 μL of DMSO was added into each well after removing the MTT solution. The value was measured at 540nm on a microplate reader. The ability of against mature *S. aureus* biofilm:

$$\%clearance\ rate = \frac{OD^{sample}}{OD^{control}} \times 100$$

Antimicrobial resistance assay

After MIC assay, the bacteria was obtained from the previous passage 0.5×MIC concentration and cultured in fresh medium at 37℃ until a logarithmic phase growth. The MIC of each passage was determined the same as the normal MIC assay procedure. The development of drug resistance was measured by the ratio of every generation to the first generation.

Drug toxicity assay

Hemolysin activity: The half hemolysis concentration (HC_{50}) was used to assess the cytotoxicity of the ruthenium polypyridines complexes. The **Ru3** was diluted to the different concentration (0.78-100 μg/mL) with PBS by two-fold serial dilution. Other compounds were diluted to the final concentration of 250 μg/mL with PBS in the 1.5 mL EP tubes. The rabbits red blood cells (RBCs) were washed with PBS for three times. Then, 40 μL RBCs were added into each tube, and incubate at 37℃ for 30 minutes. After centrifugation, the supernatant (200 μL) was transferred to a 96-well plate and measured at 543 nm on a microplate reader. The hemolytic rate was calculated by the follow:

$$\%hemolysis = \frac{OD^{sample} - OD^{nagetive\ control}}{OD^{positive\ control} - OD^{nagetive\ control}} \times 100$$

Acute toxicity assay in mice: The acute toxicity test was performed using male Kunming mice. The mice were divided into 5 group (n =6 each group). The **Ru3** was diluted by DMSO: Tween-80: 0.5% CMC-Na = 2: 1: 17 to the final concentration of 150, 100, 50 mg/kg, and vancomycin was diluted to 150 mg/kg as the same. The control group was treated with a solution of DMSO: Tween-80: 0.5% CMC-Na = 2: 1: 17. 0.5 mL of compounds were administrated intragastric injection to each mouse. Then, the abnormal behavior and death of the mice were observed within 72h.

In vivo toxicity assay in *Galleria mellonella*: The *G. mellonella* (purchased from Tianjin Huiyude Biotech Company) model was used to evaluate the toxicity of the **Ru3** in vivo. The Ru3 and Polymyxin B were diluted to the high concentration (128 mg/kg and 256 mg/kg) with DMSO. The *G. mellonella* were randomly divided into five groups (n=8 per group). 5μL of compounds were injected into the *G. mellonell*a by the microsyringes. The survival rates of *G. mellonella* were valued within 5 days at 28℃. The negative control was treated with DMSO.

In vivo anti-infective assay

Galleria mellonella infection assay: The logarithmic phase growing *S. aureus* were washed with PBS for 3 times, and diluted to OD_{600nm}=0.3 as working suspension. Each group of 8 larvae was injected with 5μL of the bacterial suspension for 1h. The **Ru3** and Vancomycin were diluted to the final concentration of 10 mg/kg and 20 mg/kg with DMSO. Then, 5μL of compounds were injected into the *G. mellonell*a. The survival of the *G. mellonella* were observed within 7 days at 28℃. The negative control was treated with DMSO only.

Mice skin-infection assay: Female Kunming mice were used in the *S. aureus* infected skin wound model. After removing the fur from the back of the mouse, a 8 mm full-thickness wounds was generated using a biopsy punch and surgical scissors. The logarithmic phase growing *S. aureus* were washed with PBS for 3 times, and diluted to OD_{600nm}=1.0 as working suspension. The wounds were infected by a 50 μL bacterial suspension per wound for 24 h. Then, the successfully infected mice were randomly divided into four groups (n=5 per group): saline, 50 μg/mL of **Ru3**, 100 μg/mL of **Ru3**,100 μg/mL of Vancomycin. The **Ru3** and Vancomycin were diluted to desired concentration by cream base and applied to the wound of the mice three times a day. The evolution of the wound size and body weight were recorded daily. After the treatment of 10 days, the mice were sacrificed and the wounds were obtained by the surgical scissors. After grinding, the homogenate was plated on agar plates to determine CFU. The wounds and viscera were fixed with 4% paraformaldehyde overnight. After that, the samples were embedded in paraffin and sectioned. Then the sections were stained with hematoxylin & eosin.

4. The spectrum of Ligand and Ruthenium Complexes

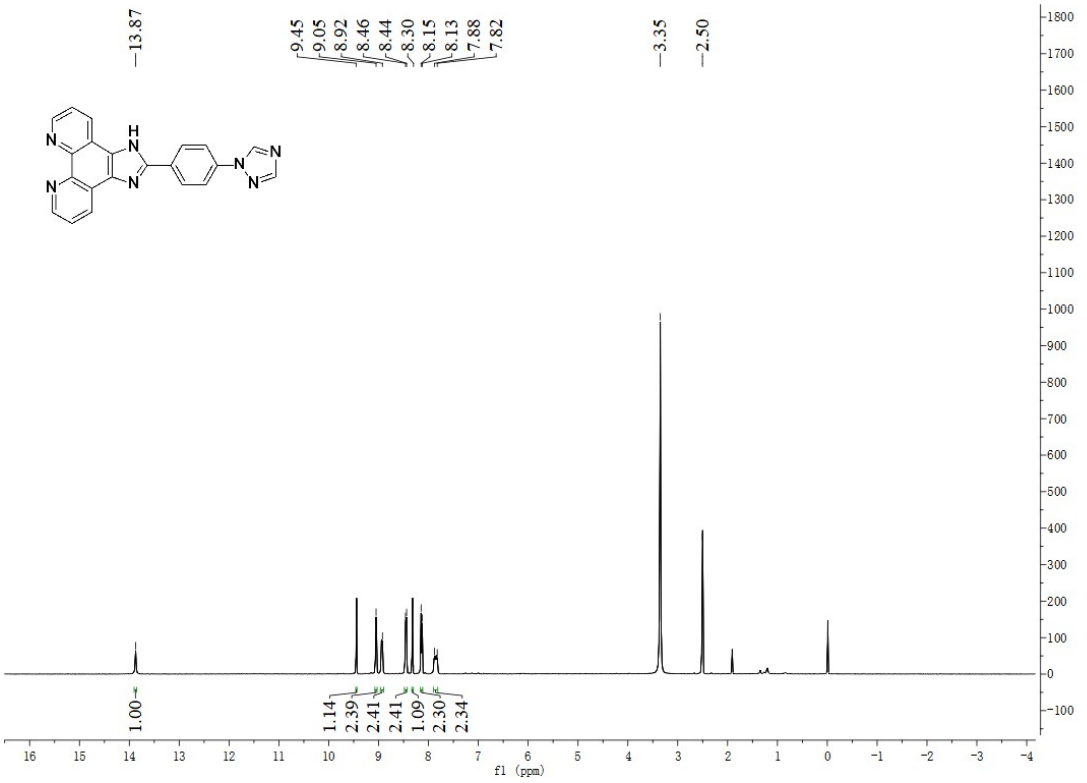

Figure S1. ^{1}H-NMR spectrum of **TPIP** in DMSO, 400MHz.

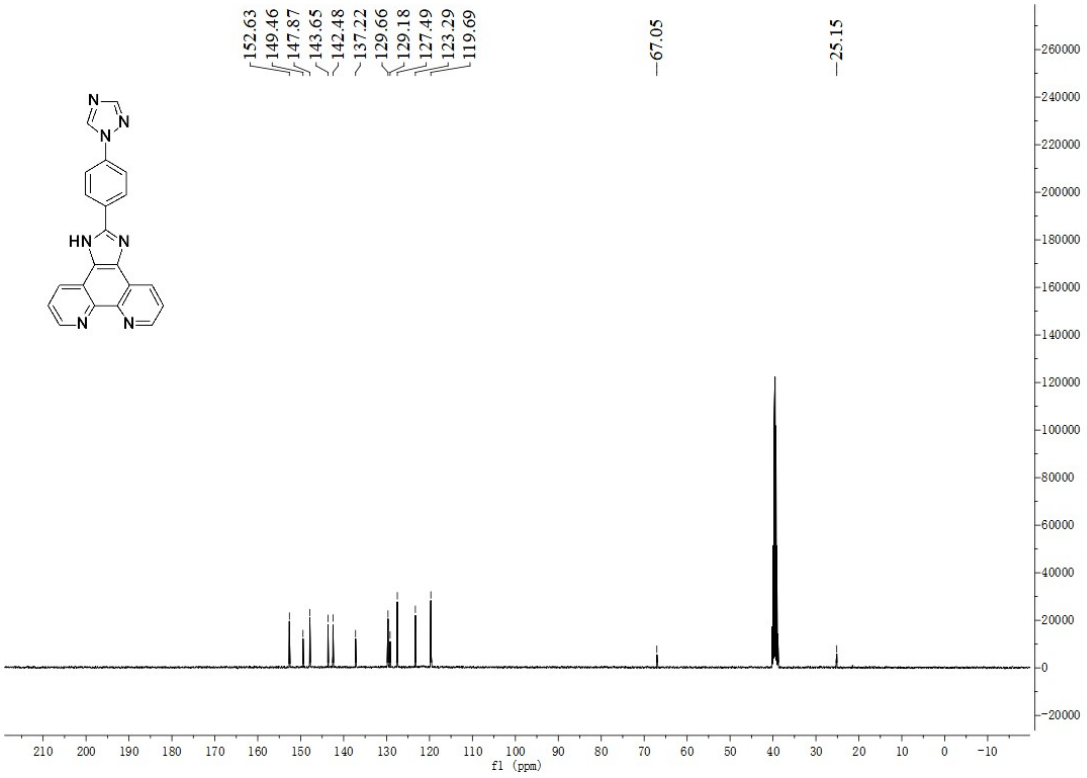

Figure S2. ^{13}C-NMR spectrum of **TPIP** in DMSO, 101MHz.

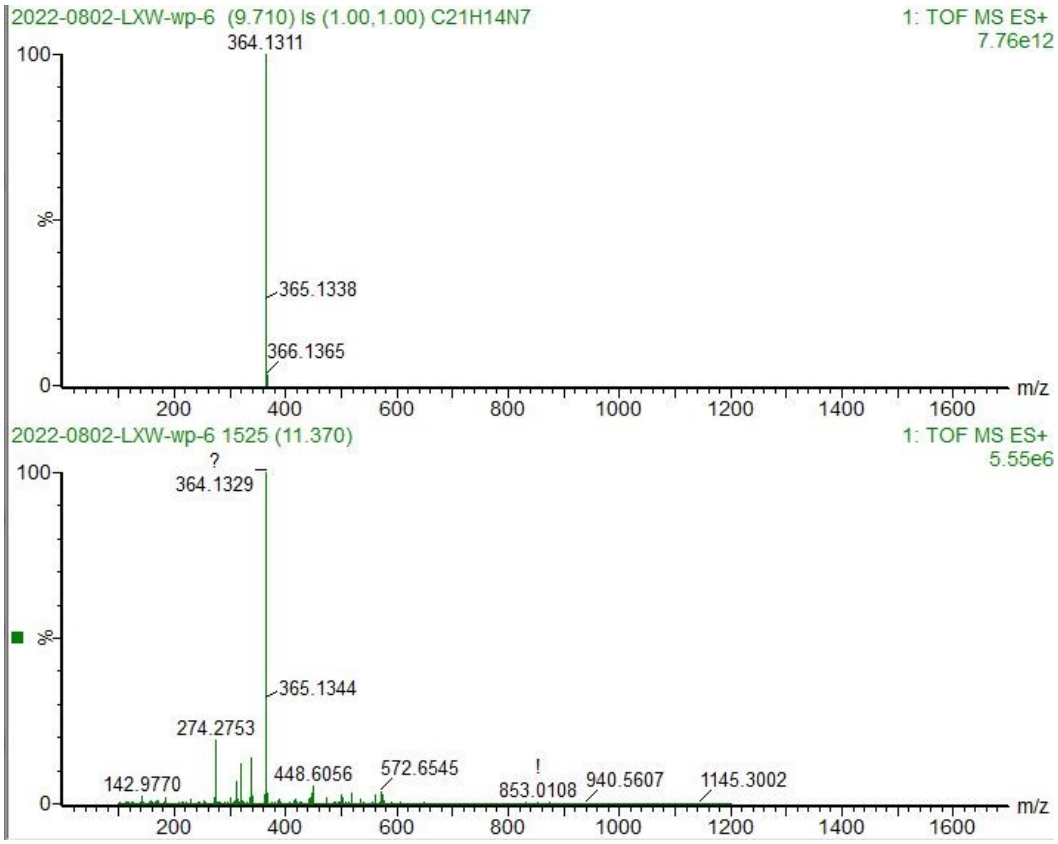

Figure S3. Representative HNMR-MS of **TPIP.**

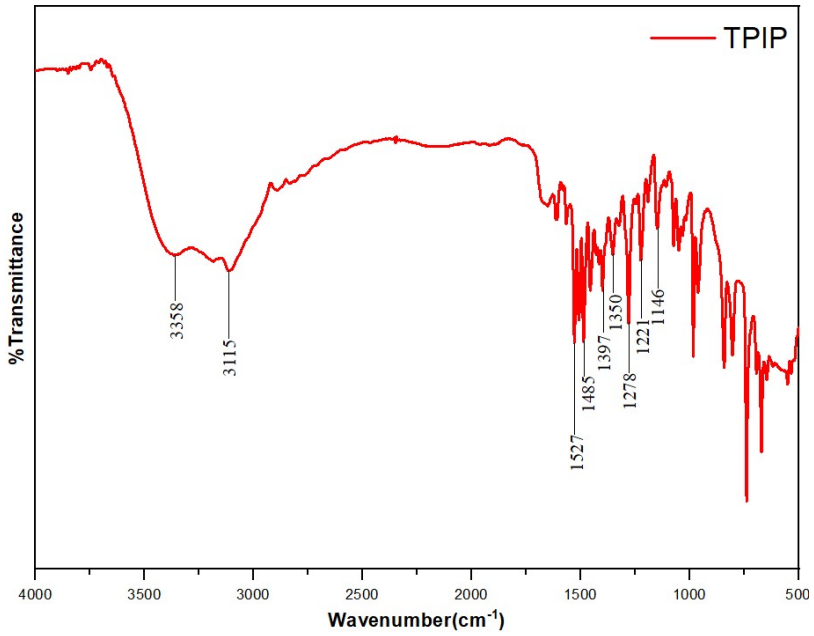

Figure S4. FTIR spectra of **TPIP.**

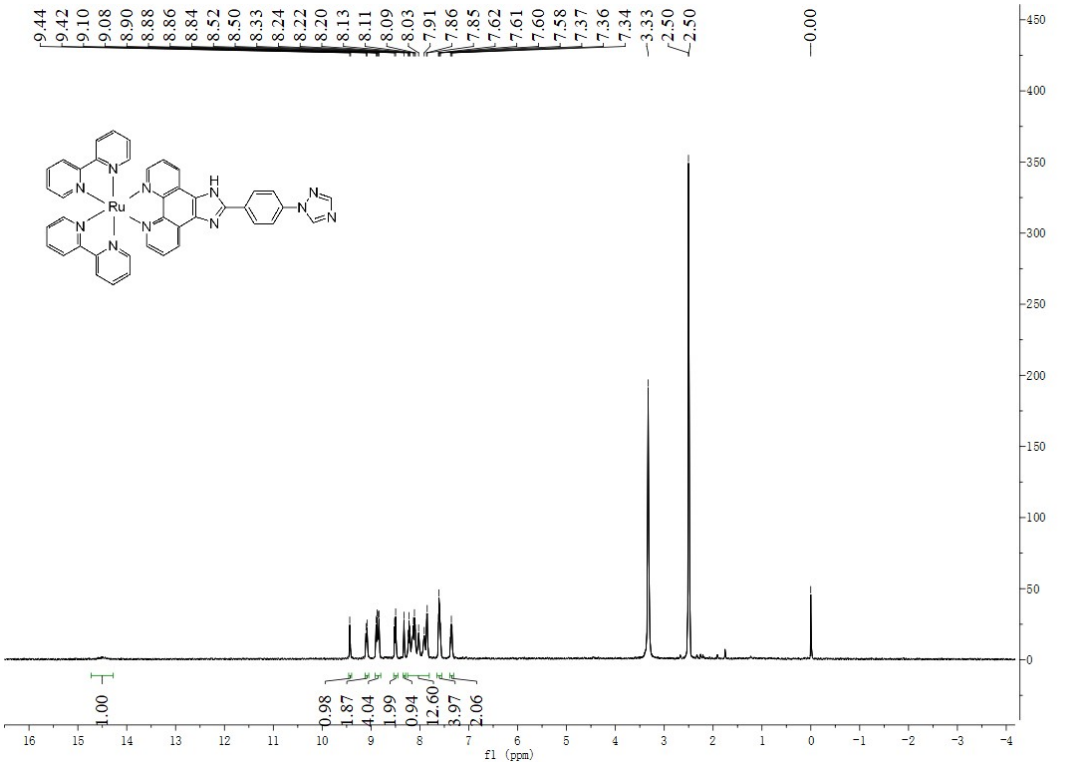

Figure S5. ^{1}H-NMR spectrum of **Ru1** in DMSO, 400MHz.

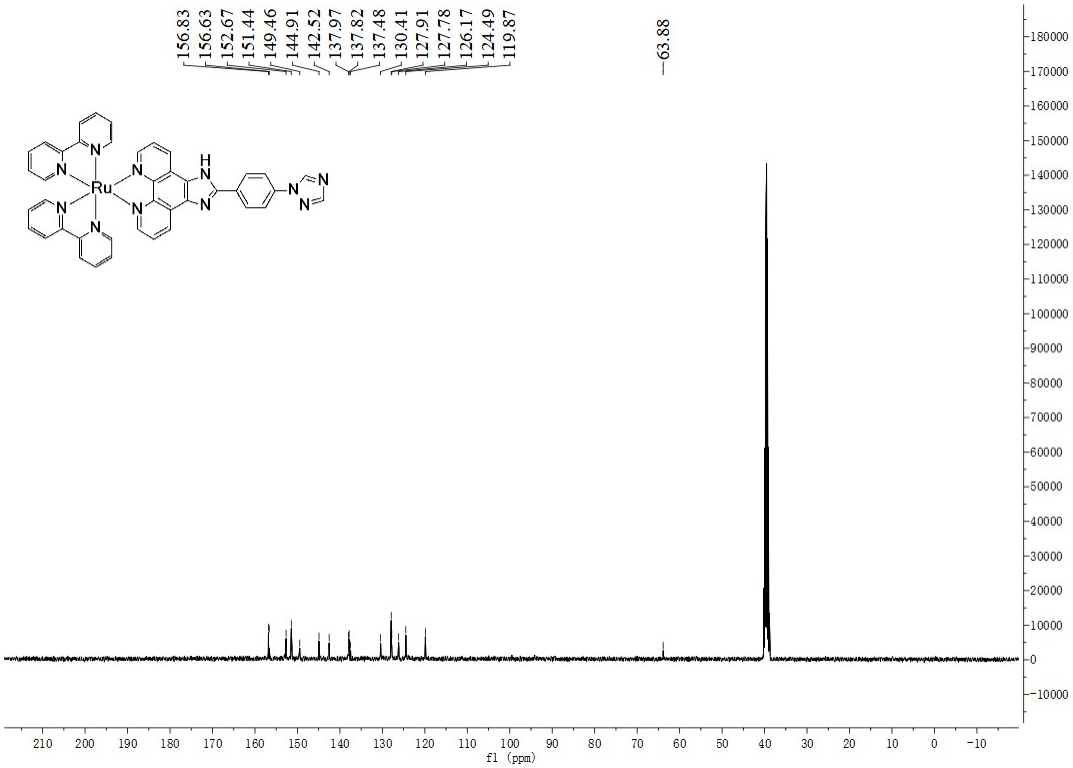

Figure S6. ^{13}C-NMR spectrum of **Ru1** in DMSO, 101MHz.

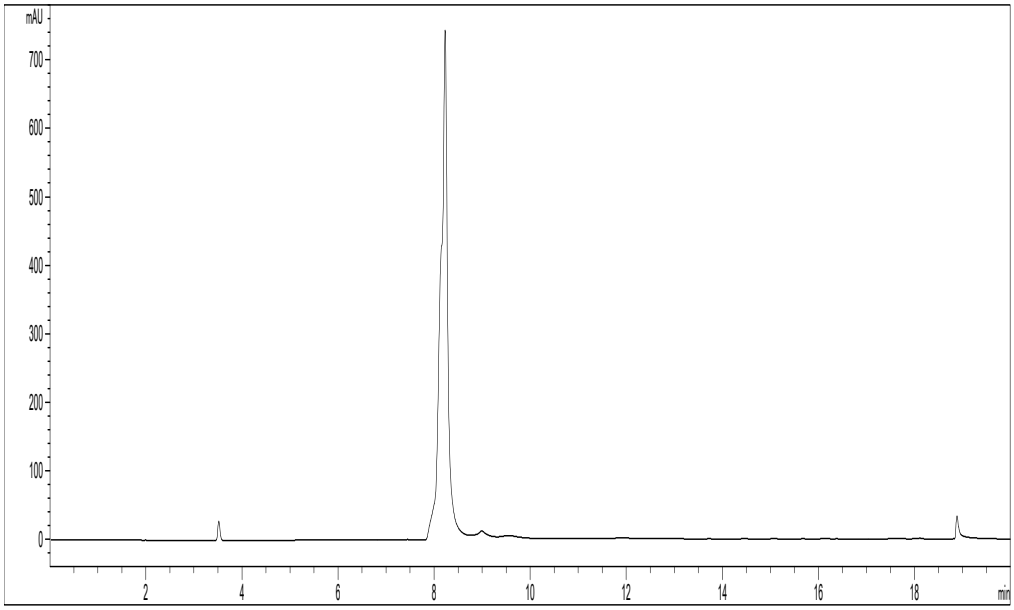

Figure S7. HPLC chromatogram of **Ru1** (290 nm).

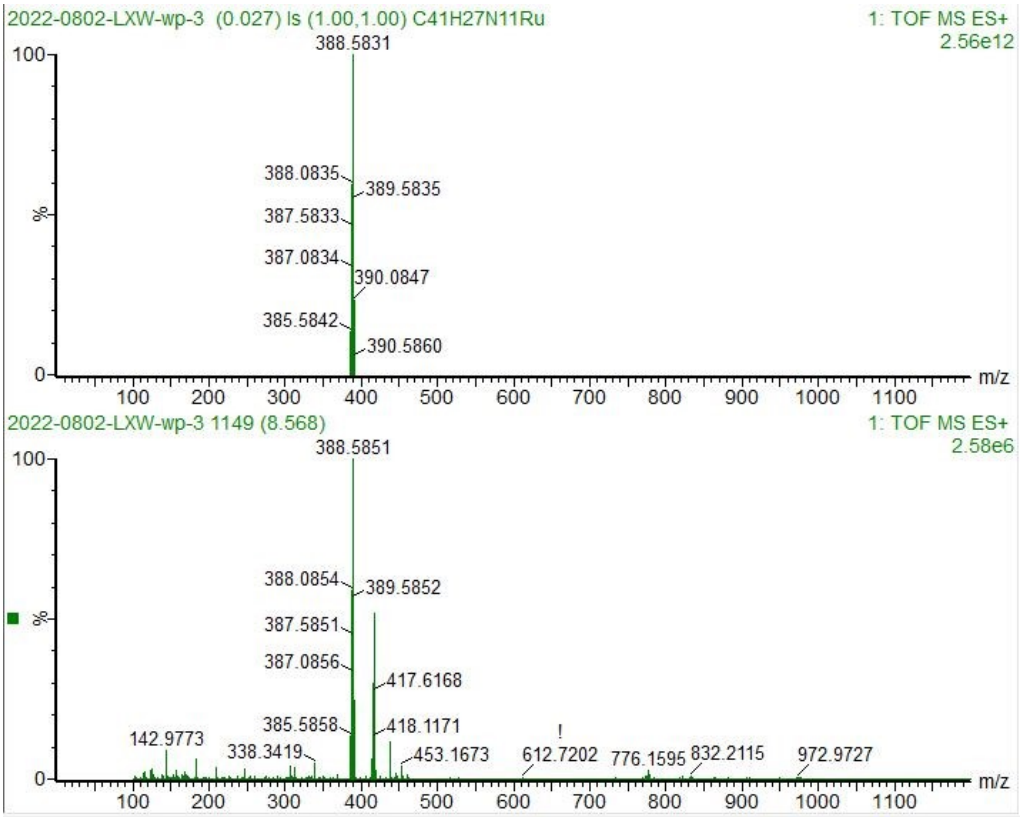

Figure S8. Representative HNMR-MS of **Ru1.**

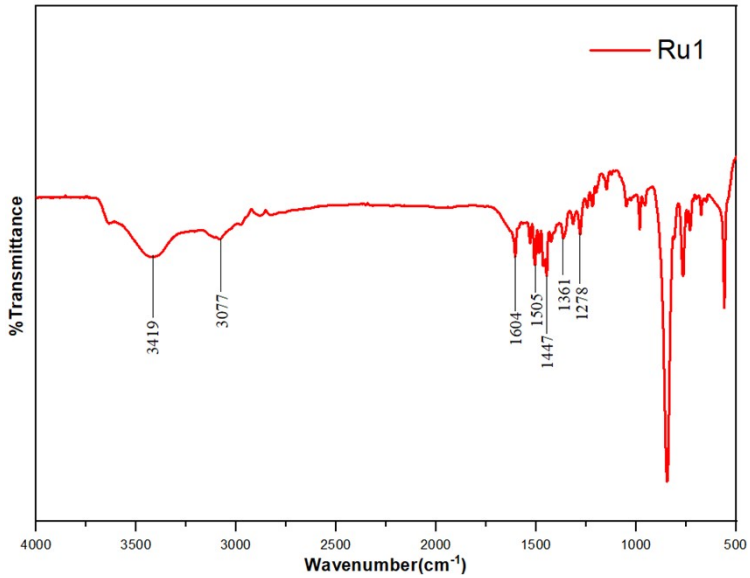

Figure S9. FTIR spectra of **Ru1.**

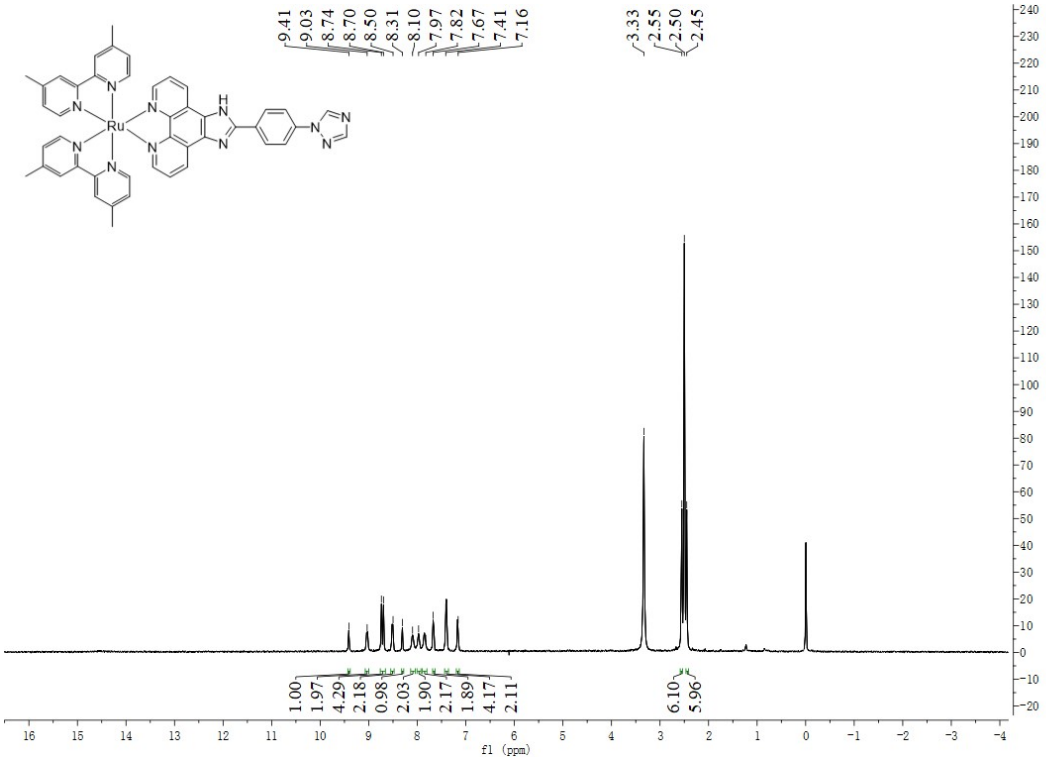

Figure S10. ^{1}H-NMR spectrum of **Ru2** in DMSO, 400MHz.

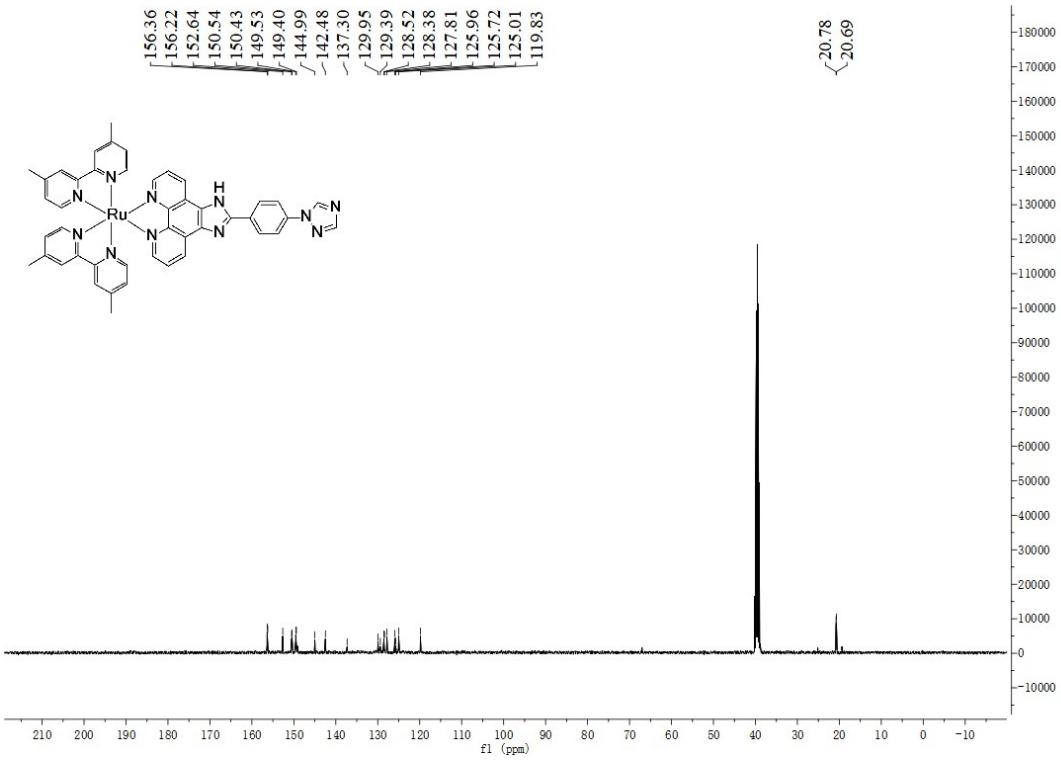

Figure S11. ^{13}C-NMR spectrum of **Ru2** in DMSO, 101MHz.

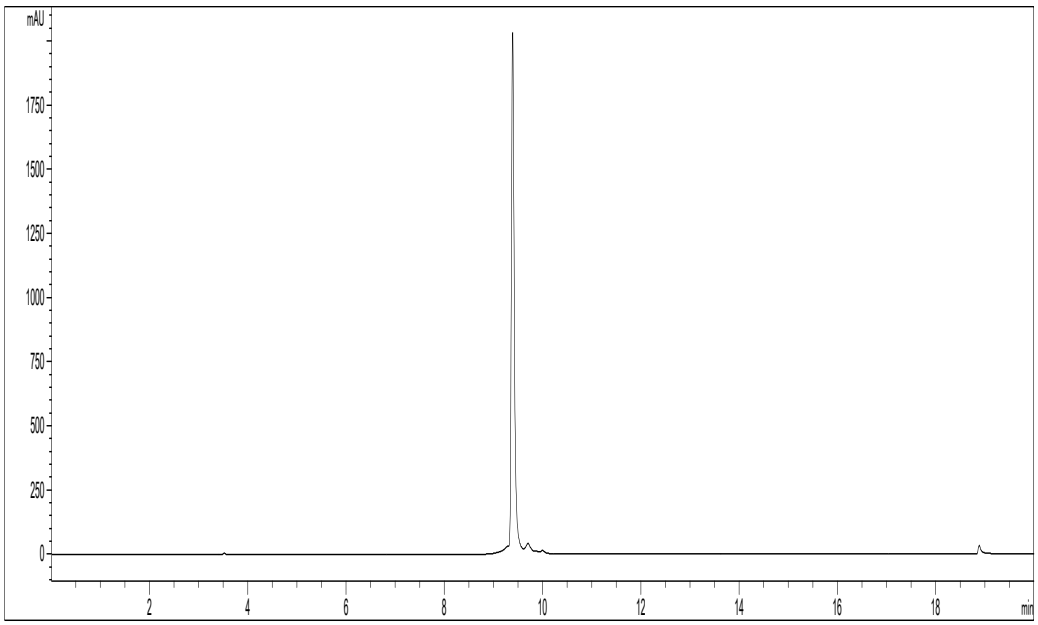

Figure S12. HPLC chromatogram of **Ru2** (290 nm).

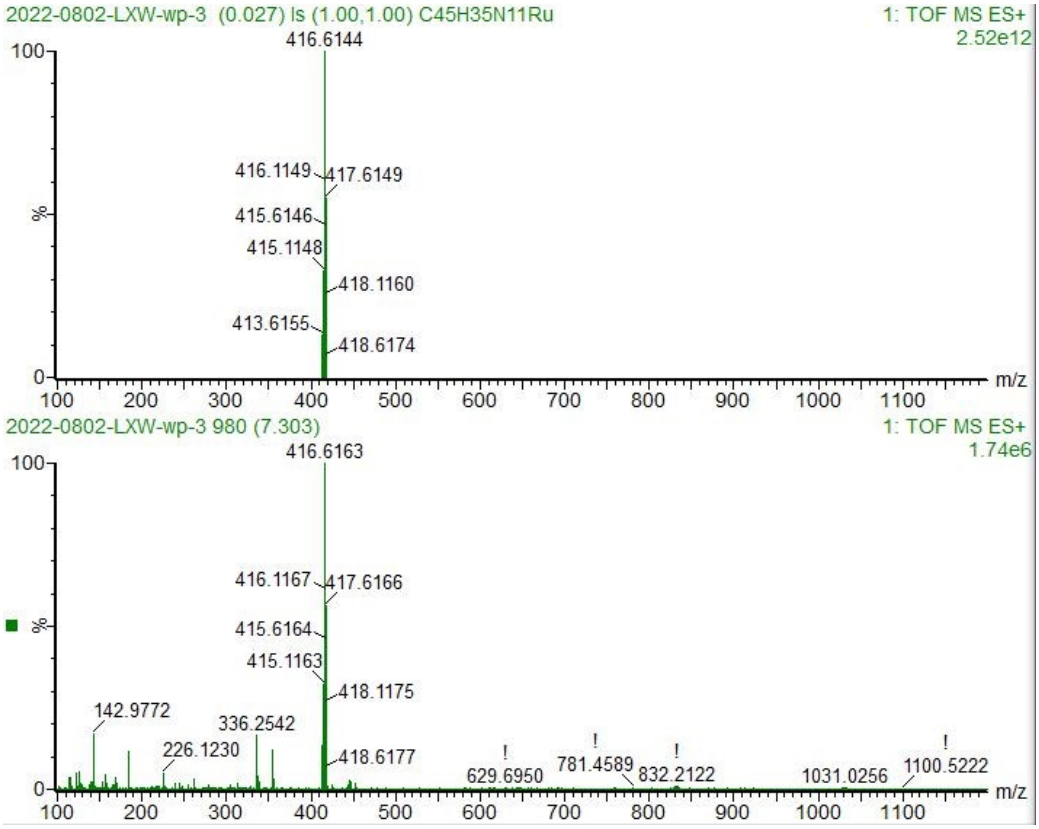

Figure S13. Representative HNMR-MS of **Ru2**.

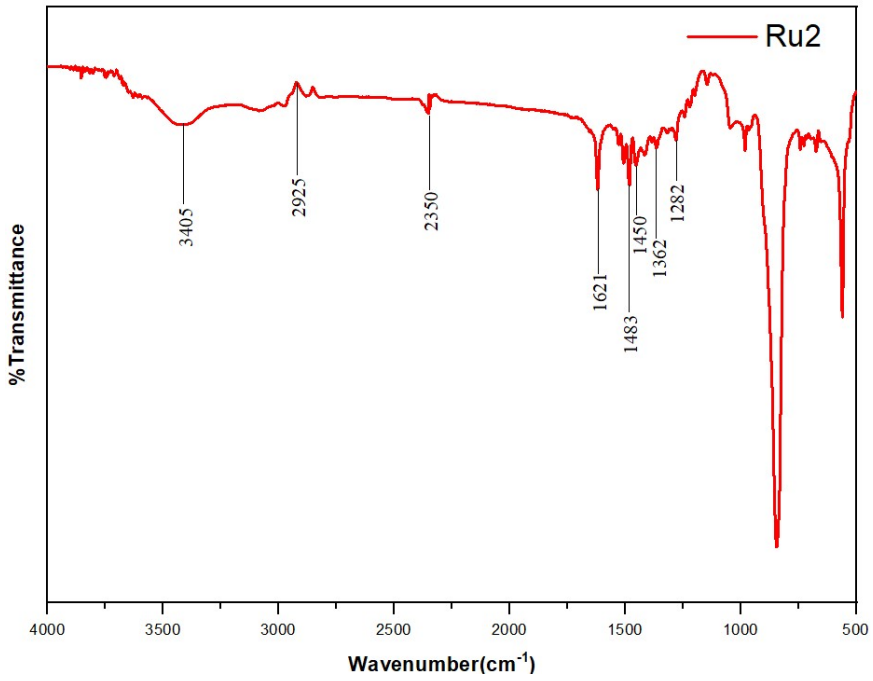

Figure S14. FTIR spectra of **Ru2**.

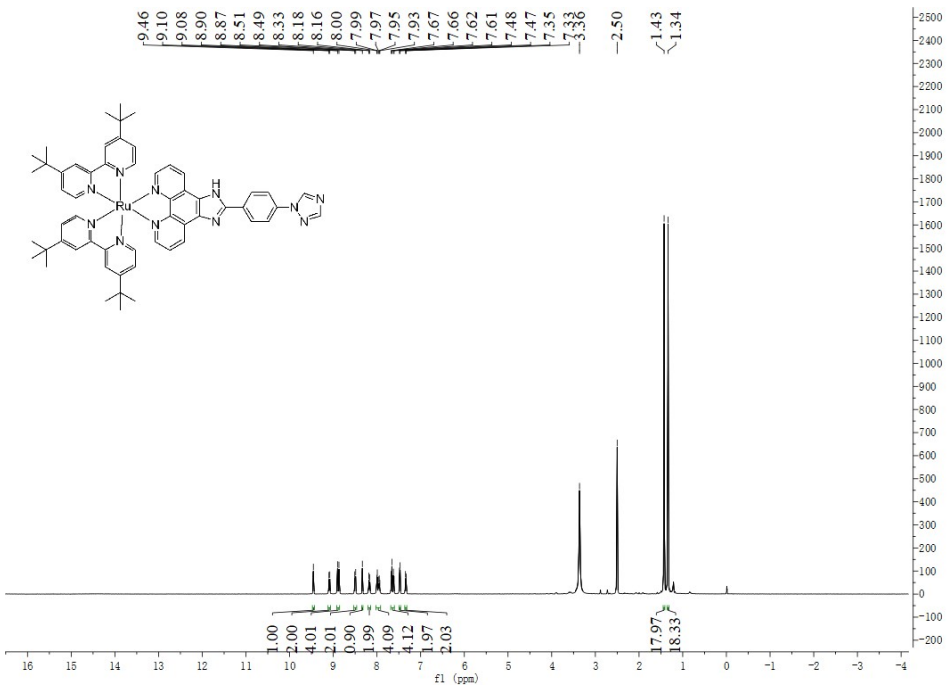

Figure S15. ^{1}H-NMR spectrum of **Ru3** in DMSO, 400MHz.

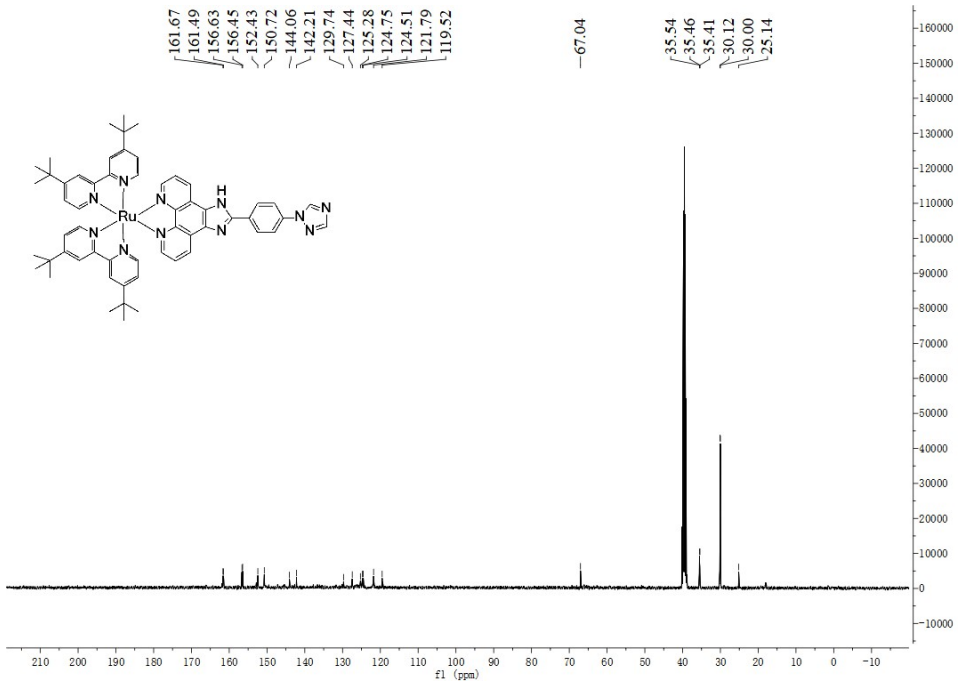

Figure S16. ^{13}C-NMR spectrum of **Ru3** in DMSO,101MHz.

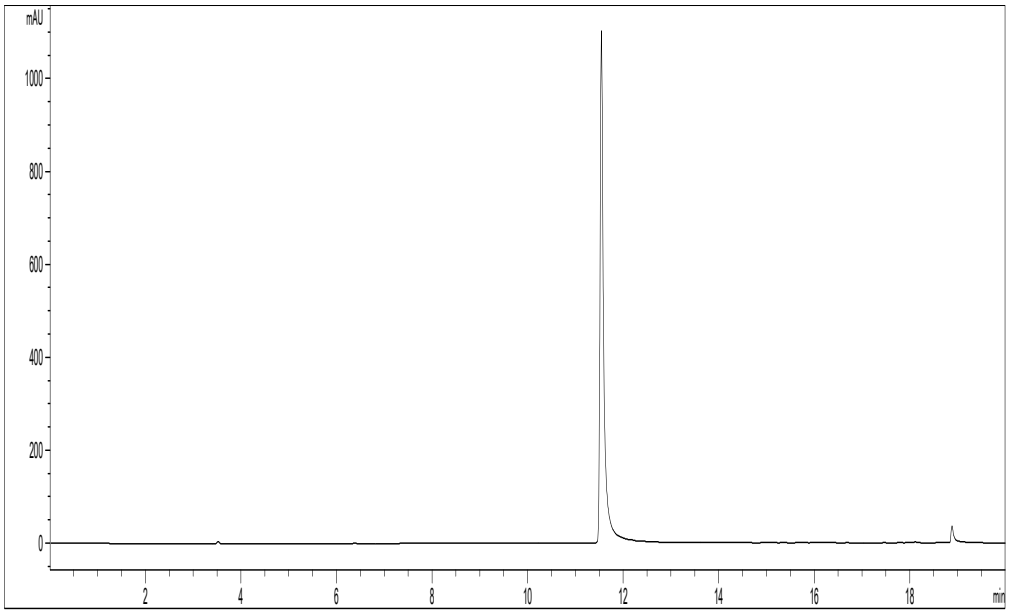

Figure S17. HPLC chromatogram of **Ru3** (290 nm).

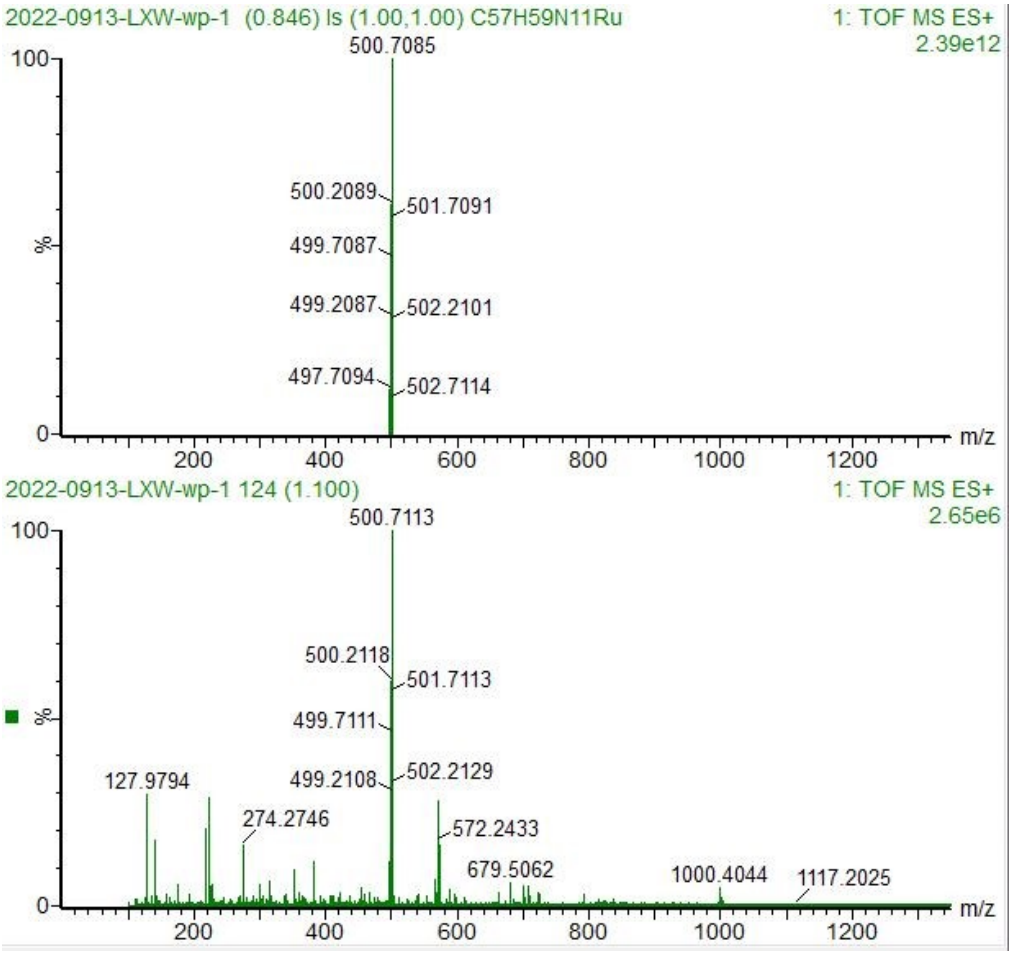

Figure S18. Representative HNMR-MS of **Ru3**.

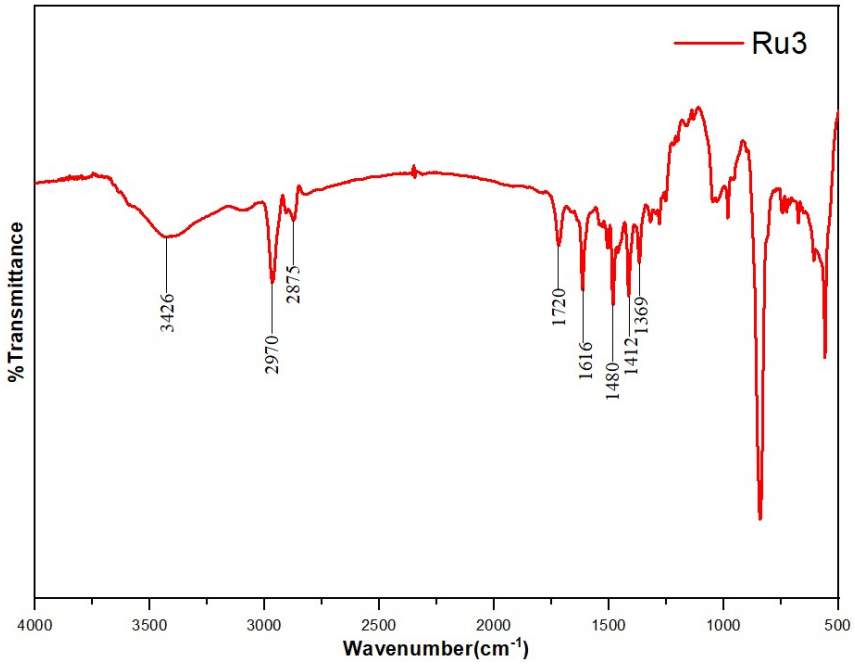

Figure S19. FTIR spectra of **Ru3**.

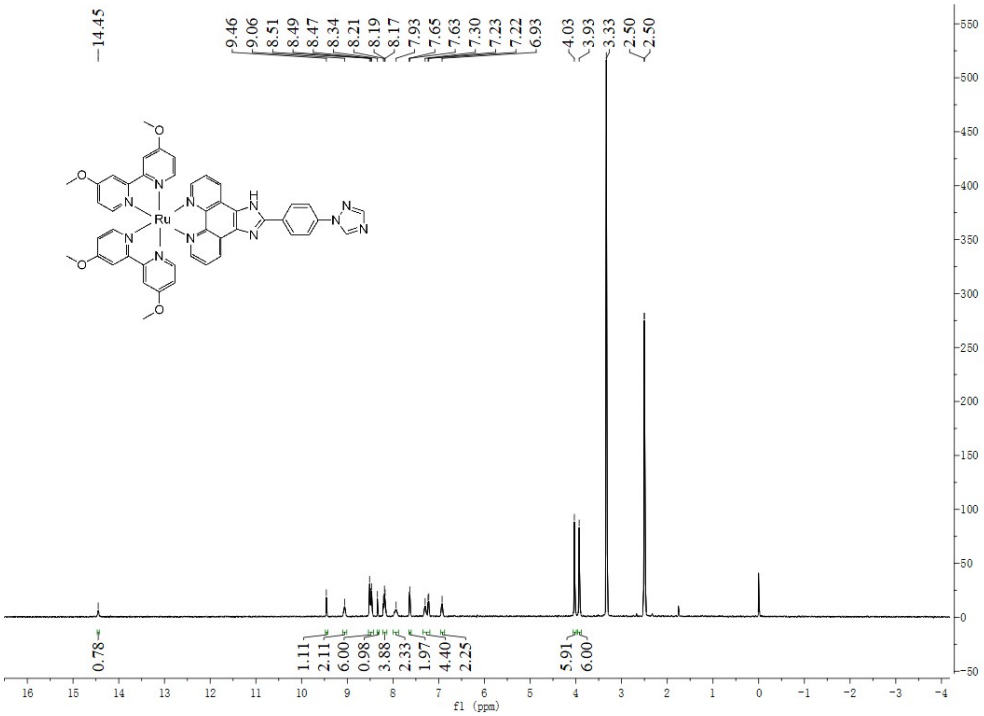

Figure S20. ^{1}H-NMR spectrum of **Ru4** in DMSO, 400MHz.

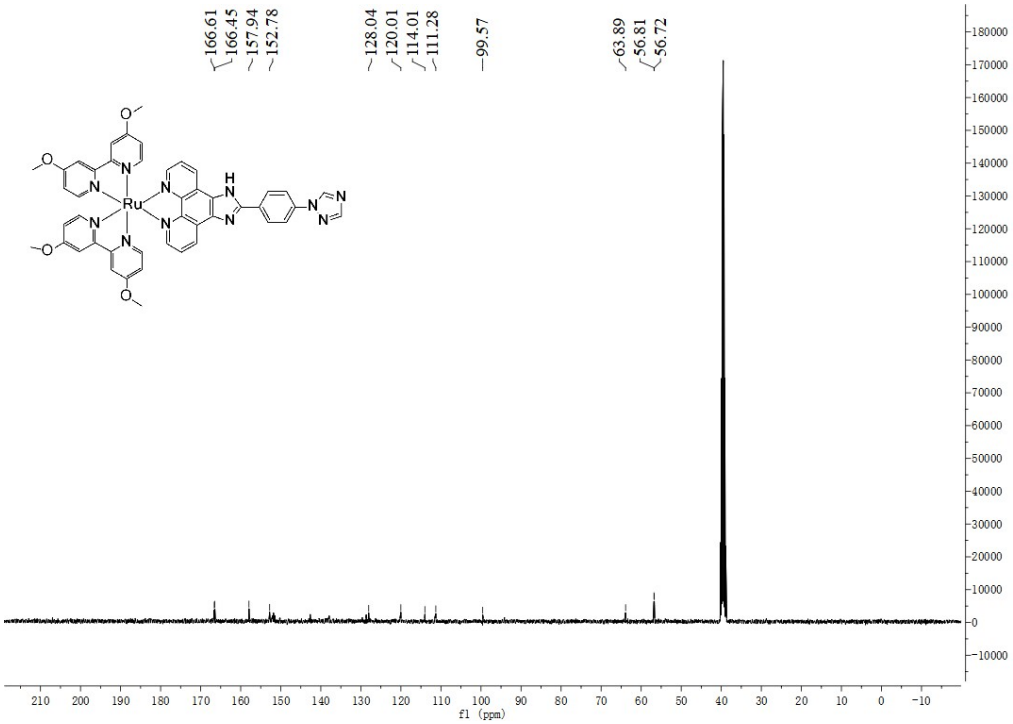

Figure S21. ^{13}C-NMR spectrum of **Ru4** in DMSO, 101MHz.

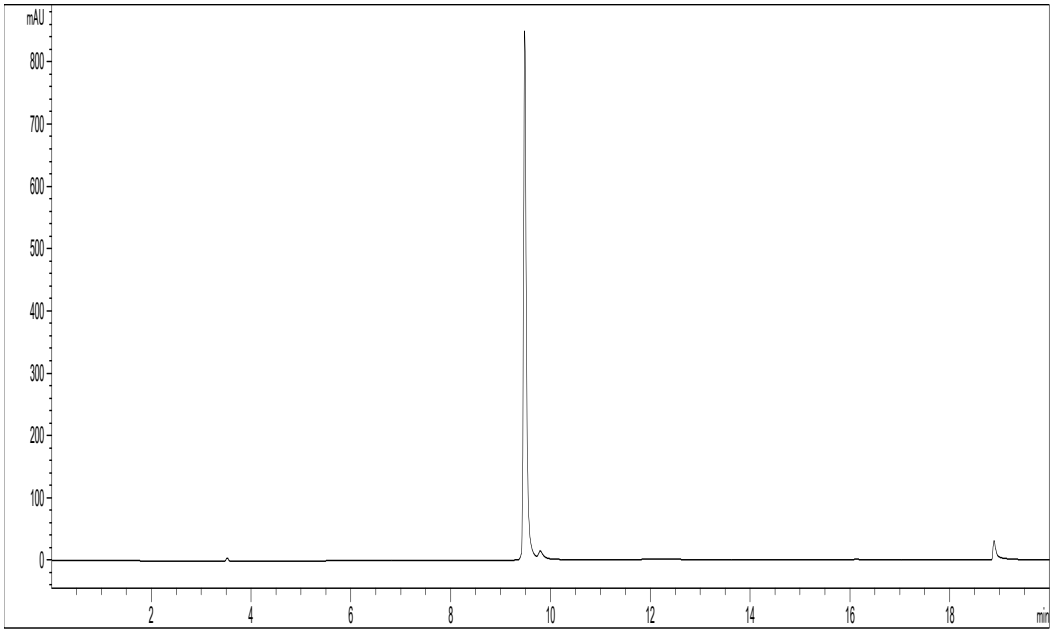

Figure S22. HPLC chromatogram of **Ru4** (290 nm).

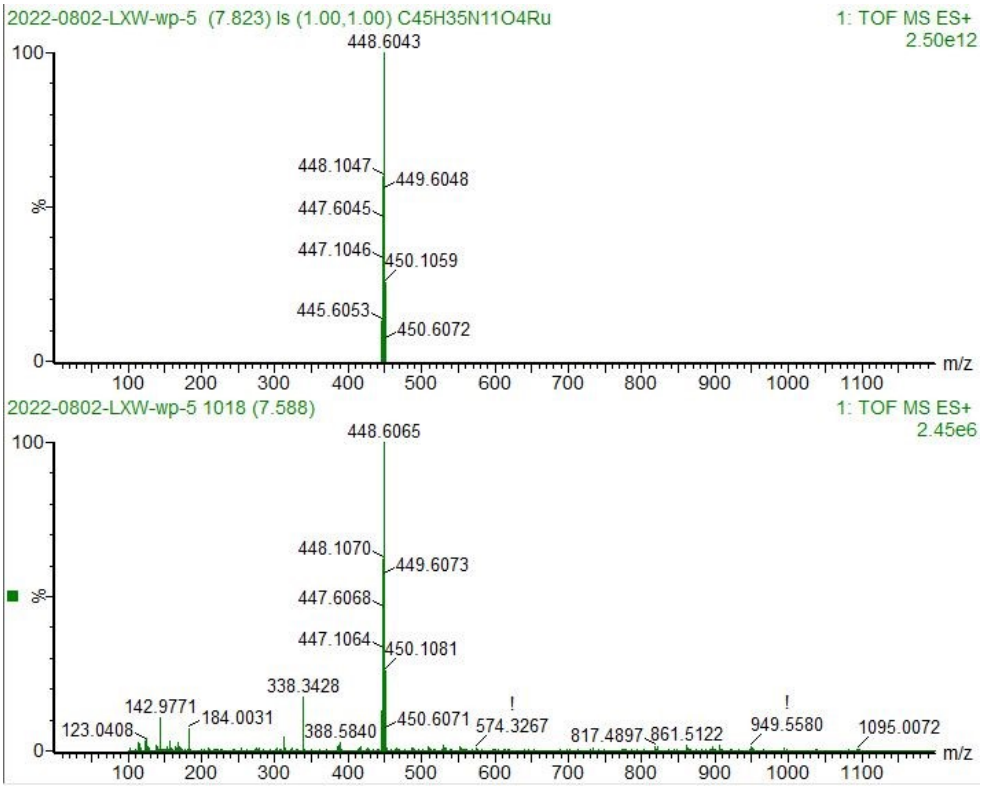

Figure S23. Representative HNMR-MS of **Ru4**.

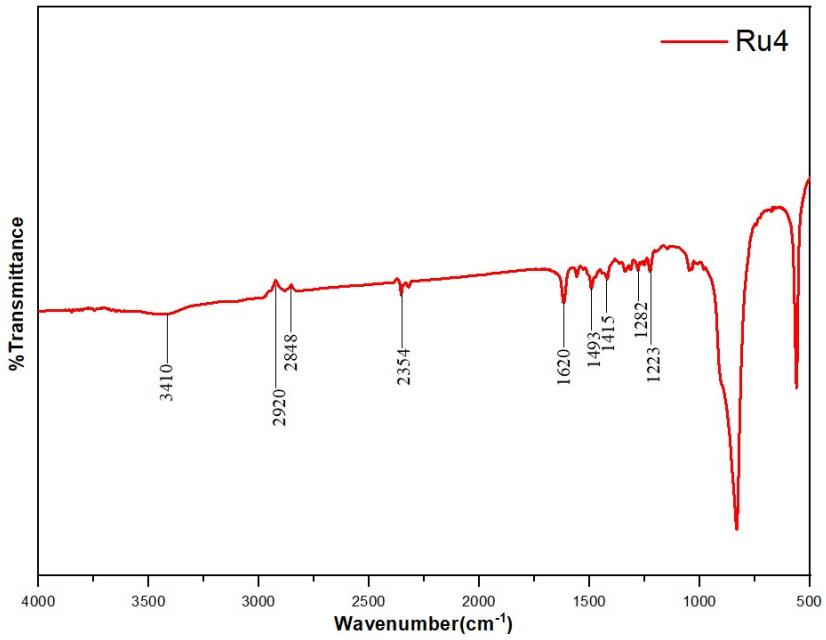

Figure S24. FTIR spectra of **Ru4**.

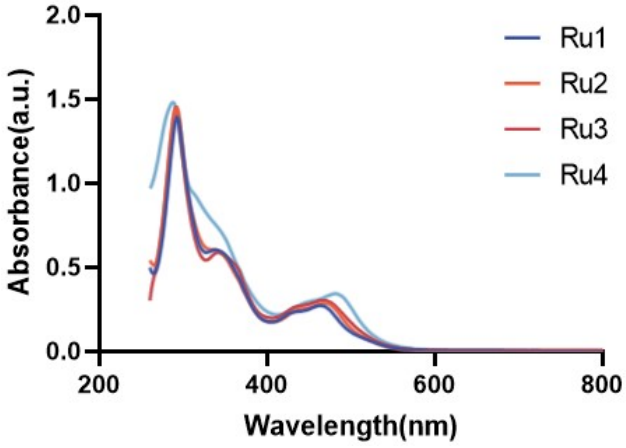

Figure S25. UV-Vis spectrum of **Ru1**, **Ru2**, **Ru3**, **Ru4** in DMSO ,20 μ g/mL.

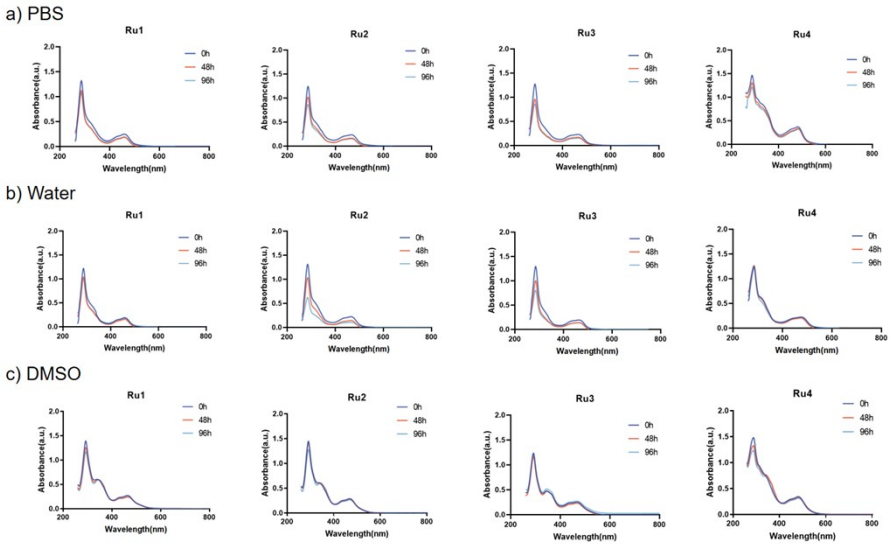

Figure S26. The stability of **Ru1**, **Ru2**, **Ru3**, **Ru4** in a) PBS, b) water, c) DMSO ,20 μg/mL.

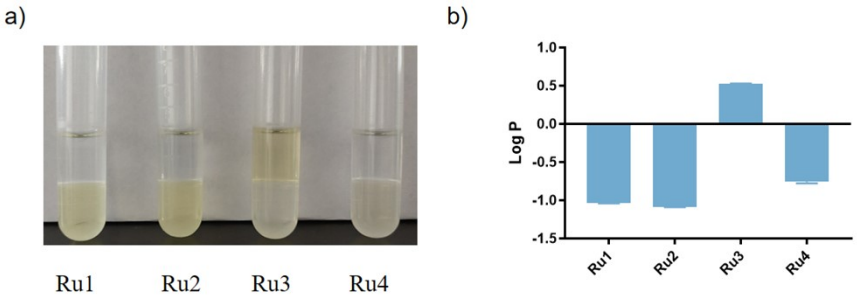

Figure S27. The lipid water partition coefficient of four complexes.

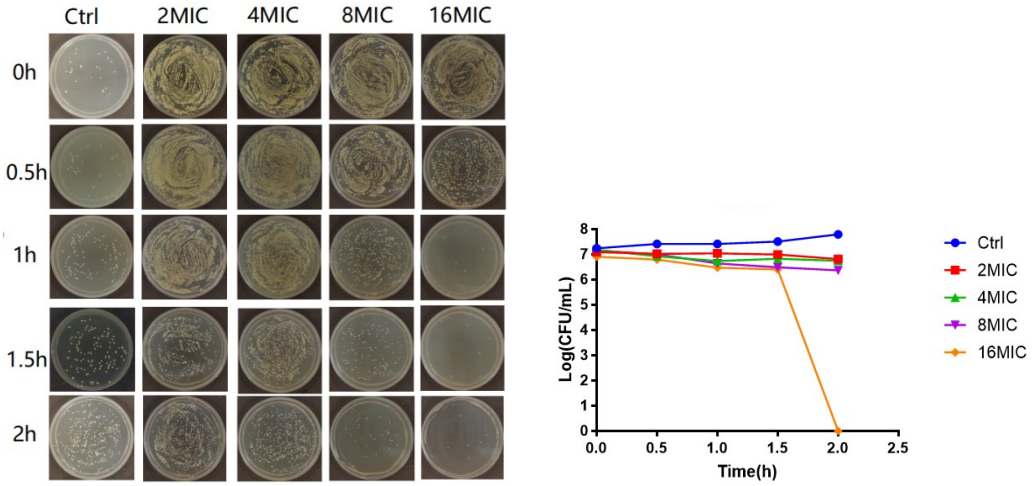

Figure S28. The time-killing curves of **Ru2** with 2×MIC, 4×MIC, 8×MIC, 16×MIC against *S. aureus*. Reverses Osmosis water was used as controls.