Electronic Supplementary Material (ESI) for ChemComm.

A Photoactivatable Znsalen Complex for Super-Resolution Imaging of Mitochondria in Living Cells

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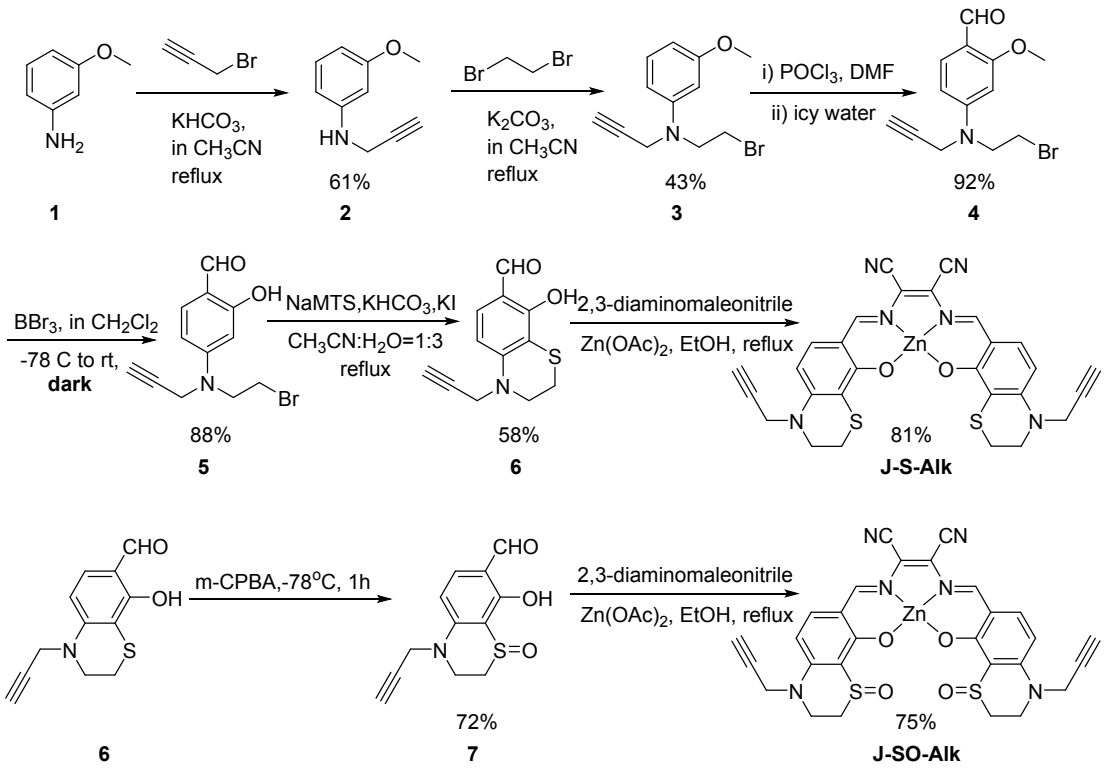
Contents

1. General experimental information

All solvents and chemicals for synthesis were purchased from Alfa Aesar and J&K and used as received without further purification, unless otherwise specified. Cellular imaging tracker was purchased from Invitrogen (Life Technologies)..

The ^{1}H NMR spectroscopic measurements were carried out using a Bruker-400 NMR at 400 MHz with tetramethysilane (TMS) as internal reference. Electrospray ionization (ESI) mass spectra were performed on a Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (Bruker, USA), positive-ion mode. The steady-state absorption spectra were obtained with an Agilent 8453 UV-*vis* spectrophotometer in 1 cm path length quartz cells. Single-photon luminescence spectra were recorded using fluorescence lifetime (Edinburgh Instrument FLS920) and steady state spectrophotometer (Hitachi F7000). Quantum yields of one photon emission of all the synthesized compounds were measured relative to the fluorescence of Rhodamine B (Φ=0.65) in ethanol. FT-IR spectra were taken on a Nicolet iN10 MX Fourier Transform Infrared Spectrometer. Confocal fluorescence images of living cells were performed using Nikon A1R-si Laser Scanning Confocal Microscope (Japan), equipped with lasers of 405/488/543/638 nm. STORM imaging was conducted by an Olympus IX71 inverted microscope equipped with a 100×, 1.45 NA oil objective (Olympus PLAN APO). The fluorescence signals were acquired using an electron-multiplying charge coupled device (EMCCD) camera (Andor iXon DV-897 BV). Data analysis were fitted with a 2D Gaussian using MLE_GPU algorithm.

2. Synthesis and characterization



Compound **2**

A reaction mixture of compound **1** (4.0 g, 32.0 mmol), 3-bromoprophyne (3.8 g, 32.0 mmol) and K_2CO_3 (4.4 g, 32.0 mmol) in 50 mL acetonitrile was refluxed under nitrogen for 12 h. After evaporation, the residue was extracted with CH_2Cl_2, washed and dried with anhydrous Na_2SO_4. Then the concentrated liquid was further purified by column chromatography to give yellow oil (3.2 g, 61%).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.33(1H,s), 3.79(3H,s), 3.91(2H,s), 4.09(1H,s), 6.34(2H,t), 6.44(1H,d), 7.20(1H,t)

Compound **3**

A reaction mixture of compound **2** (3.2 g, 22.4 mmol), 1,2-dibromoethane (44.0 g, 224 mmol) and $KHCO_3$ (2.7 g, 27.2 mmol) in 50 mL acetonitrile was refluxed under nitrogen for 12 h. After evaporation, the residue was extracted with CH_2Cl_2, washed and dried with anhydrous Na_2SO_4. Then the concentrated liquid was further purified by column chromatography to give yellow oil (2.3 g, 43%).

^{1}H NMR ($CDCl_3$, 400 MHz), δ : 2.96（s, 3H), 3.49 (t, 2H, *J*=15.3Hz), 3.69 (t, 2H, *J*=15.3Hz), 4.91 (s, 3H), 6.20 (m, 3H), 7.01 (m, 1H).

Compound **4**

$POCl_3$ (0.79 mL, 8.6 mmol) was added slowly in anhydrous DMF (5 mL) in the ice-water bath and stirred for 30 min. Then compound **3** (2.3 g, 8.6 mmol) dissolved in DMF was added in drops. The mixture was slowly warmed to room temperature and stirred overnight. The reaction solution was poured into ice, stirred for a few minutes, and filtered to give light brown solid (2.3 g, 92%).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.31(1H,s), 3.56(2H,t), 3.89(2H,tri), 4.18(2H,s), 6.26(1H,s), 6.41(1H,dd), 7.78(1H,dd), 9.61(1H,s), 10.21(1H,s)

Compound **5**

4 (2.3 g, 7.8 mmol) was dissolved in 10 mL refreshed CH_2Cl_2, and boron tribromide (1 mL, 10.0 mmol) was added at -78 ℃. The mixture was warmed slowly to room temperature and stirred for 12 h. Cold methanol was added to quench extra boron tribromide. After evaporation and extraction, the residue was purified by column chromatography to give yellow oil (1.9 g, 88%).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.29(1H,s), 3.54(2H,t), 3.86(2H,tri), 4.17(2H,s), 6.26(1H,s), 6.41(1H,dd), 7.39(1H,dd), 9.61(1H,s), 11.51(1H,s)

Compound **6**

A mixture of 4-((2-bromoethyl)(methyl)amino)-2-hydroxybenzaldehyde (**5**, 1.9 g, 6.7 mmol), sodium methanethiosulfonate (NaMTS, 6.7 g, 50 mmol) and KBr (1.6 g, 13.4 mmol) in acetonitrile (50 mL) was stirred and refluxed for 24 h. After evaporation, the residue was dissolved in CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$ solution followed by water. The organic phase was then dried over anhydrous Na_2SO_4,

filtered, and concentrated in vacuum yielding yellow oil, which was purified by column chromatography to give yellow solid (0.91g, 58%).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.29(t, 1H), 3.06(m, 2H), 3.79(m, 2H), 4.13(s, 2H), 6.51(d, 1H), 7.21(d, 1H), 9.56(s, 1H), 11.98(s, 1H)

J-S-Alk

A reaction mixture of compound **6** (400.0 mg, 1.7 mmol), 2,3-diaminomaleonitrile (92.7 mg, 0.85 mmol) and $Zn(OAc)_2 \cdot 2H_2O$ (188.4 mg, 0.85 mmol) in 15 mL ethanol was refluxed under nitrogen for 24 h. The system turned dark brown and precipitate formed. After cooling to the room temperature and evaporating the solvent, the mixture was filtered and the solid was washed in turn by ethanol, ethyl acetate and petroleum ether. After dried under reduced pressure, compound **J-S-Alk** was obtained as brown black solid (418 mg, 81 %).

^{1}H NMR (d6-DMSO, 400 MHz) δ 3.00(t, 2H), 3.27(m, 4H), 3.63(m, 4H), 4.28(d, 4H), 6.35(d, 2H), 7.08(d, 2H), 8.18(s, 2H)

Compound **7**

1, 2, 3, 4-tetrahydro-4-methyl-6-carboxaldehyde-7-methoxy-1, 4-benzothiazine (**6**, 466 mg, 2 mmol) was dissolved in refreshed dichloromethane at -78 ^oC. m-chloroperoxybenzoic acid (mCPBA (70%), 494 mg, 2 mmol) was added slowly and the mixed system was stirred for an hour. After washing with $NaHCO_3$ solution and brine, the organic phase was then dried over anhydrous Na_2SO_4 and purified by column chromatography to give white solid (358 mg, 72%).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.36(t, 1H), 2.64(td, 1H), 3.20(dt, 1H), 3.66(dt, 1H), 4.26(m, 3H), 6.56(d, 1H), 7.50(d, 1H), 9.62(s, 1H), 12.58(s, 1H)

J-SO-Alk

A reaction mixture of compound **7** (249.0 mg, 1.0 mmol), 2,3-diaminomaleonitrile (54 mg, 0.5 mmol) and $Zn(OAc)_2 \cdot 2H_2O$ (109.7 mg, 0.5 mmol) in 10 mL ethanol was refluxed under nitrogen for 24 h. The system turned dark brown precipitate formed. After cooling to the room temperature and evaporating the solvent, the mixture was filtered and the solid was washed in turn by ethanol, ethyl acetate and petroleum ether. After dried under reduced pressure, compound J-S-Alk was obtained as brown black solid (237 mg, 75 %).

^{1}H NMR (d6-DMSO, 400 MHz) δ 2.53(t, 2H), 3.02(d, 2H), 3.37(t, 2H), 3.66(dt, 2H), 3.89(td, 2H),4.28(d, 4H), 6.44(d, 2H), 7.45(d, 2H), 8.22(s, 2H)

3. Photophysical properties

3.1 Quantum yield determination

Quantum yields of one photon emission of **J-S-Alk** and **J-SO-Alk** were measured with Rhodamine B as reference (Φ=0.65).[1] The one photon fluorescence measurements were performed in 1 cm quartz cells with 1 μM compound in DMSO on a fluorescence lifetime and steady state spectrophotometer (Edinburgh Instrument FLS920) equipped 450 W Xenon light, slits 2.5 × 2.5. The values of fluorescence quantum yield, Φ (sample), were calculated according to equation as following[2]:

$$\frac{\Phi_{sample}}{\Phi_{ref}} = \frac{OD_{ref} I_{sample} d^2_{sample}}{OD_{sample} I_{ref} d^2_{ref}} \quad (Equation.\ \text{S1})$$

I: integrated emission intensity.

OD: optical density at the excitation wavelength.

d: the refractive index of solvents. d_{DMSO}=1.478; $d_{Ethanol}$=1.361

3.2 Determination of the two-photon absorption cross section

The two-photon absorption spectra of **J-SO-Alk** were determined over a broad spectral region (750 nm to 860 nm) by the typical two-photon induced fluorescence method relative to Rhodamine B as standard. The two-photon fluorescence data were acquired using a Tsunami femtosecond Ti: Sapphire laser (pulse width ≤100 fs, 80 MHz repetition rate, tuning range 710–880 nm Spectra Physics Inc., USA). The two-photon fluorescence measurements were performed in a 1 cm quartz cell with 1×10^{-5} mol/L sample dissolved in DMSO and the excitation power density is set to be 200 mW. The two-photon absorption cross section of sulfonium ZnSalens (δ_{sample}) was calculated at every 10 nm wavelength from 750 nm to 860 nm according to equation as following[3]:

$$\delta_{sample} = \delta_{ref} \cdot \frac{\Phi_{ref} \cdot C_{ref} \cdot I_{sample} \cdot d_{sample}}{\Phi_{sample} \cdot C_{sample} \cdot I_{ref} \cdot d_{ref}} \quad (2)$$

δ_{ref} : Two-photon absorption cross section of the reference (Rhodamine B), which was read out from the previous literature.

Φ : Quantum yield of sample and reference.

I: Integrated emission intensity.

C: Concentration of each sample.

d: The refractive index of solvents. d_{DMSO}= 1.478, d_{EtOH} = 1.361

4. In vitro experiments

4.1 Cell culture

All HeLa cells were incubated in complete medium (Dulbecco's modified Eagle's Medium, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin) at 37 °C in atmosphere containing 5% CO_2.

4.2 Cell cytotoxicity

HeLa cells were seeded in flat-bottomed 96-well plates, 1×10^4 cells per well, with 200 μL complete culture media for 24 h. After washed with PBS for three times (200 μL×3), the cells were incubated with appropriate concentrations of J-S-Alk or J-SO-Alk. All stock solutions were prepared in DMSO（2 mM）and diluted with complete media. After cultured for 24 h, the cells were washed with PBS three times (200 μL×3). 10 μL Cell Counting Kit-8 (CCK-8) solution and 90 μL PBS were added per well simultaneously. After 2 hours, the absorbance at 450 nm was read by 96-well plates reader. The viability of Hela cells was calculated by the following equation:

$$CV=（As-Ab）/（Ac-Ab）\times100\%$$

CV stands for the viability of cells，As, Ac and Ab stand for the absorbance of cells containing **Zn Salens**, cell control(0 μM **Zn Salens**) and blank control (wells containing neither cells nor **Zn Salens**).

4.3 Co-localization assay

HeLa cells were placed onto 0.1 mM poly-D-lysine coated glasses in complete media and the cells were incubated for 24 h. A stock solution of **J-S-Alk** in chromatographic grade, anhydrous DMSO was prepared as 2 mM. The solution was diluted to a final concentration of 2 μM by complete growth medium. Stock solution of MitoTracker Green FM was prepared as 1 mM, and the stock solution was diluted to the working concentrations in complete medium (100 nM).

After incubation of 2 μM **J-S-Alk** for one hour, cells were washed with PBS buffer twice and turned to CLSM. Images were taken under conditions as follows: 60× immersion lens with a resolution of 1024×1024 and a speed of 0.5 frame per second, 543 nm excitation wavelength and 552 to 617 nm detector slit, 20% laser power for dye, and 5% laser power for MitoTracker (ex: 488 nm, em: 505-560 nm). Differential interference contrast (DIC) and fluorescent images were processed and analyzed using ImageJ. The Pearson's Coefficient was calculated by ImageJ.

4.4 Two-photon confocal microscopy imaging

Two photon fluorescence microscopy images were performed on a modified Olympus Fluoview FV1000MPE microscope system equipped with an excitation light laser provided by a modelocked Ti: sapphire laser, (Mai Tai, Spectra-Physics Inc., USA). The microscopy settings were as follows: 60× immersion water objective, a resolution of 512×512, 820 nm excitation wavelength, red slit for **J-S-Alk** and **J-SO-Alk**, 30 % laser power (10 mW). HeLa cells were placed onto 0.1 mM poly-D-lysine coated glasses in complete media and the cells were incubated for 24 h. HeLa cells were treated with 2 μM of **J-S-Alk** or **J-SO-Alk** for 1 h and washed with prewarmed PBS buffer three times before photoirradiation at 820 nm.

4.5 STORM setup and data analysis

STORM imaging of **J-S-Alk** was performed as previously described.[4, 5] We used an Olympus IX71 inverted microscope equipped with a 100×, 1.45 NA oil objective (Olympus PLAN APO). The fluorescence signals were acquired using an electron-multiplying charge coupled device (EMCCD) camera (Andor iXon DV-897 BV). The maximum power near the rear pupil of the objective was 2.9 W/cm^2 for the 405-nm laser (LASOS) and 1.2 kW/cm^2 for the 561-nm laser (Cobolt Jive). The intensity of the 405-nm laser was set so that it only activated a few molecules in each frame. Data analysis and super-resolution image reconstruction were performed as previously described. Briefly, a wavelet transform algorithm with a proper threshold was used for single-molecule detection, and localizations of the molecules were determined by finding local maxima with a mask of 5 × 5 pixels. Then the raw images of molecules in the fit windows (7 × 7 pixels) were background-subtracted, and the pixel values were converted to numbers of photons. After that, all molecules were fitted with a 2D Gaussian using MLE_GPU algorithm to obtain the number of photons (N) and the s.d. of the point spread function (s). The background noise per pixel (b) was determined by taking the s.d. of the intensity (in photons) of an illuminated area in a raw image where no single molecule is visible. Finally, the 2D localization precision for each molecule was calculated according to the following equation:

$$\sigma^2 = \frac{S^2 + \alpha^2/12}{N} + \frac{8\pi S^4 b^2}{\alpha^2 N^2}$$

5. Figures and spectra

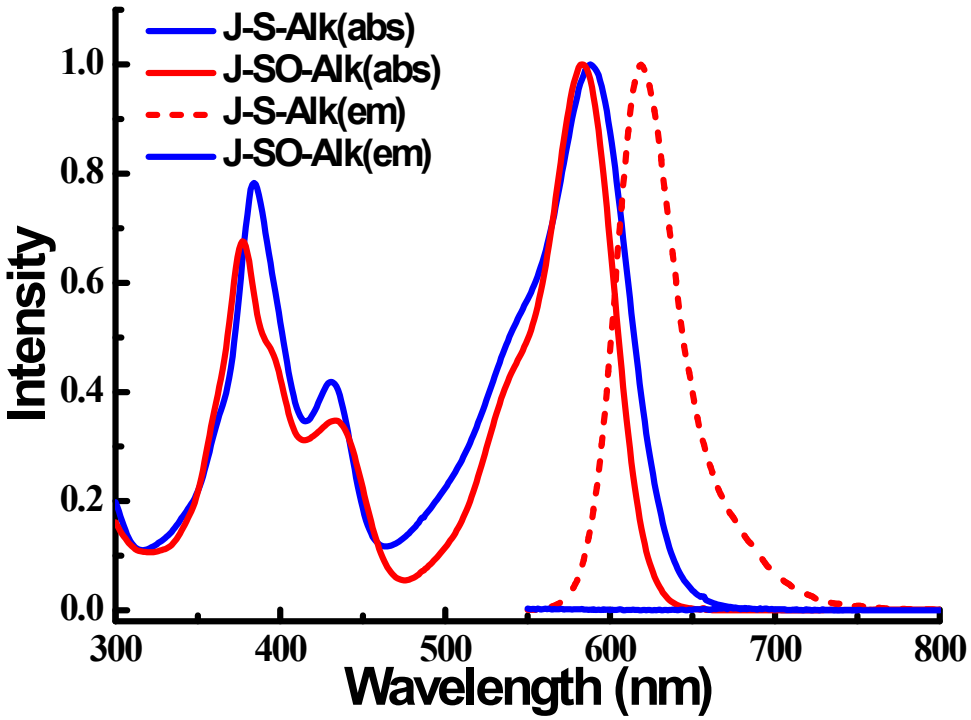


Figure S1 Absorption and emission spectra of **J-S-Alk** and **J-SO-Alk** in DMSO.

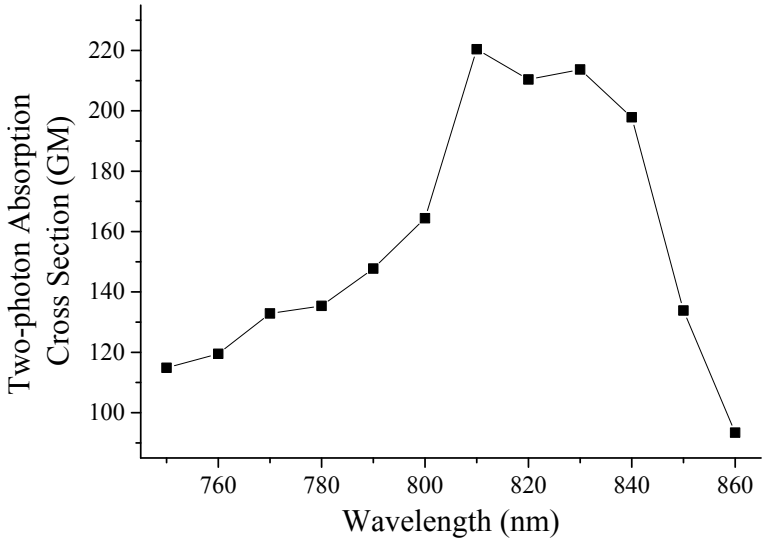


Figure S2 Two-photon absorption cross-sections of **J-SO-Alk** from 750 nm to 860 nm in DMSO referenced by Rhodamine B

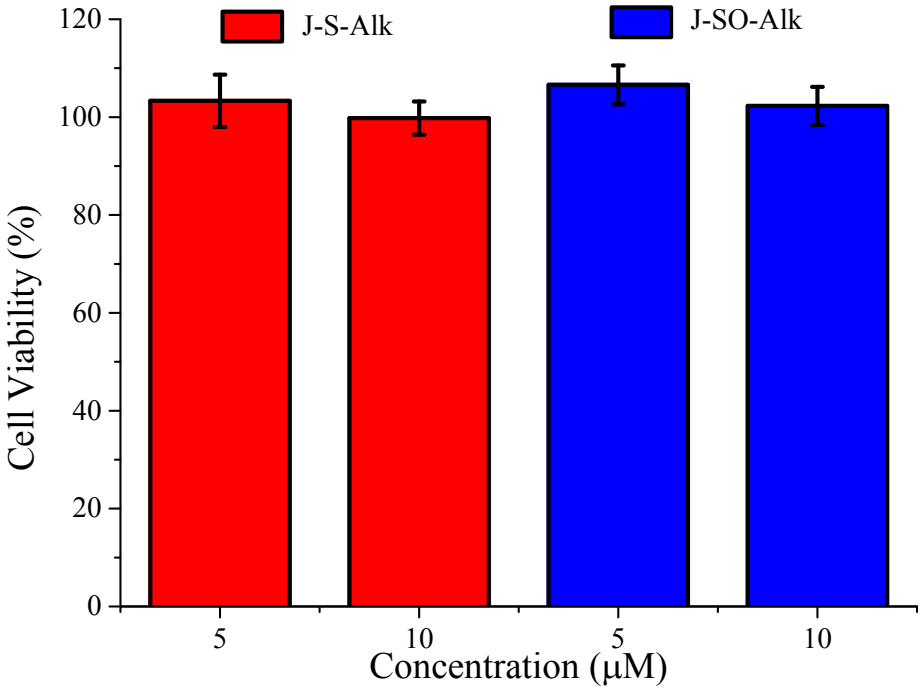


Figure S3 Cell viability of HeLa cells in the presence of **J-S-Alk** and **J-SO-Alk** using CCK-8 assay.

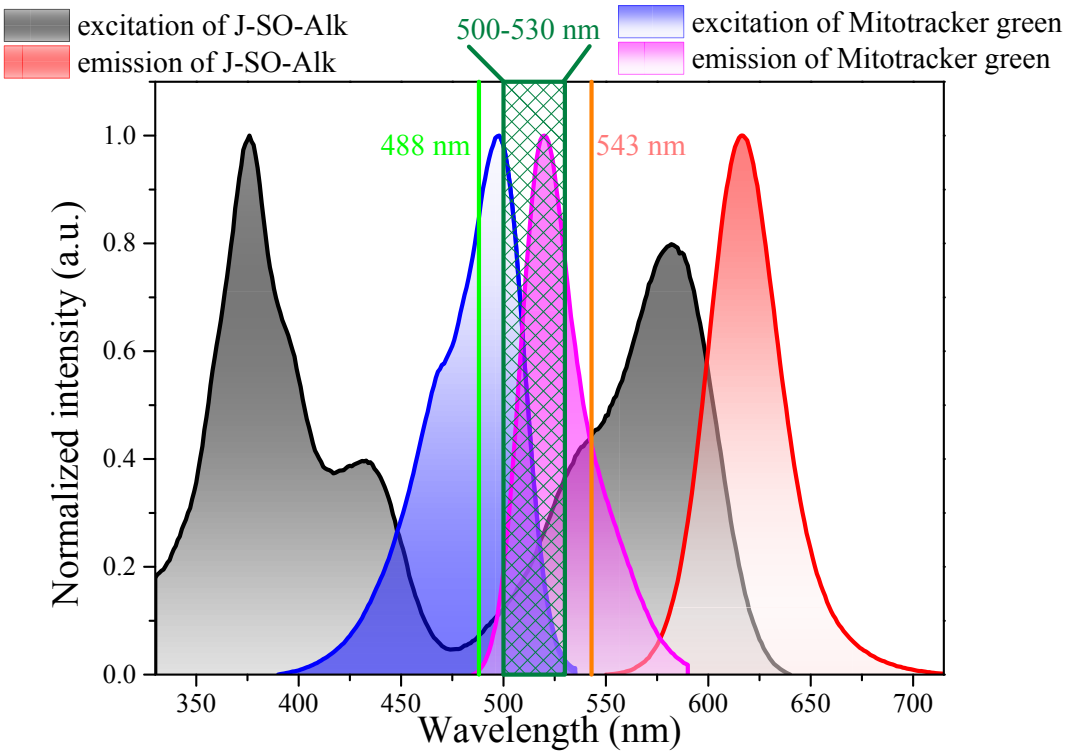


Figure S4 Excitation and emission spectra of J-SO-Alk and MitoTracker green in DMSO.

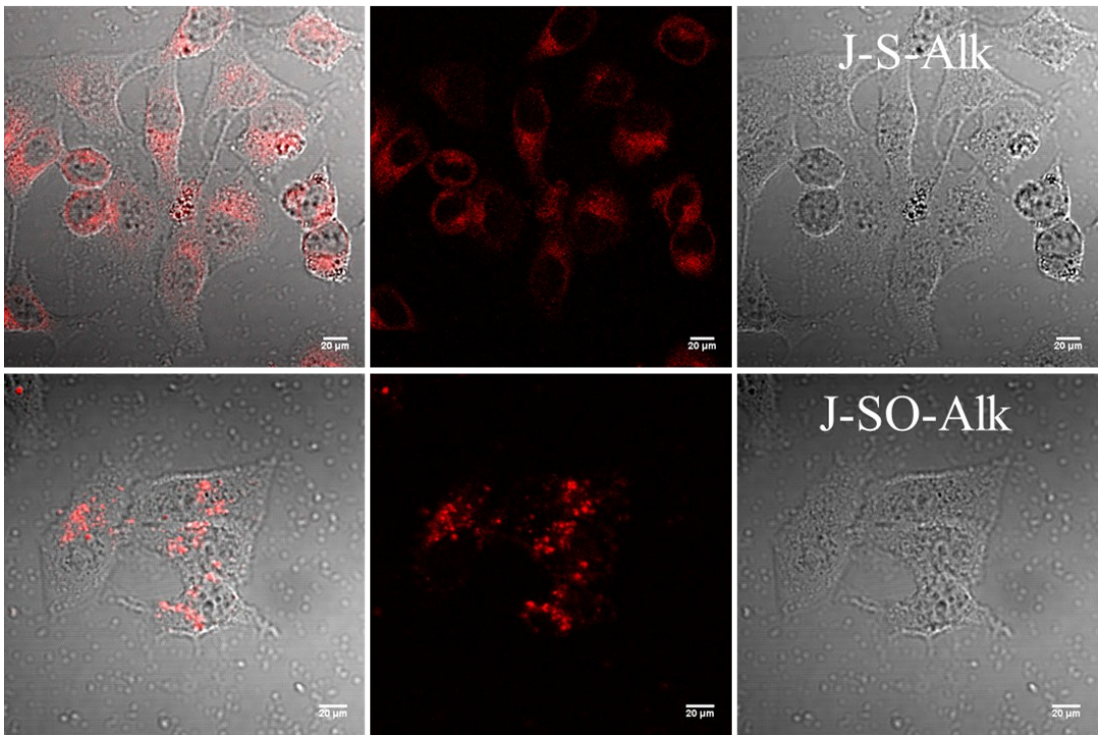


Figure S5 Two-photon images of 2 μM of **J-S-Alk** and **J-SO-Alk** in HeLa cells.

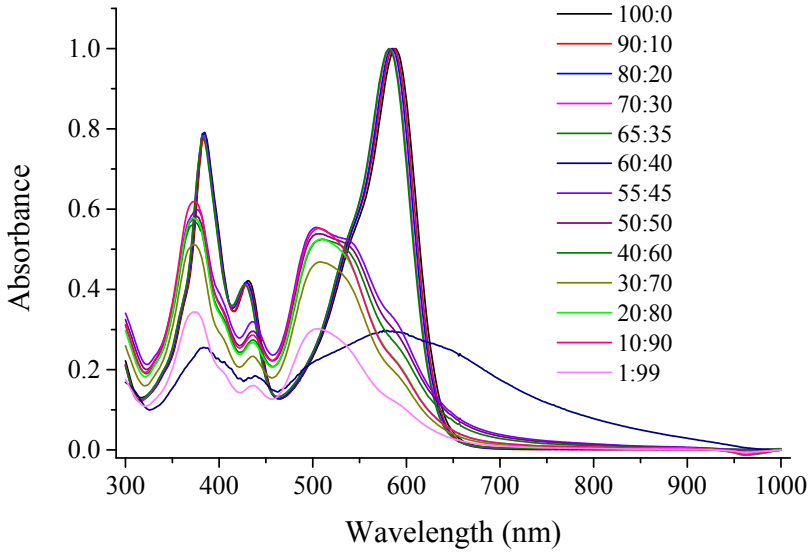


Figure S6 UV-vis spectra of **J-S-Alk** (20 μM) in the mixed solution of H_2O/DMSO. The content of H_2O was from 0% to 99%.

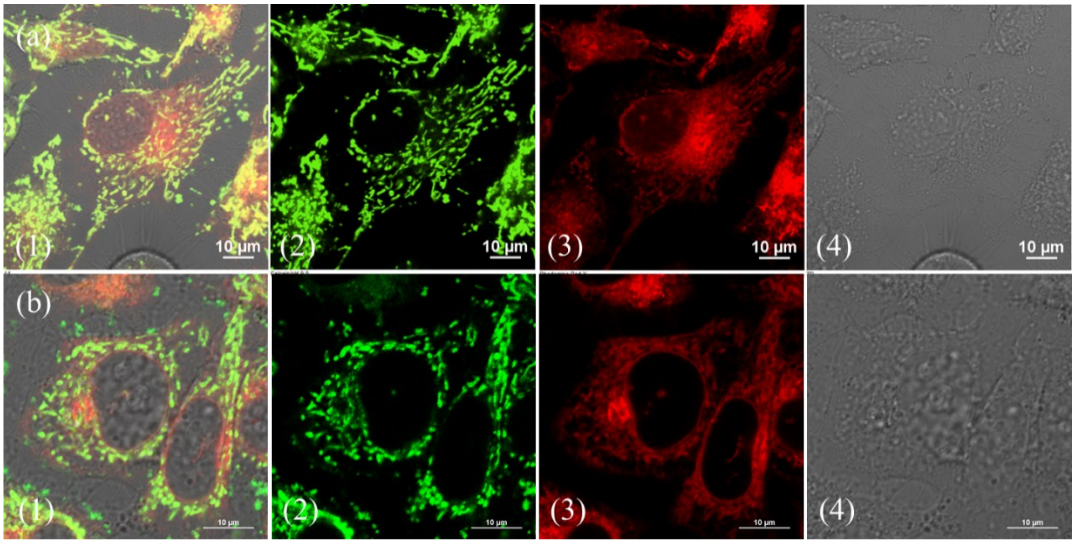
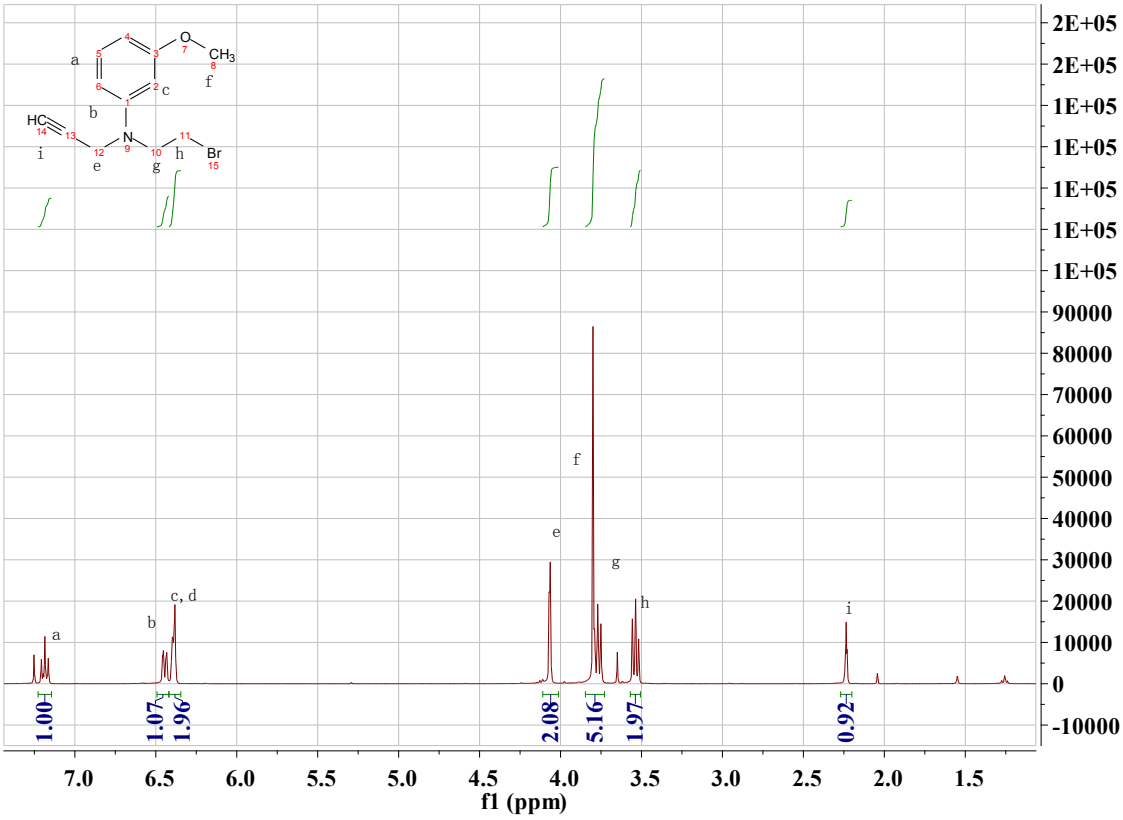


Figure S7 Co-localization analysis of **J-S-Alk** in (a) absence and (b) presence of pyridine with MitoTracker green: (1) Merged confocal fluorescence image of (2), (3) and (4); (2) Confocal fluorescence image of MitoTracker Green; (3) Confocal fluorescence image of photoactivated **J-S-Alk**; (4) Differential interference contrast (DIC) image. Scale bar = 10 μm.

^{1}H NMR



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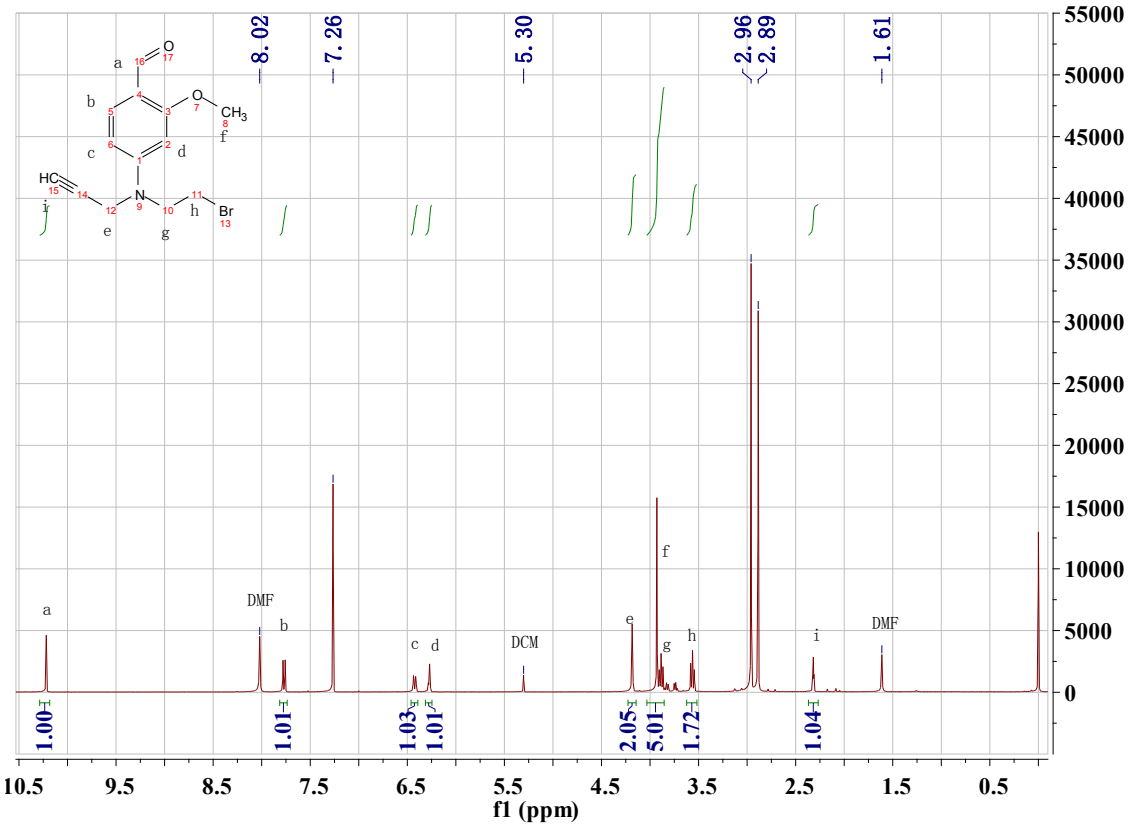
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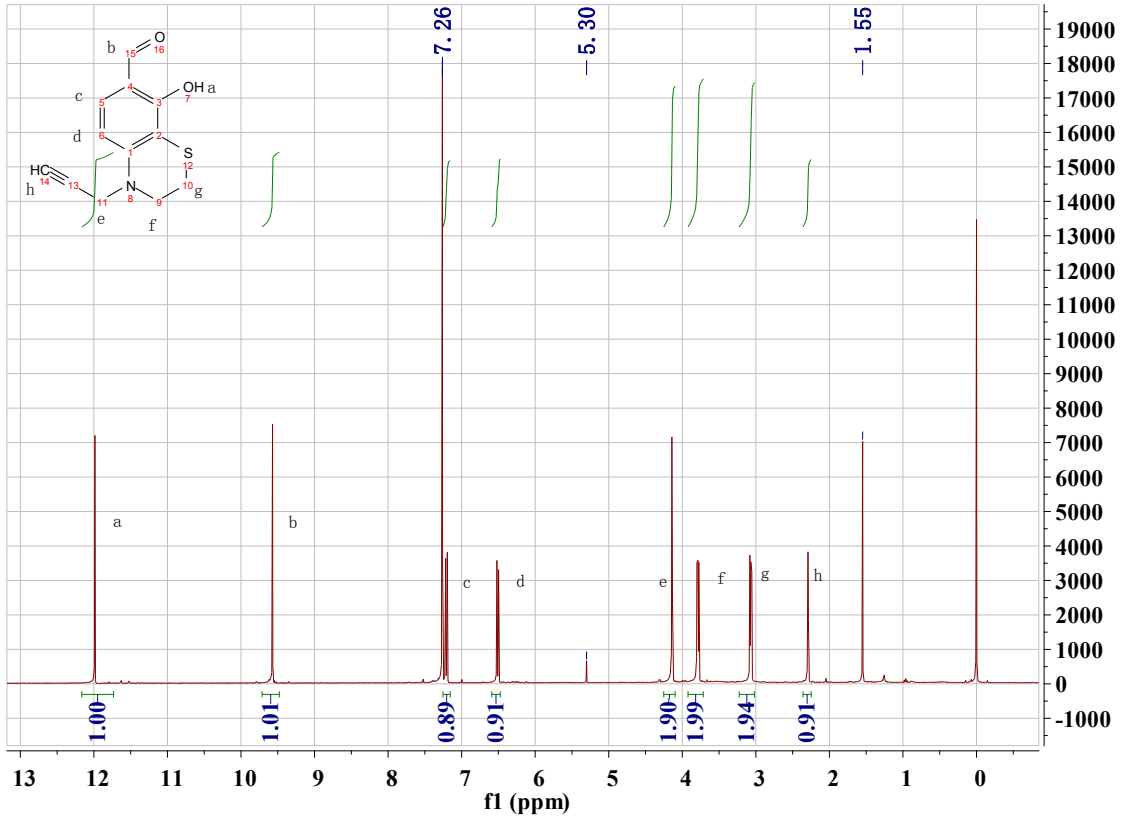
^{1}H NMR



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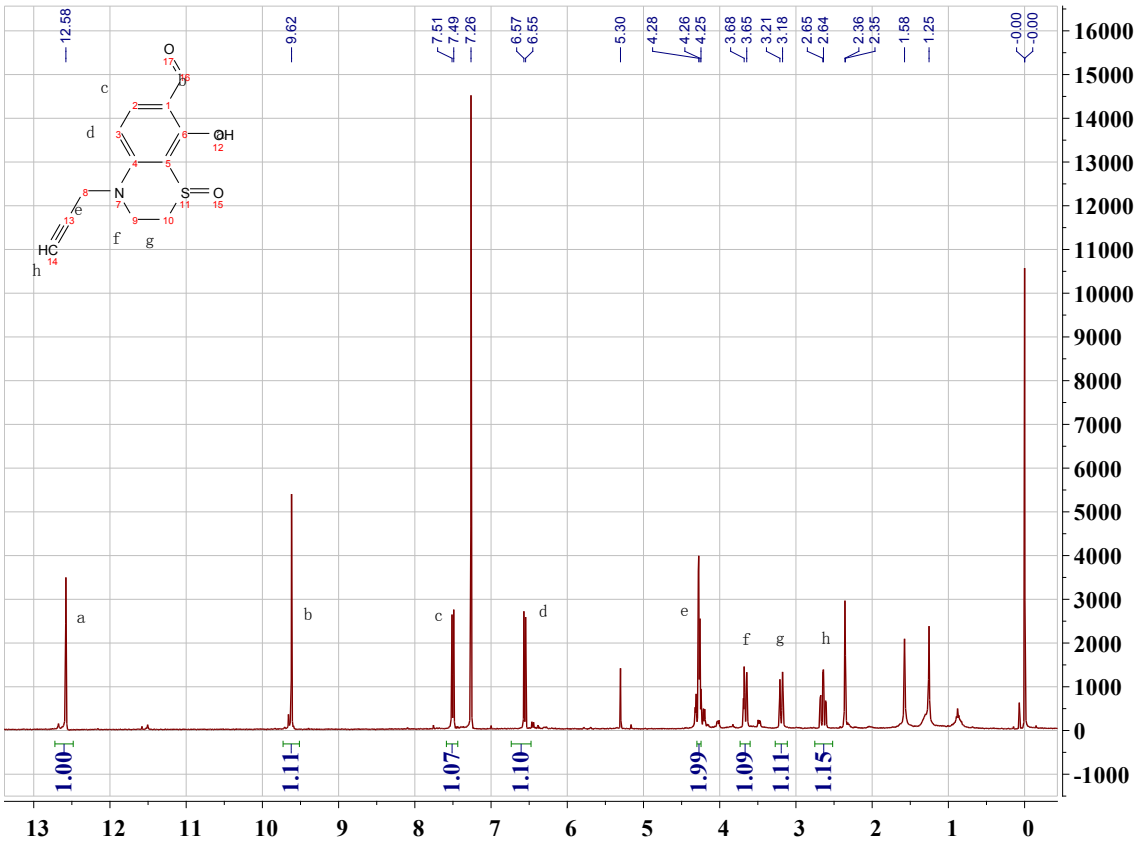
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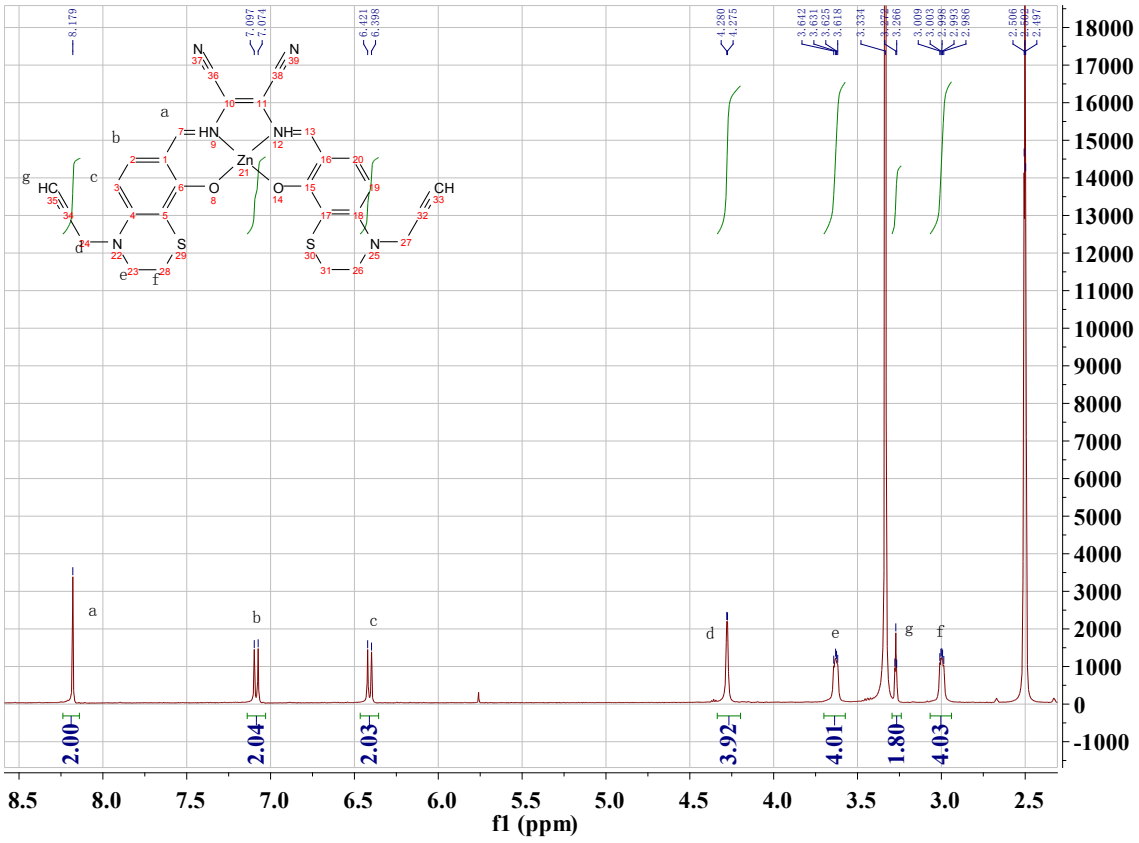
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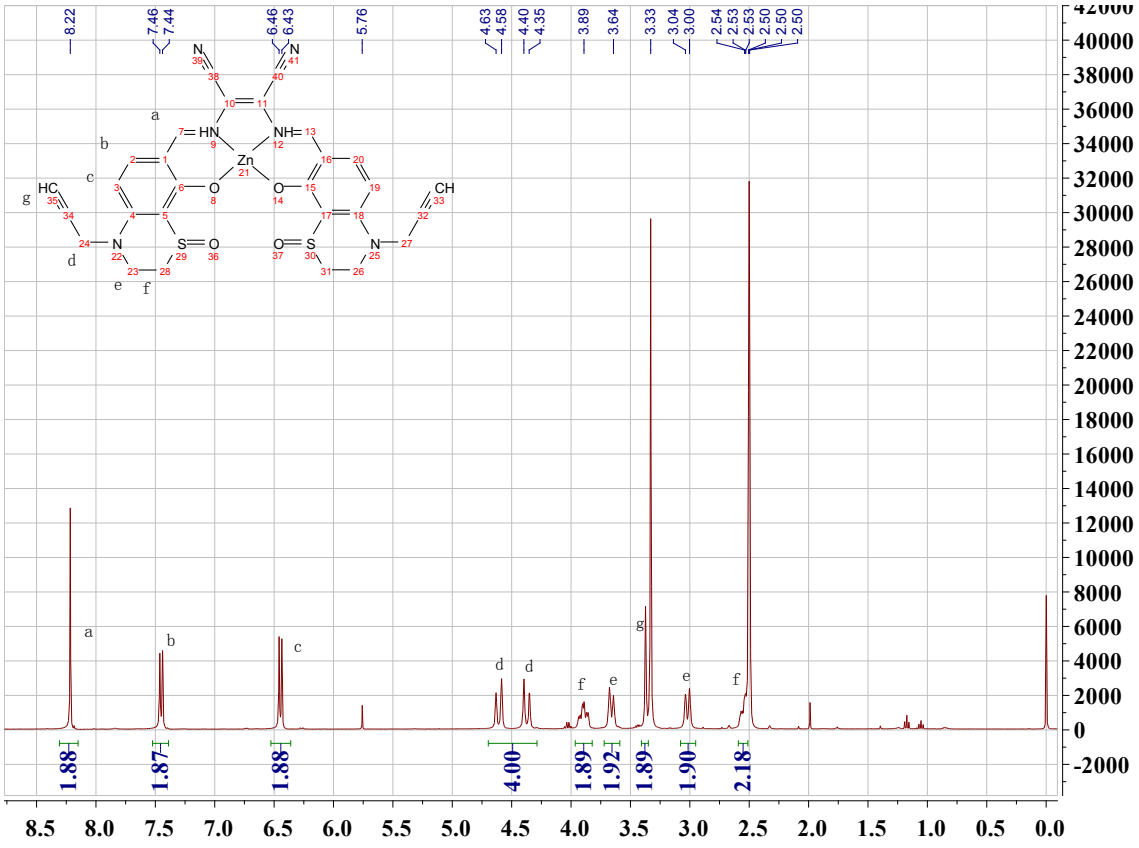
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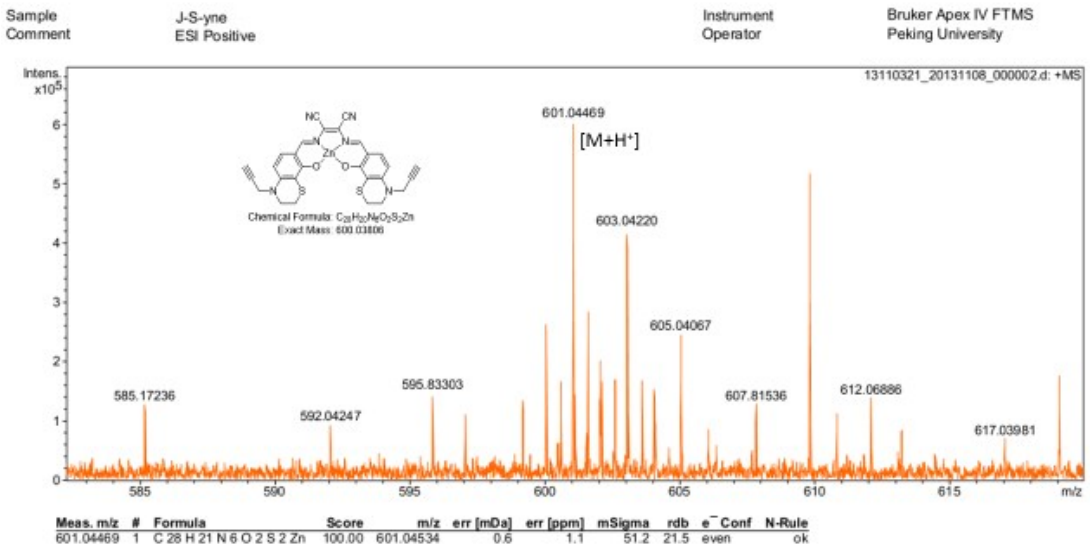

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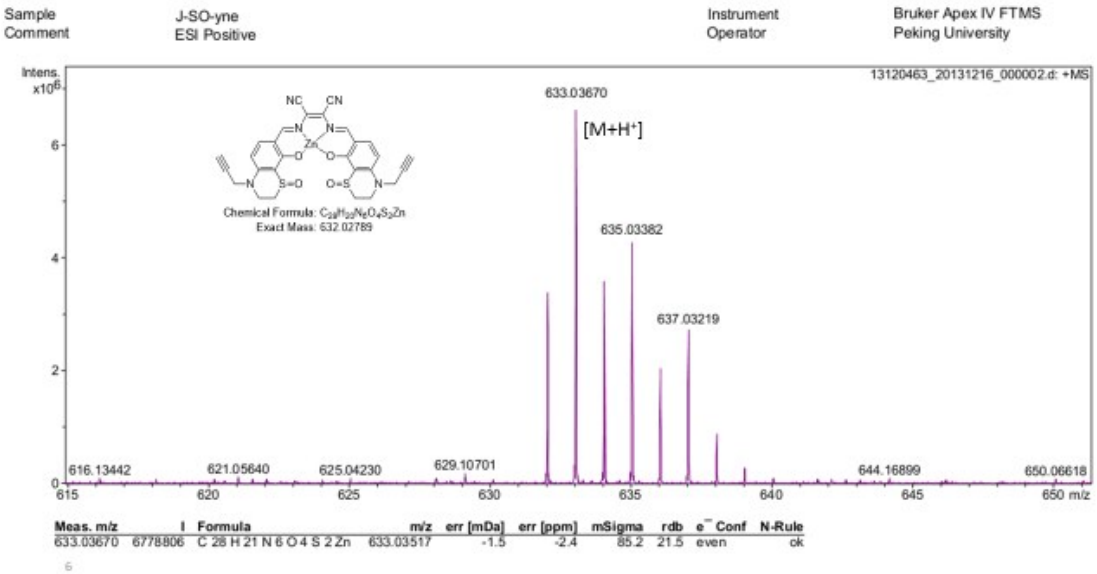
HR-MS



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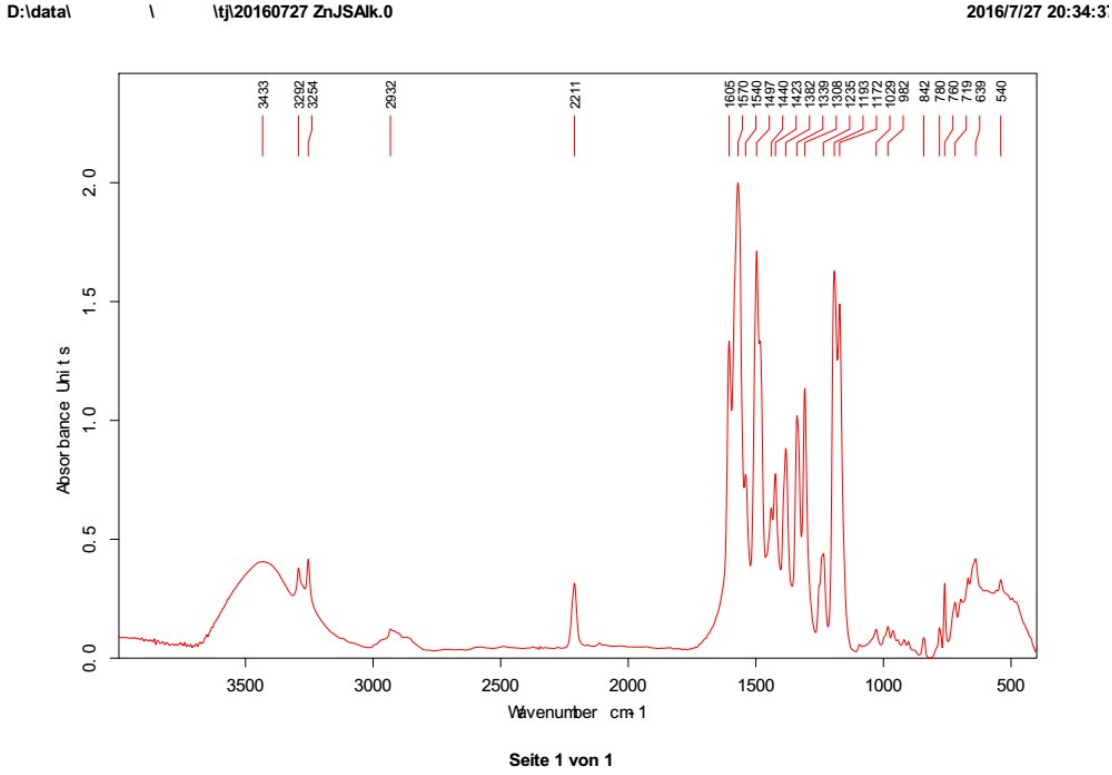
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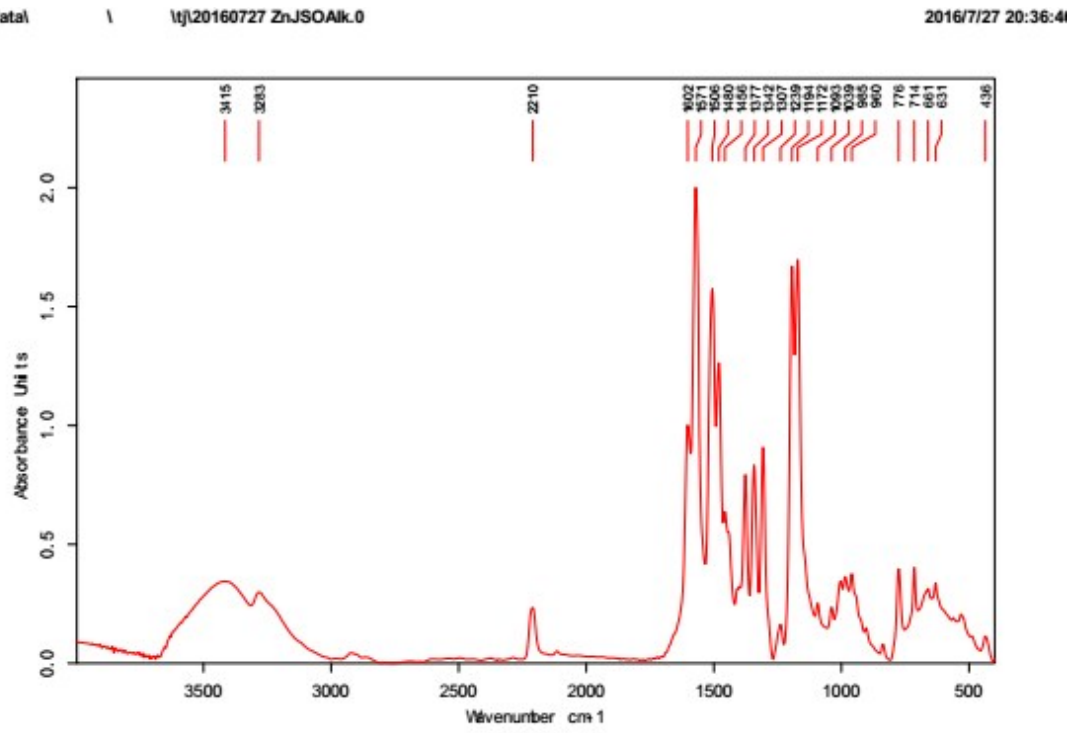
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1377
1342
1307
1239
1194
1172
1093
1039
985
960
776
714
661
631
436
Absorbance Units
0.0
0.5
1.0
1.5
2.0
3500
3000
2500
2000
1500
1000
500
Wavenumber cm-1
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6. Refference

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