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# Two-photon excitable boron complex based on tridentate imidazo[1,5-*a*]pyridine ligand for heavy-atom-free mitochondria-targeted photodynamic therapy

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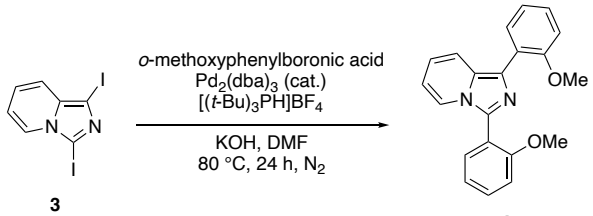
## General Information

NMR spectra were recorded on a JEOL resonance JNM-ECZ-400 operating 400 MHz for $^{1}$H- and 100 MHz for $^{13}$C-NMR spectroscopy, respectively, in $CDCl_3$ or DMSO-$d_6$ solutions. Chemical shifts are reported in parts per million (ppm) relative to TMS or residual solvent peaks as an internal standard. Melting points were measured on a Yanaco MP-J13 micro melting-point apparatus and uncorrected. High-resolution mass spectra (HRMS) were recorded in a ThermoFisher Exactive. HPLC analysis was performed on a JASCO HPLC system (JASCO PU-4180 pump, MD-4010, and CD-1595 detector). Chiral column CHIRALPAC IB (Daicel Corp.) was used for HPLC analysis. UV/vis spectra were recorded on a Shimadzu UV-1600. Fluorescence spectra were recorded on a Shimadzu RF-1500. Absolute PL quantum yields were recorded on a Hamamatsu Photonics Quantaurus-QY Plus C13534-21. Fluorescence lifetimes were recorded on a Horiba DeltaFlex. Fluorescence images of HeLa cells were observed using an OLYMPUS IX71 equipped with color CCD camera DP72 (OLYMPUS). UVA-LED irradiation was conducted using NCSU033B (NICHIA Corp., $\lambda$ = 365 nm, $I_F$ = 350 mA).

## Experimental Procedures

**Preparation and characterization of 4:**



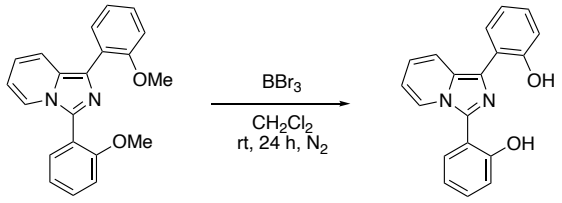

**3** **4**
58% yield

To a 30 mL of DMF solution containing 1,3-diiodoimidazo[1,5-*a*]pyridine (**3**, 1.1108 g, 3.0 mmol), which was prepared according to the literature,[S1] were added *o*-methoxyphenylboronic (1.0107 g, 6.6 mmol), $Pd_2(dba)_3$ (0.1385 g, 0.15 mmol), tri-*tert*-butylphosphonium tetrafluoroborate (0.0884 g, 0.3 mmol), and potassium hydroxide (1.1211 g, 19.8 mmol) at room temperature, and the mixture was stirred for 24 hours at 80 °C under nitrogen atmosphere. After the reaction mixture was poured into water and extracted with ethyl acetate, the organic layer was washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residual mixture was subjected to chromatography on silica gel using mixtures of ethyl acetate and hexane (1 : 2) as eluents, and 1.74 mmol (0.5736 g) of **4** was separated in 58% yield.

**Compound 4**:

Brown solid; mp 151.0–152.0 °C; $^{1}$H NMR ($CDCl_3$) $\delta$: 3.82 (s, 3H), 3.89 (s, 3H), 6.52 (t, $J$ = 6.6 Hz, 1H), 6.74 (dd, $J$ = 9.3, 6.6 Hz, 1H), 7.01–7.12 (m, 4H), 7.31 (t, $J$ = 7.3 Hz, 1H), 7.45 (t, $J$ = 7.3 Hz, 1H), 7.57–7.61 (m, 2H), 7.69 (d, $J$ = 6.6 Hz, 1H), 7.82 (d, $J$ = 6.6 Hz, 1H); $^{13}$C NMR ($CDCl_3$) $\delta$: 55.4, 55.5, 110.96, 111.03, 111.8, 118.2, 119.2, 120.3, 120.9, 121.1, 123.1, 124.1, 128.0, 128.36, 128.40, 130.6, 131.6, 132.9, 136.1, 156.2, 157.3; HRMS (ESI) *m*/*z* calcd for $C_{21}H_{18}O_2N_2$+H 331.1441, found 331.1433.

**Preparation and characterization of 2:**



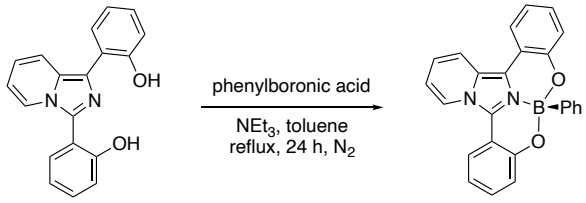

**4** **2**
71% yield

To a 8 mL of dichloromethane solution containing **4** (0.2624 g, 0.79 mmol) was added boron tribromide (1 mol/L solution in dichloromethane, 2.4 mL, 2.4 mmol) at 0 °C under nitrogen atmosphere, and the mixture was stirred for 24 hours at room temperature. After the reaction mixture was poured into water and extracted with dichloromethane, the organic layer was washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residual mixture was subjected to chromatography on silica gel using mixtures of ethyl acetate and hexane (1 : 2) as eluents, and 0.56 mmol (0.1685 g) of **2** was separated in 71% yield.

**Compound 2**:

Brown solid; mp 152.0–153.0 °C; $^1$H NMR (DMSO-$d_6$) δ: 6.83–6.87 (m, 1H), 6.93–6.97 (m, 2H), 7.01–7.05 (m, 2H), 7.10 (dd, *J* = 8.2, 0.9 Hz, 1H), 7.16 (ddd, *J* = 8.3, 7.2, 1.6 Hz, 1H), 7.42 (ddd, *J* = 8.3, 7.4, 1.7 Hz, 1H), 7.54 (dd, *J* = 7.7, 1.7 Hz, 1H), 7.87–7.90 (m, 2H), 8.12 (d, *J* = 9.4 Hz, 1H), 10.40 (br, 1H), 12.11 (br, 1H); $^{13}$C NMR (DMSO-$d_6$) δ: 113.4, 115.7, 116.3, 116.7, 118.6, 119.0, 119.3, 119.6, 120.8, 124.2, 126.0, 126.5, 127.6, 128.2, 131.1, 131.5, 134.2, 155.3, 155.5; HRMS (ESI) *m*/*z* calcd for $C_{19}H_{14}O_2N_2$+H 303.1128, found 303.1122.

**Preparation and characterization of 1:**




**2** **1**
90% yield

To a 5 mL of toluene solution containing **2** (0.0911 g, 0.3 mmol) were added phenylboronic acid (0.0466 g, 0.36 mmol) and triethylamine (0.14 mL, 0.9 mmol) at room temperature, and the mixture was stirred for 24 hours at room temperature under reflux and nitrogen atmosphere. After the reaction mixture was concentrated *in vacuo*, the residual mixture was subjected to chromatography on silica gel using mixtures of dichloromethane and hexane (2 : 1)as eluents. As a result, 0.27 mmol (0.1067 g) of **1** was separated in 90% yield.

**Compound 1**:

Yellow solid; mp 281.0–282.0 °C; $^1$H NMR (DMSO-$d_6$) δ: 6.93–7.04 (m, 6H), 7.08–7.14 (m, 3H), 7.18–7.25 (m, 2H), 7.30 (dd, *J* = 9.5, 6.6 Hz, 1H), 7.36–7.40 (m, 1H), 7.96 (dd, *J* = 7.7, 1.4 Hz, 1H), 8.15 (dd, *J* = 7.9, 1.1 Hz, 1H), 8.37 (d, *J* = 9.5 Hz, 1H), 9.01 (d, *J* = 7.3 Hz, 1H); $^{13}$C NMR (DMSO-$d_6$) δ: 112.8, 116.3, 117.5 118.2, 119.5, 119.6, 119.67,

119.73, 120.1, 123.7, 124.27, 124.33, 124.4, 125.6, 126.6, 127.1, 127.7, 130.0, 130.5, 132.1, 154.6, 156.5; HRMS (ESI) *m*/*z* calcd for $C_{25}H_{17}O_2N_2B$+H 389.1456, found 389.1452.

**Single crystal X-ray structure analysis:**

Crystal data of **1** (Recrystallized from dichloromethane and hexane), monoclinic space group $P2_1/n$, $a$ = 11.3434(3) Å, $b$ = 14.0488(4) Å, $c$ = 13.7721(4) Å, $\beta$ = 107.982(8)°, $V$ = 2087.53(13) Å$^3$, $Z$ = 4, $\rho$ = 1.367 Mg/m$^3$, in the final least-squares refinement cycles on $F^2$, the model converged at $R_1$ = 0.0820, *wR2* = 0.1917, and GOF = 1.053 for 3797 reflections CCDC2085924.

**HPLC analysis of 1:**

The racemic **1** was analyzed by HPLC using a chiral column, CHIRALPAL IB (Daicel Corp.); eluent: hexane/chloroform = 3/1 (v/v), flow 0.5 mL/min. The 373 nm CD signals showed the opposite of optical rotation and the retention times were 50.9 min and 61.7 min, respectively. This result is shown in **Fig. S1**. Although the absolute configurations of both enantiomers of **1** were not determined, it was calculated that the (*R*)-**1** and (*S*)-**1** show the positive and negative ECD signals at around 370 nm, respectively (**Fig. S8**). The ECD spectra are also shown in **Fig. S2**.

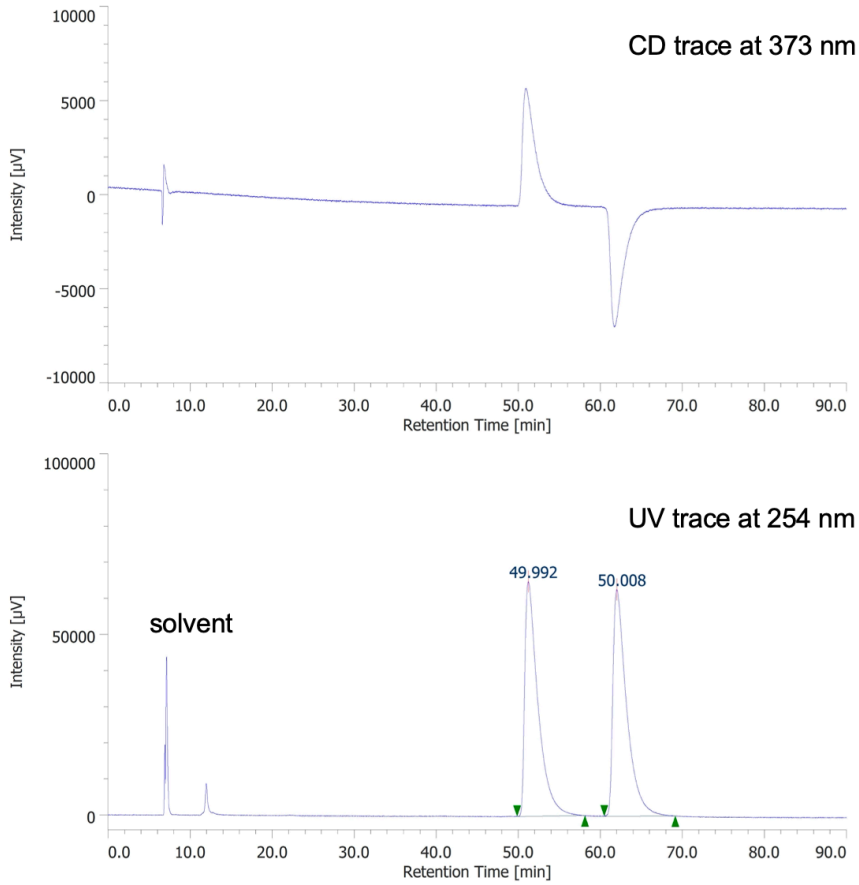



**Fig. S1** UV trace at 254 nm (upper) and CD trace at 373 nm (lower) of **1**.

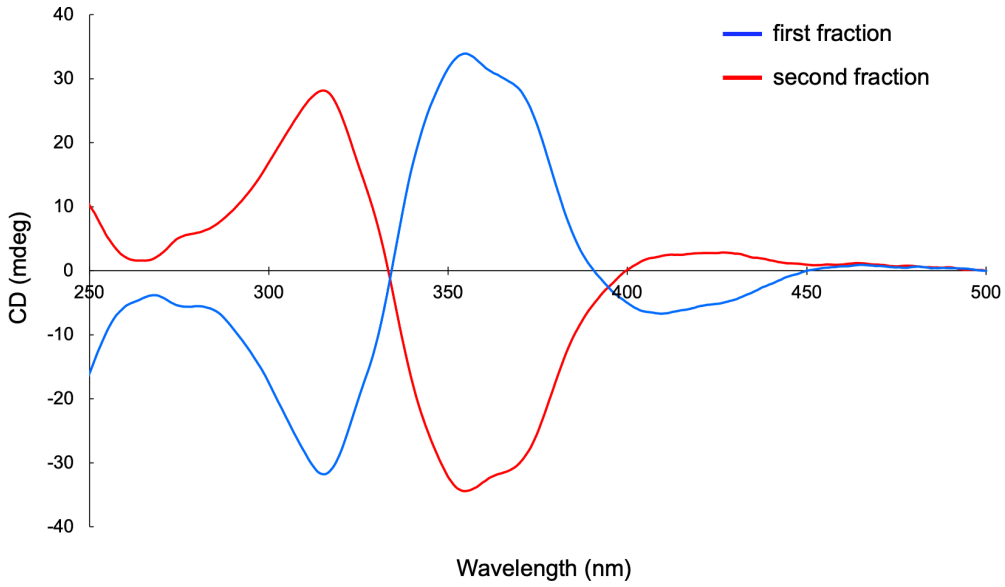



**Fig. S2** Experimental ECD spectra of **1**.

**Absorption and fluorescence spectra of 1:**

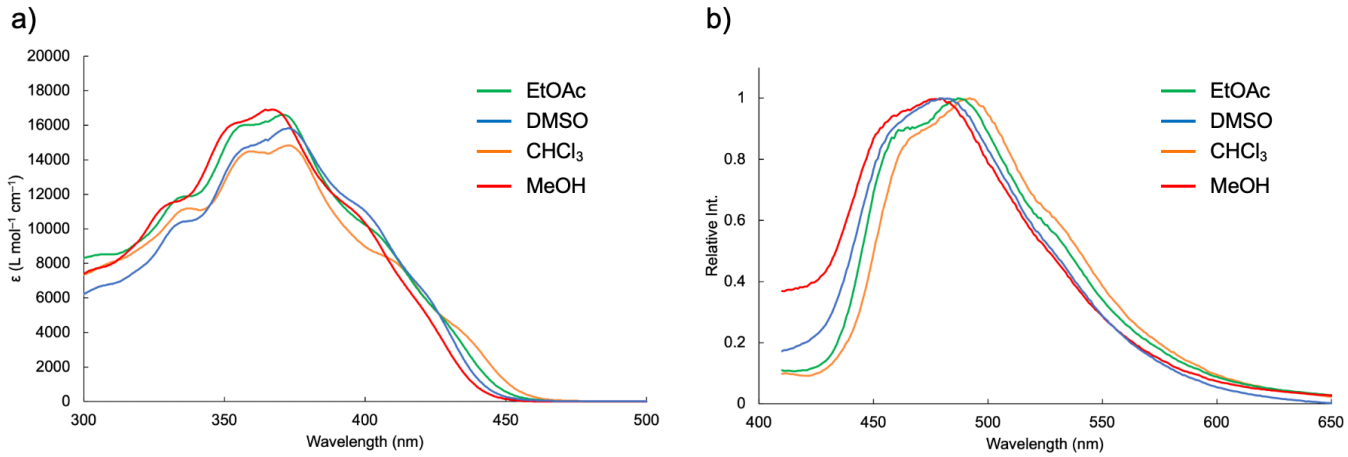



**Fig. S3** a) Absorption and b) PL spectra of **1** in various organic solvents.

**Table S1** Photophysical data of **1** in various organic solvents at room temperature.

| Solv. | $\lambda_{abs,\,max}$ (nm) | $\lambda_{em}$ (nm) | SS (nm)[a] | $\Phi_{em}$[b] | $\tau_f$ (ns) | $K_r \times 10^8$ ($s^{-1}$)[c] | $K_{nr} \times 10^8$ ($s^{-1}$)[d] |
|---|---|---|---|---|---|---|---|
| DMSO | 373 | 479 | 106 | 0.34 | 5.02 | 0.68 | 1.31 |
| EtOAc | 371 | 487 | 116 | 0.24 | 4.41 | 0.54 | 1.72 |
| $CHCl_3$ | 373 | 492 | 119 | 0.20 | 3.53 | 0.57 | 2.27 |
| MeOH | 367 | 480 | 113 | 0.24 | 4.53 | 0.53 | 1.68 |

[a]Stokes shift.

[b]Absolute PL quantum yields.

[c]The radiative decay rates ($K_r$) were calculated from equation, $K_r = \Phi/\tau$.

[d]The nonradiative decay rates ($K_{nr}$) were calculated from equation, $K_{nr} = (1-\Phi)/\tau$.

**Transient absorption spectra:**

Transient absorption spectra were measured by means of femtosecond pump–probe experiments. Light source was an amplified mode-locked Ti:sapphire laser (Solistice, Spectra-Physics). The second harmonic was used for excitation pulse. Transient absorption spectra were probed by delayed pulses of a femtosecond white-light continuum generated by focusing a signal light (at 1.3 μm) generated by an optical parametric amplifier (OPA) (TOPAS, Light Conversion Ltd.) into a $CaF_2$ plate. Probe light was detected by a C-MOS detector (Hamamatsu, PMA-20). The temporal resolution was ca. 150 fs. This experimental setup and the result are shown in **Fig. S4** and **Fig. 3**, respectively.

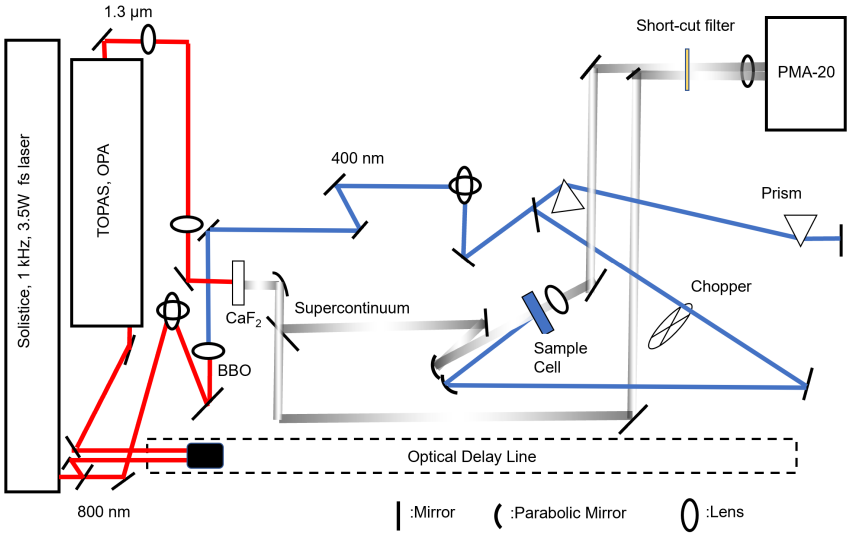



**Fig. S4** Experimental setup for femtosecond transient absorption spectra.

**Cytotoxicity against HeLa cells:**

HeLa cells were cultured in EMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin G and streptomycin) at 37 °C under a 5% $CO_2$ atmosphere in 96-well flat bottom plate. After overnight cultivation, HeLa cells were incubated with a dilution series of **1** for 1 hour or 24 hours. The viability was evaluated using CCK-8 (Dojindo). The absorbance at 450 nm ($A_{450}$) was measured by a microplate reader (TECAN Infinite M200). The $A_{450}$ from independent 3 wells were used to calculate percentage of cell viability according to the formula below and show with standard deviation:

Percentage of cell viability = $A_{450}$ of treated cells / $A_{450}$ of untreated cells × 100 (%)

These results are shown in **Fig. S5**.

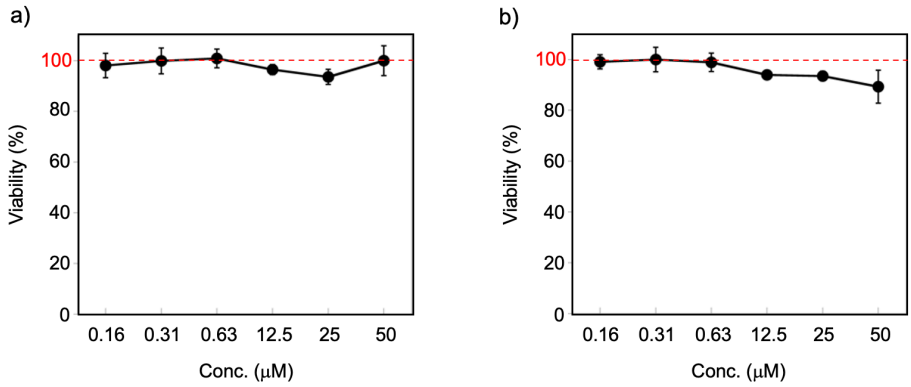


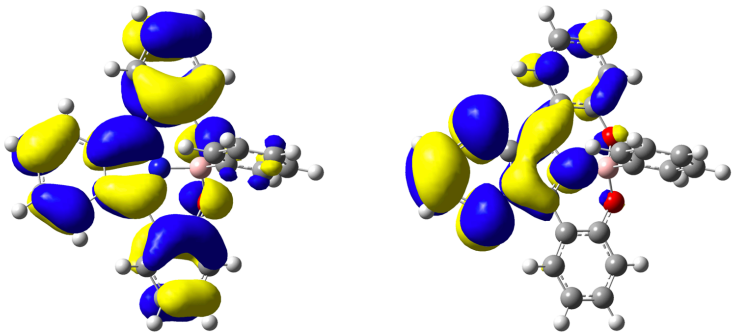

**Fig. S5** The viabilities of HeLa cells incubated with various concentrations of **1** after a) 1 hour for acute toxicity assay and b) 24 hours for subacute toxicity assay.

**Incubation of HeLa cells with 1 and a mitochondrial marker:**

HeLa cells were incubated with **1** and a mitochondrial marker (MitoTracker Red®, ThermoFisher Scientific) at 37 °C for 1 hour under a 5% $CO_2$ atmosphere. The fluorescence images were observed through the WU filter for the detection of cyan fluorescence indicating **1** and the WIY filter for the detection of red fluorescence indicating the mitochondrial marker using an OLYMPUS IX 71 inverted microscope. The bright images were also observed at the same field. These images are shown in **Fig 4a**.

**TD-DFT calculation:**

Time-dependent density function theory (TD-DFT) calculation was conducted at B3LYP/6-31G(d) level.[S2] The HOMO of **1** was delocalized over the entire 1,3-diarylated imidazo[1,5-*a*]pyridine ligand, whereas the LUMO of **1** was mainly localized at the centered imidazo[1,5-*a*]pyridine moiety. In addition, it was found that the perpendicular oriented phenyl ring at boron atom has little contribution to HOMO and LUMO. These results are shown in **Fig. S6**. The calculated absorption and ECD spectra are also shown in **Fig. S7**, **S8** and **S9**, respectively. A half-width at half height maximum of 0.333 eV was used for the absorption and ECD simulations.



HOMO of **1**
(–5.44 eV)

LUMO of **1**
(–2.13 eV)

**Fig. S6** HOMO and LUMO of **1** estimated by DFT calculations at the B3LYP/6-31G(d) level.

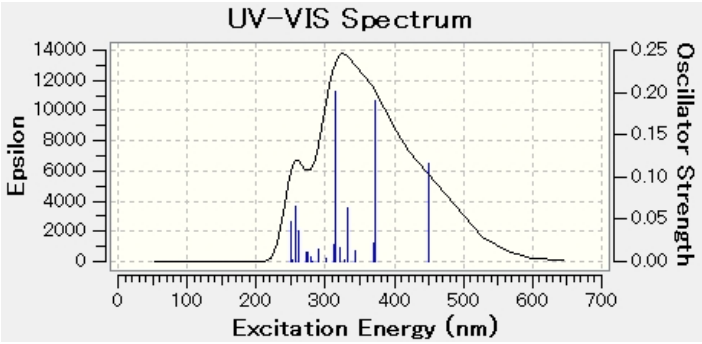



**Fig. S7** Calculated absorption spectrum of **1**.

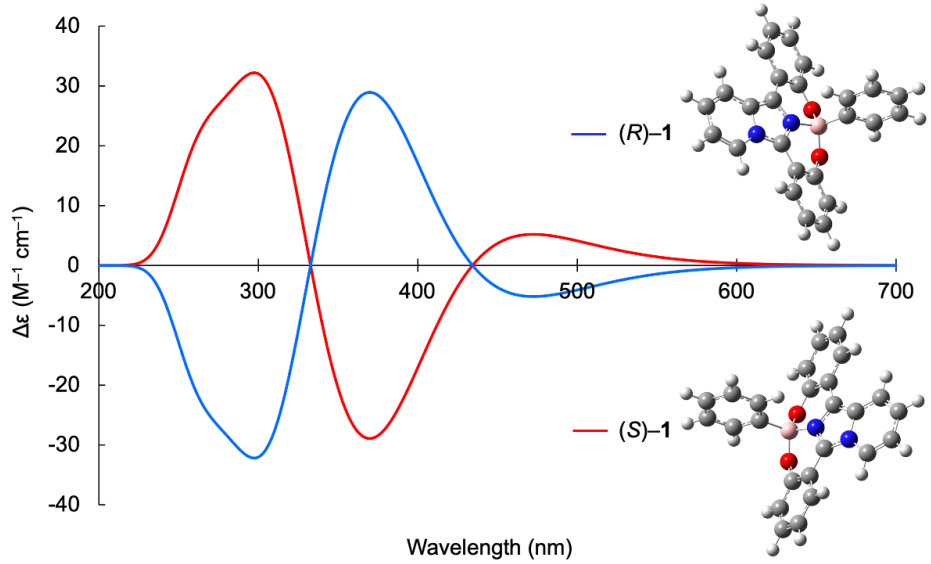



**Fig. S8** Calculated ECD spectra of **1**.

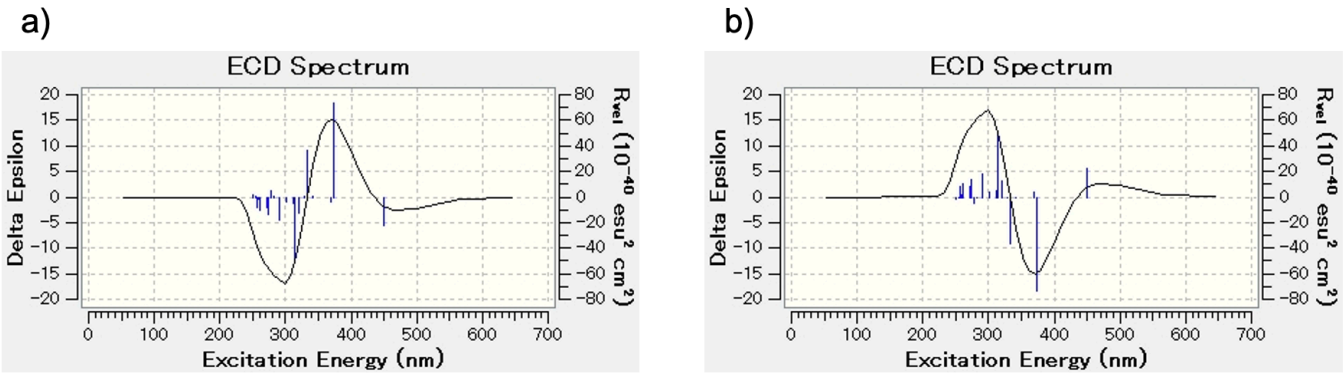



**Fig. S9** Calculated ECD spectra with the bar spectra of a) (*R*)-**1** and b) (*S*)-**1**.

**Photocytotoxicity:**

HeLa cells were incubated with **1** (final concentration: $5.0 \times 10^{-5}$ mol $L^{-1}$) at 37 °C for 1 hour under a 5% $CO_2$ atmosphere. After washing the cells, the stained HeLa cells were irradiated with an UVA-LED (365 nm, 1.1 W) at 37 °C under a $CO_2$ atmosphere. The viability was evaluated using CCK-8 (Dojindo). The absorbance at 450 nm ($A_{450}$)

was measured by a microplate reader (TECAN Infinite M200). The $A_{450}$ from independent 3 wells were used to calculate percentage of cell viability according to the formula below and show with standard deviation:

Percentage of cell viability = $A_{450}$ of treated cells / $A_{450}$ of untreated cells × 100 (%)

These results are shown in **Fig. 4b**.

**JC-1 assay:**

HeLa cells were incubated with **1** (final concentration: $5.0 \times 10^{-5}$ mol $L^{-1}$) at 37 °C for 1 hour under a 5% $CO_2$ atmosphere. After the UVA-LED irradiation, the cells were incubated with JC-1 dye (JC-1-Mitochondrial membrane potential assay kit; abcam) at 37 °C for 20 minutes under a 5% $CO_2$ atmosphere. The fluorescence images were observed through the WIG filter for the detection of red fluorescence indicating polarized normal cells and the WIB filter for the detection of green fluorescence indicating depolarized damaged cells using an OLYMPUS IX 71 inverted microscope. The bright images were also observed at the same field. These images are shown in **Fig. 5**.

We also conducted the JC-1 assay using HeLa cells incubated with lower concentration of **1**. Although the mitochondrial damages of HeLa cells were not observed in the presence of $5.0 \times 10^{-7}$ mol $L^{-1}$ of **1** after 30-min irradiation, those were observed in the presence of $5.0 \times 10^{-6}$ mol $L^{-1}$ of **1**. These results are shown in **Fig. S10**.

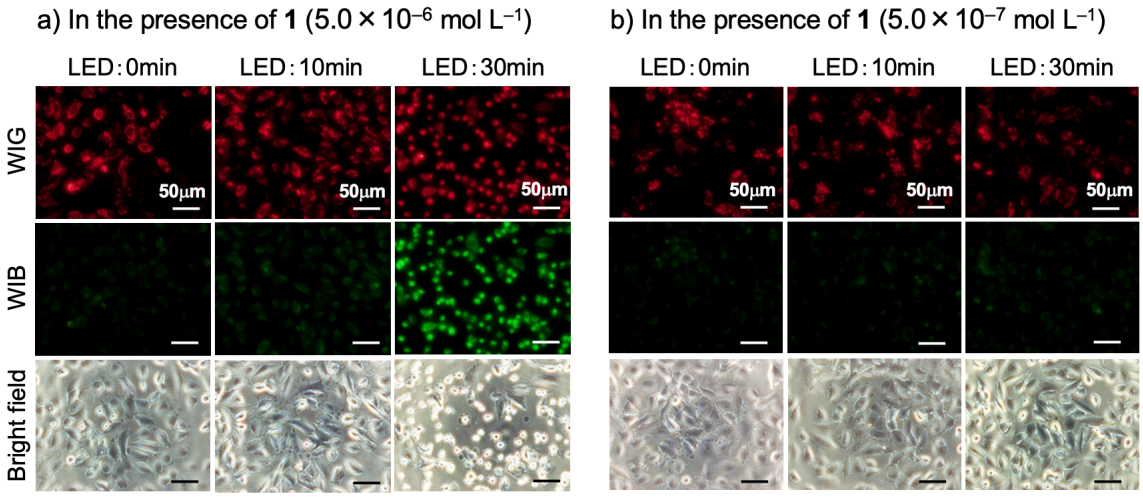



**Fig. S10** Photographs of HeLa cells incubated with the JC-1 dye in the presence of (a) $5.0 \times 10^{-6}$ mol $L^{-1}$ of **1** and (b) $5.0 \times 10^{-7}$ mol $L^{-1}$ of **1**; fluorescence images through WIG filter (upper), fluorescence images through WIB filter (middle), and bright fields (lower).

**Two-photon excitation experiment:**

To perform two-photon excitation experiment, we used a home-built multi-photon microscope, as shown in **Fig. S11**. A wavelength-tunable femtosecond optical parametric oscillator (Spectra-Physics, Inc., InSight DeepSee; tuning range, 680–1300 nm; pulse duration, ≈ 110 fs; repetition rate, 80 MHz) was used for the light source of multiphoton microscopy. The central wavelength was set to be 740 nm for two-photon excitation experiment. The output laser power was controlled by the combination of the half-wave plate and polarizer. The focal spot is two-dimensionally

scanned onto a sample by use of a pair of GMs, a pair of relay lenses, and an objective lens (Nikon, Inc., CFI Plan 50X; magnification, 50; NA, 0.9; working distance, 350 μm; oil-immersion type). To further expand the lateral imaging region, we scanned the sample position using a stepping-motor-driven translation stage every time an image was obtained by the GM optics. Forward-propagated fluorescent light was collected via the condenser lens and then was separated from the excitation laser light by a dichroic mirror (Semrock, Inc., FF640-FDi01-25x36; reflection band ($R_{avg}$ > 95%), 350–629.5 nm; transmission band ($T_{avg}$ > 93%), 652–950 nm) and an optical band-pass filter (Semrock, Inc., FF01-490/60-25; the transmission band ($T_{avg}$ > 93%), 460–520 nm). Finally, the fluorescent signal was detected by a photon-counting photomultiplier (Hamamatsu Photonics, K.K., H8259-01) connected with a pulse counter. This result is shown in **Fig. S12**. Using the above setup, two-photon image of a 280 μm by 280 μm region, composed of 256 pixels by 256 pixels, was also acquired at a rate of 0.25 image/s. This result is shown in **Fig. S13**.

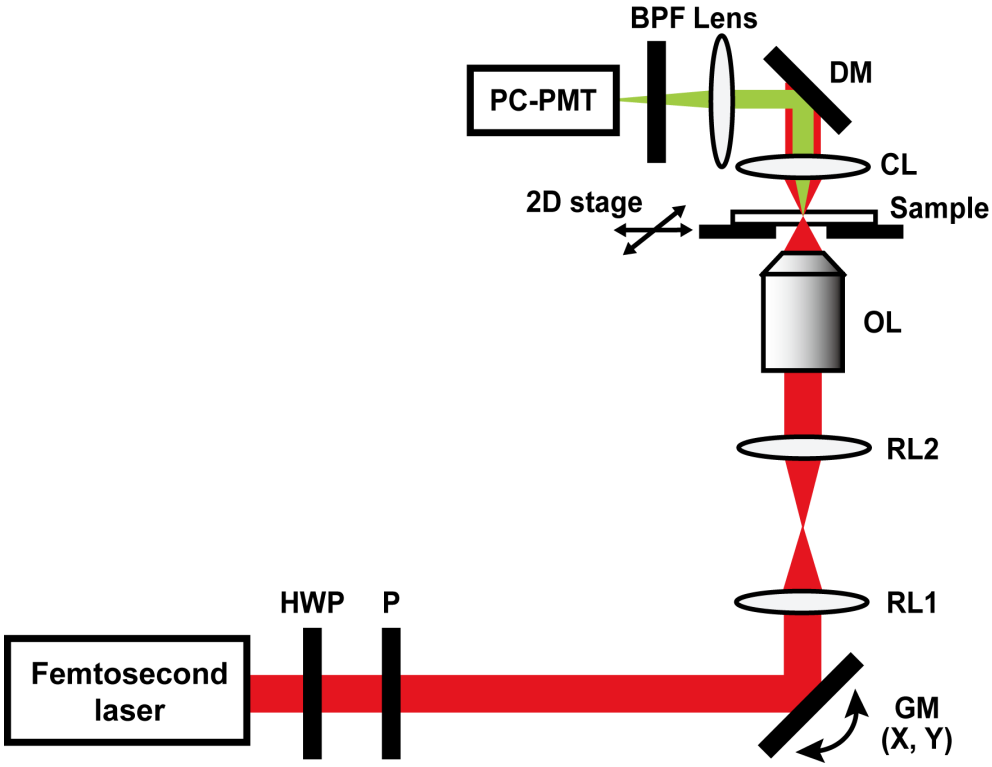



**Fig. S11** Experimental setup for multi-photon microscopy. HWP: half-wave plate; P: polarizer; GM: galvanometer mirror; RL1 and RL2: relay lenses; OL: objective lens; CL: condenser lens; DM: dichroic mirror; BPF: optical band-pass filter; PC-PMT: photon-counting photomultiplier.

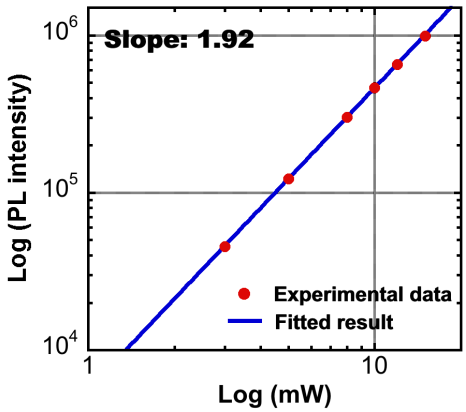



**Fig. S12** Log-log plots of fluorescence intensity of **1** vs. input laser power at 740 nm. Fluorescence emission was observed at 490±30 nm.

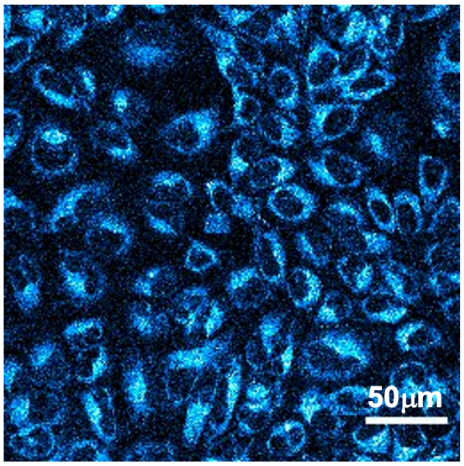



**Fig. S13** Two-photon imaging of HeLa cells stained by **1** excited at 740 nm. Fluorescence emission was observed at 490±30 nm.

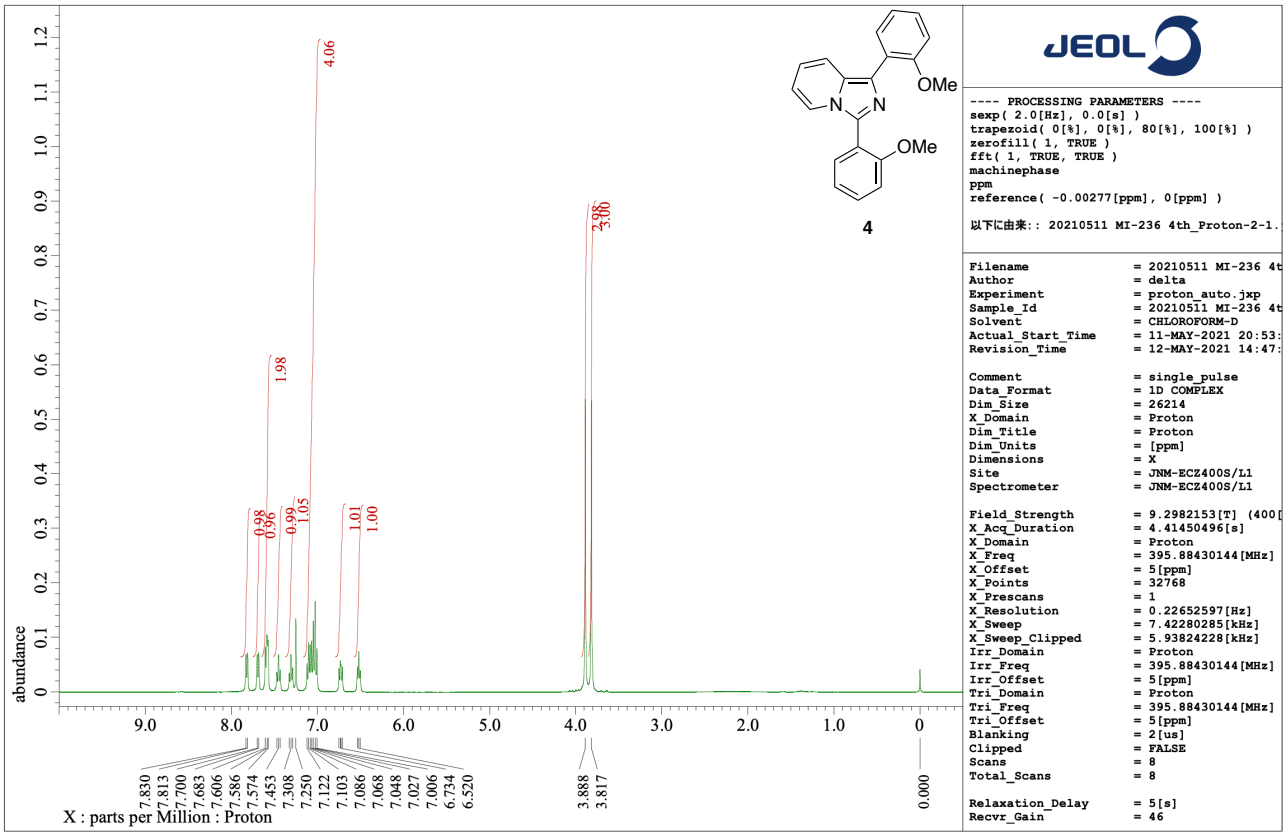
## Reference

S1 S. Fuse, T. Ohuchi, Y. Asawa, S. Sato, H. Nakamura, *Bioorg. Med. Chem. Lett.* **2016**, *26*, 5887.

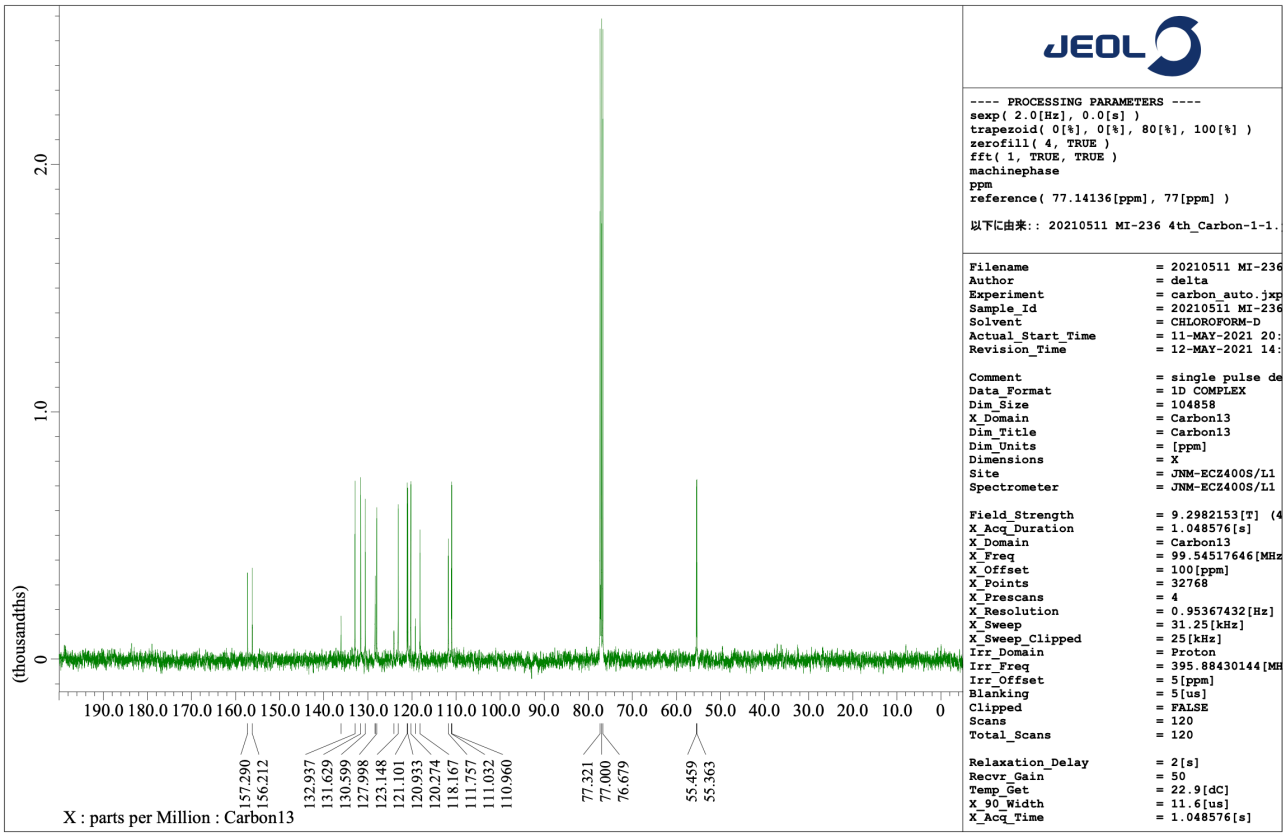
S2 Gaussian 09, Revision B.01, M. J. Frisch, et al., Gaussian, Inc., Wallingford CT, 2011.

## Compound 4

$^{1}$H NMR




$^{13}$C NMR



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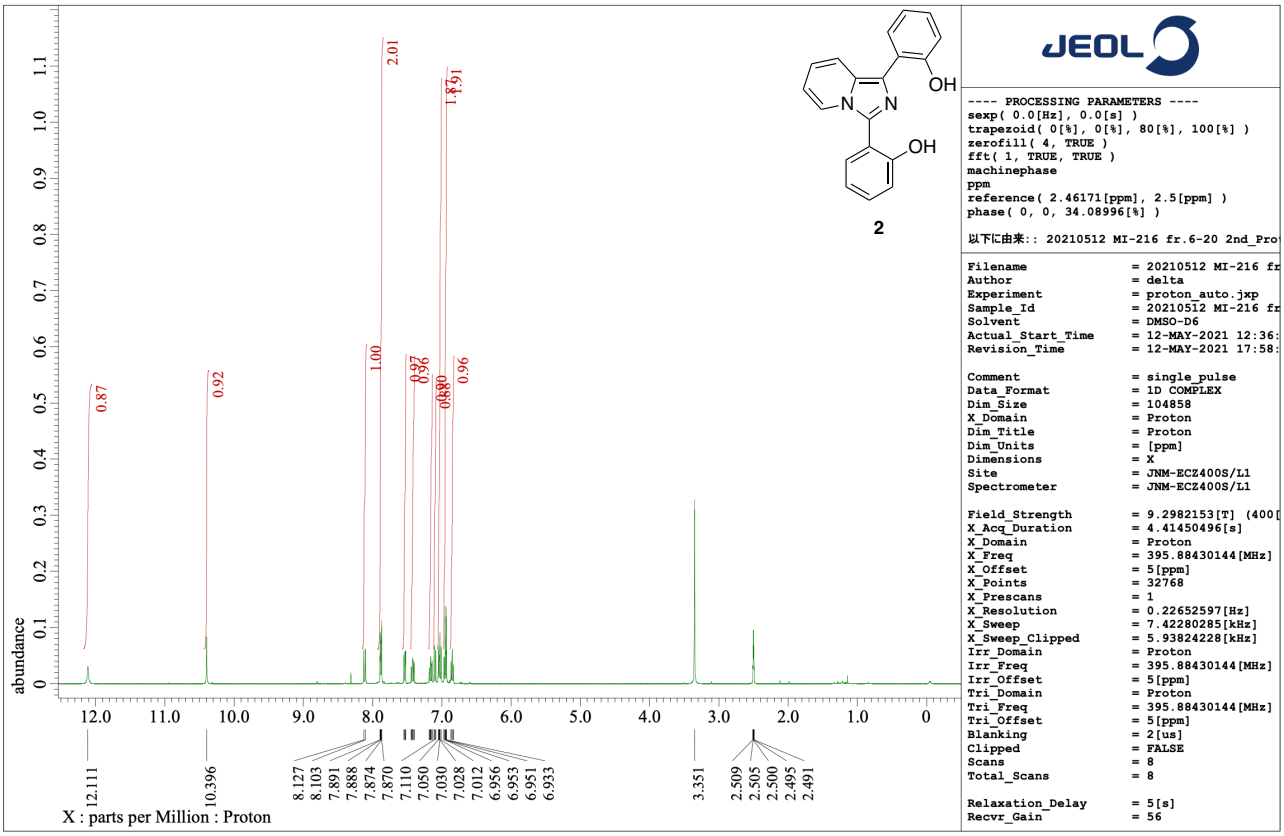
$^{1}$H NMR

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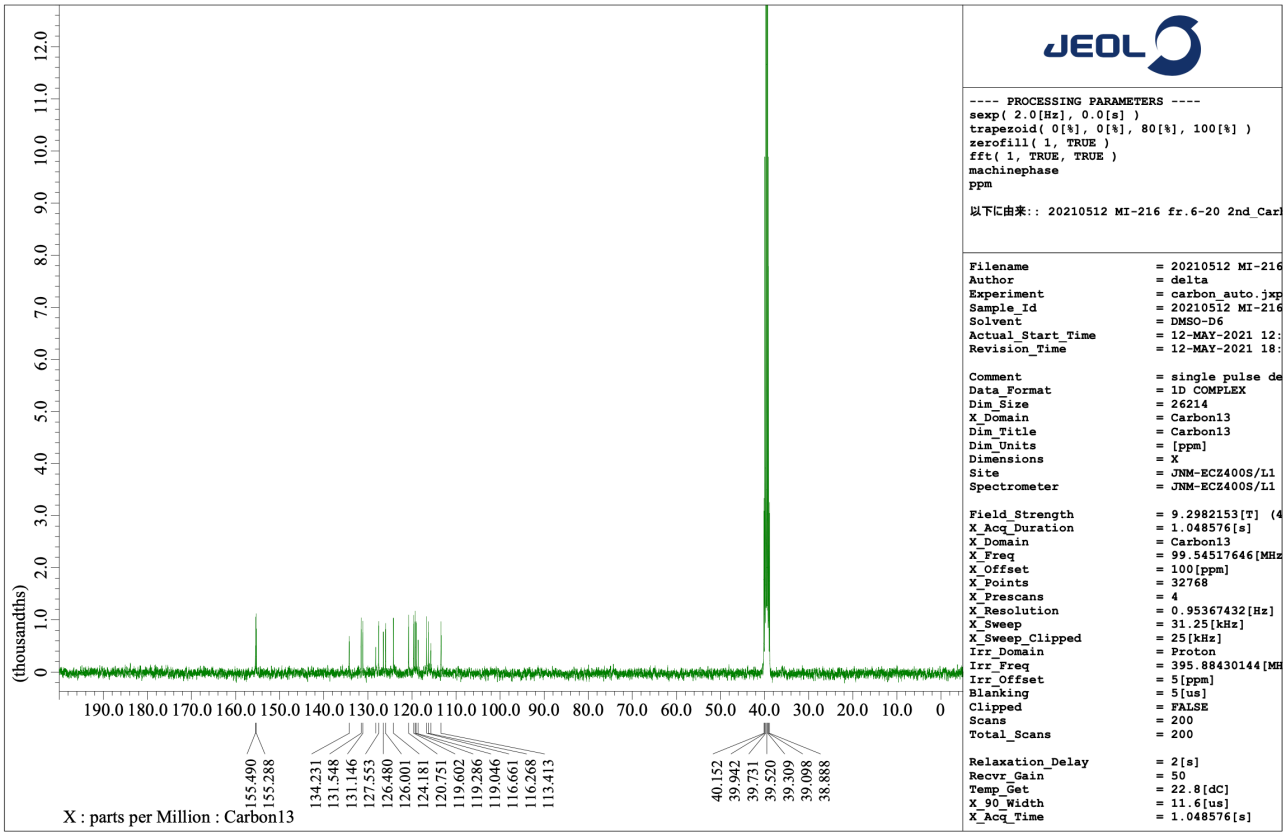
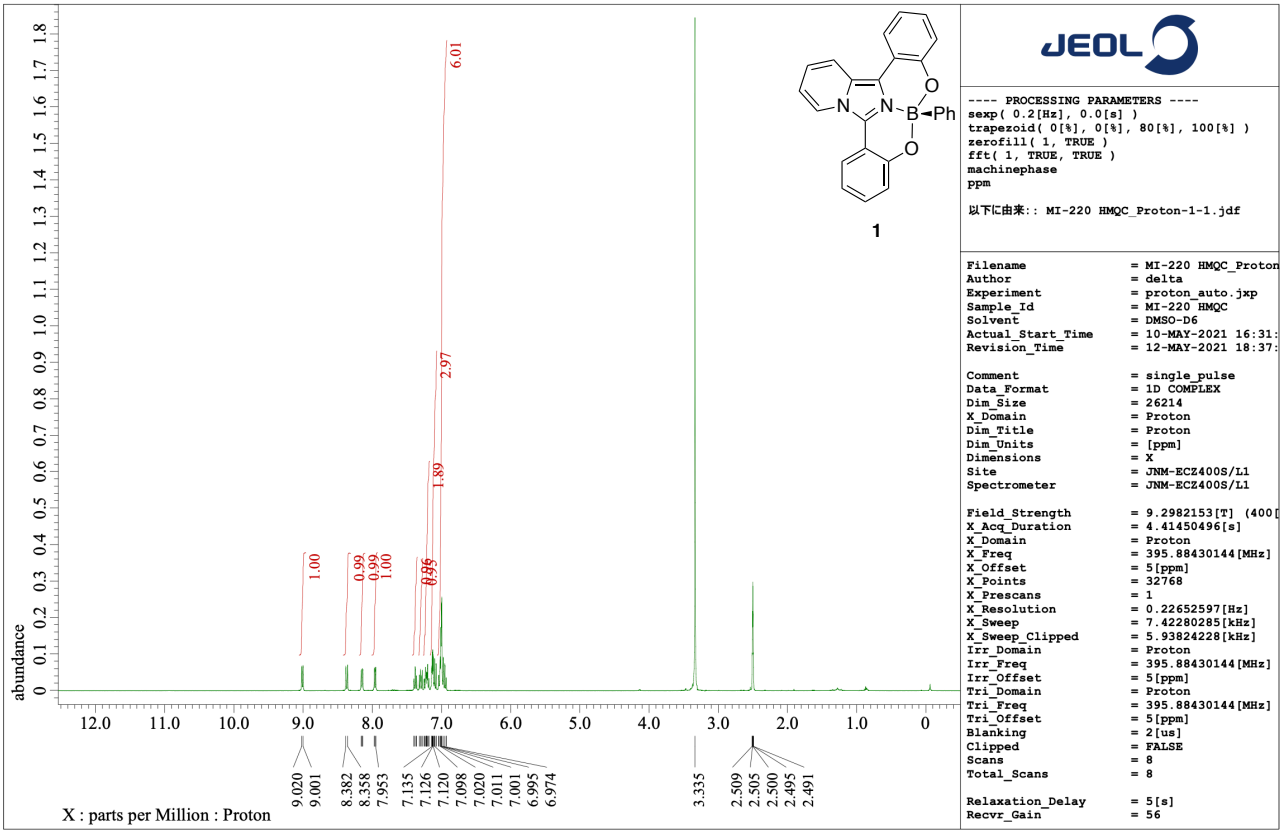


## Compound 1

$^{1}$H NMR




$^{13}$C NMR