

Supporting Information

Photorelease of a Metal-Binding Pharmacophore from a Ru(II) Polypyridine Complex

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EXPERIMENTAL SECTION

Compound Design

The geometry of the lead compound was determined using density-functional theory (DFT) calculations and excited states were determined using time-dependent DFT calculations with the Gaussian 09 software package.¹ The calculations were performed using the hybrid functional PBE0 in conjunction with the Los Alamos LANL2 effective core potential and the corresponding double-zeta basis set for the ruthenium ion. The other atoms were described using the Pople double-zeta basis set with a single set of polarization functions on non-hydrogen atoms (6-31G(d)). Solvent effects were included using a polarizable continuum model (PCM). The structures of all calculated molecules correspond to ground state minima on the ground state potential energy surfaces with no imaginary frequencies present. The structure of the *N*-terminal domain of PA endonuclease from the influenza H1N1 virus (PDB: 6E6V)² was prepared using the Molecular Operating Environment (MOE, Version: 2019.0102)³ software package by removal of the bound ligand, removal of water molecules, and calculated protonation state for protein residues. The optimized three-dimensional structures were docked using the GOLD software package. The manganese ions within the active site were predefined to have an octahedral geometry within GOLD. The binding pose was energetically minimized and evaluated.

Instrumentation and Methods

All reagents were obtained from commercial sources and used without further purification. Solvents were dried over molecular sieves if necessary. Reactions containing a ruthenium salt or complex were performed with the exclusion of light. The progress of the reactions was monitored using glass-backed silica TLC with an impregnated fluorescent indicator. Column chromatography was performed on a CombiFlash Rf Teledyne ISCO system. NMR spectra were recorded at apparatus from the nuclear magnetic resonance facility located in the Department of Chemistry and Biochemistry at the University of California, San Diego. ¹H and ¹³C NMR spectra were measured on a 300 MHz or 500 MHz NMR spectrometer. The spectra were analyzed by chemical shifts (δ) in parts per million (ppm) referenced to tetramethylsilane (δ 0.00) ppm using the residual proton solvent peaks as internal standards and coupling constants (J) in Hertz (Hz). The multiplicity of the peaks is abbreviated as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). Mass spectra were recorded at the molecular mass spectrometry facility located in the Department of Chemistry and Biochemistry at the

University of California, San Diego. High resolution mass spectrometry (HR-MS) were measured with an Agilent 6230 time-of-flight mass spectrometer using a jet stream electrospray ionization source (ESI). The jet stream source was operated under positive ionization mode with the following parameters: VCap: 3500V; fragmentor voltage: 160 V; nozzle voltage: 500 V; drying gas temperature: 325 °C, sheath gas temperature: 325 °C, drying gas flow rate: 7.0 L/min; sheath gas flow rate: 10 L/min; nebulizer pressure: 40 psi. For analytic HPLC the following system was used: Agilent 1200 series degasser and pump system with an Agilent Pursuit XRs 5C18 (250×4.6 mm) column. The solvents (HPLC grade) were millipore water (solvent A) and acetonitrile (solvent B). The following solvent gradient was used: 0-3 min: isocratic 100% A (0% B); 3-17 min: linear gradient from 100% A (0% B) to 0% A (100% B); 17-20 min: isocratic 0% A (100% B). Absorption spectra were measured with a PerkinElmer Lambda 25 UV-vis spectrometer. Emission spectra with a BioTek Synergy H4 spectrometer and excitation spectra with a Molecular Devices Spetramax Gemini XPS spectrometer.

Synthesis and Characterization

Ru(dimethyl sulfoxide)₄(Cl)₂ (1)

This compound was prepared using a previously published method with slight modifications.⁴ RuCl₃ (500 mg, 2.41 mmol) was suspended in EtOH (50 mL) and heated to reflux for 5 h. During this time, the solid completely dissolved and the color of the solution turned to green. The solvent was removed under reduced pressure. The residue was dissolved in dry DMSO (3 mL) and the mixture heated at 150 °C for 3 h. During this time the solution becomes more fluid and color changed over orange to yellow. The solution was cooled down to room temperature and dry acetone (20 mL) was added. The mixture was kept at room temperature overnight. The yellow solid was collected by vacuum filtration and washed with acetone (20 mL) and Et₂O (20 mL). Yield: 969 mg (2.00 mmol, 83%). ¹H NMR (300 MHz, D₂O): δ 3.49 (s, 6H), 3.47 (s, 6H), 3.39 (s, 6H), 2.72 (s, 6H). HRMS (*m/z*): [M-Cl]⁺ calcd. for C₈H₂₄ClO₄RuS₄, 408.9281; found, 408.9278.

Ru(2,2'-bipyridine)₂(Cl)₂ (2)

This compound was prepared using a previously published method with slight modifications.⁵ Ru(dimethyl sulfoxide)₄(Cl)₂ (2, 200 mg, 0.41 mmol), 2,2'-bipyridine (128 mg, 0.82 mmol) and LiCl (170 mg, 4.00 mmol) were suspended in dry DMF (20 mL). The solution was heated

to reflux for 4 h under nitrogen atmosphere. After this time, the solution was cooled down and acetone (200 mL) was added. The crude product was kept overnight in the freezer. The precipitate was collected by vacuum filtration. The solid was washed thoroughly with H₂O and Et₂O. Yield: 150 mg (0.31 mmol, 76%). ¹H NMR (300 MHz, CD₃SO): δ 9.97 (d, *J* = 5.6 Hz, 2H), 8.64 (d, *J* = 8.1 Hz, 2H), 8.48 (d, *J* = 8.1 Hz, 2H), 8.06 (t, *J* = 7.8 Hz, 2H), 7.77 (t, *J* = 6.6 Hz, 2H), 7.68 (t, *J* = 7.8 Hz, 2H), 7.51 (d, *J* = 5.6 Hz, 2H), 7.10 (t, *J* = 6.6 Hz, 2H). HRMS (*m/z*): [M-Cl]⁺ calcd. for C₂₀H₁₆ClN₄Ru, 449.0103; found, 449.0104.

[Ru(2,2'-bipyridine)₂(NCCH₃)₂][Cl]₂ (3)

Ru(2,2'-bipyridine)₂(Cl)₂ (**3**, 100 mg, 0.21 mmol) was dissolved in dry CH₃CN (50 mL) under nitrogen atmosphere and silver(I) trifluoromethanesulfonate (54 mg, 0.21 mmol) was added. The reaction mixture was heated to reflux for 3 h. After this time, the solution was cooled down and the precipitate was filtered off over celite. The solvent was removed under reduced pressure. The crude product was recrystallized from CH₃CN in the freezer. The solid was dissolved in H₂O and a saturated aqueous solution of NH₄PF₆ was added. The crude product precipitated as a PF₆⁻ salt that was collected by centrifugation and washed with H₂O and Et₂O. The counter ion was exchanged to Cl⁻ by elution with MeOH from the ion exchange resin (Amberlite IRA-410). Yield: 113 mg (0.20 mmol, 95%). ¹H NMR (300 MHz, CD₃SO): δ 9.38 (d, *J* = 5.5 Hz, 2H), 8.86 (d, *J* = 8.1 Hz, 2H), 8.72 (d, *J* = 8.1 Hz, 2H), 8.39 (td, *J* = 7.7, 1.4 Hz, 2H), 8.07 (td, *J* = 7.7, 1.4 Hz, 2H), 7.95 (dd, *J* = 7.5, 5.8 Hz, 2H), 7.60 (d, *J* = 5.7 Hz, 2H), 7.40 (td, *J* = 5.8, 1.4 Hz, 2H). HRMS (*m/z*): [M-PF₆]⁺ calcd. for C₂₄H₂₂N₆RuPF₆, 641.0592; found, 641.0587.

***tert*-Butyl(pyridin-4-ylmethyl)carbamate (4)**

This compound was prepared using a previously published method with slight modifications.⁶ 4-(Aminomethyl)pyridine (1 mL, 1.07 mmol) was dissolved in dry CH₃CN (10 mL). Catalytic amounts of ZrCl₄ (23 mg, 0.10 mmol) were added to the solution. Dropwise a solution of di-*tert*-butyl dicarbonate (232 mg, 1.07 mmol) in dry CH₃CN (10 mL) was added. The reaction mixture was stirred for 60 min at room temperature under nitrogen atmosphere. The solvent was removed under reduced pressure. The residue was extracted with EtOAc (3×10 mL) against H₂O (10 mL). The organic phases were combined and washed with H₂O (10 mL) and brine (10 mL). The solvent was removed and the crude product purified by column

chromatography on silica gel using a gradient of EtOAc/Hexane (0%/100% - 20%/80%). The fractions containing the product were combined and the compound dried. Yield: 106 mg (0.51 mmol, 48%). ¹H NMR (300 MHz, CD₃SO): δ 8.49 (d, *J* = 5.1 Hz, 2H), 7.50 (t, *J* = 6.3 Hz, 1H), 7.22 (d, *J* = 5.1 Hz, 2H), 4.14 (d, *J* = 6.3 Hz, 2H), 1.40 (s, 9H). HRMS (*m/z*): [M+H]⁺ calcd. for C₁₁H₁₇N₂O₂, 209.1285; found, 209.1286.

[Ru(2,2'-bipyridine)₂(*tert*-butyl(pyridin-4-ylmethyl)carbamate)₂][Cl]₂ (5)

[Ru(2,2'-bipyridine)₂(NCCH₃)₂][Cl]₂ (**4**, 100 mg, 0.18 mmol) and *tert*-butyl(pyridin-4-ylmethyl)carbamate (**1**, 94 mg, 0.45 mmol) were dissolved in EtOH (40 mL) under nitrogen atmosphere. The reaction mixture was heated at 50 °C for 8 h. After this time, the solution was cooled down and the solvent was removed under reduced pressure. The solid was washed with Et₂O. The crude product was purified by column chromatography on silica gel using a gradient of MeOH/DCM (0%/100% - 10%/90%). The fractions containing the product were united and the compound dried. Yield: 117 mg (0.13 mmol, 72%). ¹H NMR (300 MHz, CD₃OD): δ 9.87 (d, *J* = 5.6 Hz, 2H), 8.59-8.50 (m, 4H), 8.42 (dd, *J* = 8.2, 3.7 Hz, 2H), 8.32 (d, *J* = 8.1 Hz, 2H), 8.10-8.02 (m, 2H), 7.83-7.75 (m, 4H), 7.71-7.55 (m, 2H), 7.33-7.08 (m, 6H), 4.17 (s, 4H), 1.37 (s, 18H). HRMS (*m/z*): [M-Pyridine-Cl]⁺ calcd. for C₃₁H₃₂ClN₆O₂Ru, 657.1318; found, 657.1309.

[Ru(2,2'-bipyridine)₂(pyridin-4-ylmethanamine)₂][Cl]₂ (6)

[Ru(2,2'-bipyridine)₂(*tert*-butyl(pyridin-4-ylmethyl)carbamate)₂][Cl]₂ (**5**, 100 mg, 0.12 mmol) was dissolved in DCM/MeOH (4:1, 50 mL) and a HCl solution in Et₂O (2M, 10 mL) was added. The reaction mixture was stirred at room temperature overnight. After this time, the solution was cooled down and the solvent was removed under reduced pressure. The product was purified by reverse phase column chromatography with a linear gradient (0%:100% - 100%:0% methanol/water). The fractions containing the product were united and the compound dried. Yield: 61 mg (0.10 mmol, 81%). ¹H NMR (300 MHz, CD₃OD): δ 9.90 (d, *J* = 5.7 Hz, 2H), 9.00-8.89 (m, 2H), 8.75-8.36 (m, 4H), 8.23-8.14 (m, 2H), 8.15-8.04 (m, 2H), 7.92-7.75 (m, 4H), 7.72-7.59 (m, 2H), 7.49-7.15 (m, 6H), 4.13 (s, 4H). HRMS (*m/z*): [M-Pyridine+Cl]⁺ calcd. for C₂₆H₂₄ClN₆Ru, 557.0793; found, 557.0782.

[Ru(2,2'-bipyridine)₂(3,4,5-trihydroxy-*N*-(pyridin-4-ylmethyl)benzamide)₂] [Cl]₂ (7)

3,4,5-Trihydroxybenzoic acid (60 mg, 0.17 mmol) was stirred in thionyl chloride (2 mL) for 2 hours at room temperature. Thionyl chloride was removed under reduced pressure and the resulting solid was dissolved in 2 mL of DCM. [Ru(2,2'-bipyridine)₂(pyridin-4-ylmethanamine)₂][Cl]₂ (**6**, 100 mg, 143 μmol) was dissolved in 10 mL of DCM and slowly added to the stirring solution of the acid chloride and stirred for 5 h at room temperature. The solvent was removed under reduced pressure, and the product was purified using reversed phase chromatography using a gradient of ethyl acetate/methanol (0%/100% - 100%/0%). Yield: 62 mg (62 μmol, 43%). ¹H NMR (500 MHz, CD₃OD): δ 9.96 (d, *J* = 5.5 Hz, 2H), 8.69 (d, *J* = 8.1 Hz, 2H), 8.59 (d, *J* = 5.4 Hz, 2H), 8.53 (dd, *J* = 14.6, 8.1 Hz, 3H), 8.44 (d, *J* = 8.1 Hz, 2H), 8.15 (dt, *J* = 24.1, 7.7 Hz, 3H), 7.94 – 7.81 (m, 6H), 7.72 – 7.63 (m, 3H), 7.38 (d, *J* = 5.5 Hz, 2H), 7.32 (t, *J* = 6.5 Hz, 2H), 7.25 (t, *J* = 6.5 Hz, 2H), 4.15 (s, 4H). ¹³C NMR (125 MHz, CD₃OD) δ 159.46, 158.45, 158.20, 157.91, 153.61, 152.68, 152.56, 151.90, 151.69, 143.55, 136.69, 136.48, 136.20, 135.71, 127.03, 126.97, 126.45, 125.88, 124.59, 123.63, 123.46, 123.16, 122.96, 41.07. MS (*m/z*): [M]⁺ calcd. for C₄₆H₄₀N₈O₈Ru, 466.57; found, 466.67.

3,4,5-Trihydroxy-*N*-(pyridin-4-ylmethyl)benzamide (8)

3,4,5-Trihydroxybenzoic acid (500 mg, 2.94 mmol) was stirred in thionyl chloride (2 mL) for two hours at room temperature. Thionyl chloride was removed under reduced pressure, and the resulting residue was dissolved in 2 mL of DCM. 4-(Aminomethyl)pyridine (250 mg, 2.31 mmol) was dissolved in 2 mL of DCM and slowly added to the acid chloride solution, and stirred for 2 h at room temperature. The solvent was removed under reduced pressure and the resulting residue was dissolved in 1M NaOH. The product was carefully precipitated out by the slow addition of 6 M HCl. Yield: 14 mg (52 μmol, 2%). ¹H NMR (500 MHz, CD₃OD): δ 8.60 (dd, *J* = 4.5, 1.6 Hz, 2H), 7.50 (d, *J* = 4.7 Hz, 2H), 7.05 – 7.04 (m, 3H), 4.16 (s, 2H). ¹³C NMR (126 MHz, CD₃OD): δ 169.78, 149.32, 147.91, 144.88, 137.76, 123.26, 121.62, 108.78, 41.90. HRMS (*m/z*): calcd. for [C₁₃H₁₃N₂O₄]⁺, 261.0870; found, 261.0866.

[Ru(2,2'-bipyridine)₂(pyridine)₂][Cl]₂ (9)

[Ru(2,2'-bipyridine)₂(NCCH₃)₂][Cl]₂ (**4**, 100 mg, 0.18 mmol) and pyridine (145 μL, 1.80 mmol) were dissolved in EtOH (40 mL) under nitrogen atmosphere. The reaction mixture was heated at 50 °C overnight. After this time, the solution was cooled down and the solvent was

removed under reduced pressure. The product was purified by reverse phase column chromatography with a linear gradient (0%:100% - 100%:0% methanol/water). The fractions containing the product were united and the compound dried. Yield: 71 mg (0.12 mmol, 69%). $^1\text{H NMR}$ (500 MHz, CD_3OD): δ 9.97 (d, $J = 5.6$ Hz, 2H), 8.66 (d, $J = 8.1$ Hz, 2H), 8.60 (d, $J = 5.8$ Hz, 2H), 8.54-8.49 (m, 4H), 8.42 (d, $J = 7.5$ Hz, 2H), 8.13 (dtd, d, $J = 12.4, 7.9, 1.5$ Hz, 2H), 7.90-7.79 (m, 6H), 7.70 (d, $J = 5.8$ Hz, 2H), 7.48-7.39 (m, 2H), 7.23 (t, $J = 5.7$ Hz, 2H). HRMS (m/z): $[\text{M-Pyridine+Cl}]^+$ calcd. for $\text{C}_{25}\text{H}_{21}\text{ClN}_5\text{Ru}$, 528.0527; found, 528.0519.

Aqueous Stability Tests

The stability of the compounds was assessed by HPLC analysis. Compounds were dissolved in water or phosphate buffered saline (PBS) and incubated at 37 °C for 48 h in the dark. After this time, the solution was analyzed using the following HPLC system: Agilent 1200 series degasser and pump system with an Agilent Pursuit XRs 5C18 (250×4.6 mm) column. The solvents (HPLC grade) were millipore water (solvent A) and acetonitrile (solvent B). The following solvent gradient was used: 0-3 min: isocratic 100% A (0% B); 3-17 min: linear gradient from 100% A (0% B) to 0% A (100% B); 17-20 min: isocratic 0% A (100% B).

Photoinduced Ligand Release Studied by UV-vis Spectroscopy

Compounds were dissolved in MilliQ water with an absorbance of ~0.4 at 450 nm. During time intervals between 0-10 min, changes in the absorption spectrum in the dark or upon light exposure with a LED light source (centered at 450 nm) were monitored with a PerkinElmer Lambda 25 UV-vis spectrometer. After 9 min of light exposure, the compound had been irradiated with a total light dose of 1.89 J/cm² as determined using an optical power meter.

Photoinduced Ligand Release Studied by Emission Spectroscopy

Compounds were dissolved in MilliQ water with an absorbance of ~0.4 at 450 nm. During time intervals between 0-10 min, changes in the emission spectrum upon light exposure with a LED light source (centered at 450 nm) were monitored with a Synergy H4 (BioTek) microplate reader. After 9 min of light exposure, the compound had been irradiated with a total light dose of 1.89 J/cm² as determined using an optical power meter.

Photoinduced Ligand Release Studied by HPLC

Compounds were dissolved in MilliQ water at a concentration of 1 mg/mL. The solutions were either kept in the dark or exposed to a LED light source (centered at 450 nm) for 2 or 4 min. Changes were monitored by using the following analytical HPLC system: Agilent 1200 series degasser and pump system with an Agilent Pursuit XRs 5C18 (250×4.6 mm) column. The solvents (HPLC grade) were millipore water (solvent A) and acetonitrile (solvent B). The following solvent gradient was used: 0-3 min: isocratic 100% A (0% B); 3-17 min: linear gradient from 100% A (0% B) to 0% A (100% B); 17-20 min: isocratic 0% A (100% B).

Quantum Yield of Photoinduced Ligand Release

The quantum yield of the photoinduced ligand release was studied with slight modifications from previous procedures.⁷ Solutions of compound **7** and a reference ([Ru(2,2'-bipyridine)₂(pyridine)₂]Cl₂) were prepared in water such that the absorbance at the irradiation wavelength of 450 nm was 0.2 as measured by a PerkinElmer Lambda 25 UV-visible spectrometer. The solution was irradiated at several time intervals between 0-10 min with a LED light source (centered at 450 nm). The progress of the photoinduced ligand release was monitored by using HPLC analysis using the following analytical HPLC system and conditions. The instrument used was an Agilent 1200 series degasser and pump system with an Agilent Pursuit XRs 5C18 (250×4.6 mm) column. The solvents (HPLC grade) were millipore water (solvent A) and acetonitrile (solvent B). The following solvent gradient was used: 0-3 min: isocratic 100% A (0% B); 3-17 min: linear gradient from 100% A (0% B) to 0% A (100% B); 17-20 min: isocratic 0% A (100% B). The peaks in the chromatograms were integrated to determine the amount of released ligand. The percentage of remaining starting material was plotted against the irradiation dose and the corresponding linear slope determined. The quantum yield was determined by comparison with the reference [Ru(2,2'-bipyridine)₂(pyridine)₂]Cl₂ ($\Phi = 0.20$).⁸ Using the following equation, the quantum yield of the photoinduced ligand release was calculated:

$$\Phi_{\text{sample}} = \Phi_{\text{reference}} * \frac{D_{\text{sample}}}{D_{\text{reference}}} * \frac{\text{slope}_{\text{sample}}}{\text{slope}_{\text{reference}}}$$
$$D = 1 - 10^{-A}$$

Φ = quantum yield; D = fraction of light absorbed, A = absorption

PA_N Endonuclease Expression and Purification

Expression and purification of PA_N endonuclease was performed as reported previously (*J. Med. Chem.* **2019**, *62*, 9438-9449). Pandemic H1N1 N-terminal PA (PAN) endonuclease Δ52-64:Gly truncated construct was expressed from a pET-28a parent vector containing a kanamycin-resistance reporter gene with expression inducible by the Lac 1 operon. PA_N endonuclease was expressed as an 8-histidine tagged fusion protein, cleavable by TEV protease. The transformation protocol was adapted from pET system manual (Novagen) using single competent BL21 cells. Briefly, 1 μL of 25 ng/μL recombinant plasmid was used for transformation. Cells were mixed by flicking with plasmid and were heat shocked at 42 °C for 30 sec followed by incubation on ice for 5 min. Outgrowth was plated on LB agarose plates contain 50 μg/mL kanamycin and was incubated overnight at 37 °C. One colony was scraped from the LB plate and added to 50 mL of SOC broth containing 50 μg/mL kanamycin and was incubated for 5 h at 37 °C with shaking at 125 rpm. Glycerol stocks of this culture were prepared (0.9 mL cultured media + 0.1 mL 80% glycerol) and column frozen for future expressions. SOC media (100 mL) containing 50 μg/mL kanamycin was combined with 1 mL frozen cell glycerol stock and was incubated with shaking at 200 rpm at 37 °C. When the OD₆₀₀ of this starter culture reached >2 (~5-6 h), the culture was equally divided into 6×1 L batches of expression media (TB media with added 0.2% dextrose, 0.1 mM MnCl₂, and 0.1 mM MgSO₄) containing 50 μg/mL kanamycin. Cells were grown to the beginning of log phase (OD₆₀₀ = between 0.4-0.6) at 37 °C with shaking at 200 rpm. When the OD₆₀₀ = 0.4-0.6, the cultures were cooled to room temperature over ice. Expression of PA_N endonuclease was then induced by the addition of IPTG to a final concentration of 0.1 mM. The cultures were grown with vigorous shaking (250 rpm) overnight at room temperature. After ~18 h, the cells were harvested by centrifuging at 2000g for 30 min at 4 °C. The resulting cell paste was stored at -80 °C prior to lysis. The cell paste was thawed on ice for 2 h and re-suspended 1:1 (v/v) with lysis buffer (20 mM Na₂PO₄, 500 mM NaCl, 25 mM imidazole, 1 mM MgCl₂, 2 mM dithiothreitol, 0.2% TritonX, pH=7.4) plus EDTA free protease inhibitor (1 pellet per ~50 mL lysis buffer), lysozyme (1 mg/mL), and DNase-1 (10-100 μg/mL). Further lysis was performed using a probe sonicator over 10 min with cycles of 10 sec sonication and 20 sec rest. The cell suspension was kept in a water/ice bath during sonication. The lysates were free-flowing and homogenous. The lysates were shaken at 125 rpm for 30 min on ice. Cell debris was then

pelleted by centrifugation at 10000 rpm for 45 min at 4 °C. The supernatant was decanted from the pellet and was filtered through 0.45 µm syringe filters. The resulting lysate was loaded onto 2×5 mL HisTrap HP (Pharmacia) columns that had been previously charged with Ni ions. The columns were washed with binding buffer (20 mM Na₂PO₄, 500 mM NaCl, 25 mM imidazole, pH 7.4) until fraction absorbance reached a steady baseline. The protein was then eluted over a 45 min gradient at a flow rate of 4 mL/min, from 0-100% elution buffer (20 mM Na₂PO₄, 500 mM NaCl, 500 mM imidazole, pH 7.4). PA_N endonuclease eluted between 40-60% elution buffer. SDS-PAGE analysis showed a band corresponding to PA_N endonuclease running at ~23 kDa. Fractions containing endonuclease protein were combined in a 10K MWCO dialysis bag with 2000 units of TEV protease and dithiothreitol final concentration of 1 mM. The solutions were dialyzed against dialysis buffer (100 mM NaCl, 1 mM dithiothreitol, 1 mM MnCl₂, 20 mM Tris, 5% glycerol, pH 8.0) overnight at 4 °C with two buffer exchanges. A white precipitate forms over time along the inside and outside walls of the dialysis tubing. After buffer exchange, the solution was filtered through a 0.45 µm filter and was concentrated to 5-10 mg/ml using a pressurized Amicon system. This protein was suitable for use in fluorescence quenching-based nuclease assays.

PA_N Endonuclease Enzymatic Assay

PA_N endonuclease activity assays were carried out as previously reported (*J. Med. Chem.* **2019**, *62*, 9438-9449). Assays were performed using Black Costar 96-well plates. Each well contained a total volume of 100 µL comprised of buffer (20 mM Tris, 150 mM NaCl, 2 mM MnCl₂, 0.2% TritonX100, pH 8.0), influenza PA_N endonuclease (25 nM), inhibitor (various concentrations), and fluorescent ssDNA-oligo substrate (200 nM). A single-stranded, 17-mer DNA substrate labelled with a 5'-FAM fluorophore and a 3'-TAMRA quencher ([6-FAM]AATCGCAGGCAGCACTC[TAM], Sigma-Aldrich) was employed as the substrate. All assay components were pipetted into the plate, and ultimately, the substrate was added using a multi-channel pipette, and the assay was immediately started. Samples were prepared in triplicates. Background wells consisting of all assay components except enzyme were prepared for each sample. Positive and negative controls were prepared on each plate to gauge the fluorescence signal of fully active protein and the absence of protein. Change in fluorescence of each well was measured by a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek) at 39 second intervals over 45 min at 37 °C ($\lambda_{\text{ex}} = 485 \text{ nm}$; $\lambda_{\text{em}} = 528 \text{ nm}$). The gain was set to 100. Typically, data collected between 20 and 35 min was used in the activity calculations, as

this data range had a linear slope. The slope of the fluorescence signal for each sample was background corrected, and percent inhibition was determined by normalizing the slope of the sample to that of the positive and negative controls. Dose response curves were generated for inhibitors by plotting percent inhibition versus log of the concentration for each inhibitor. A dose response curve was performed with concentrations of inhibitor between 316 μ M and 3.16 nM. The data were fit with a sigmoidal curve to determine the IC₅₀ value using Prism.

SUPPORTING FIGURES

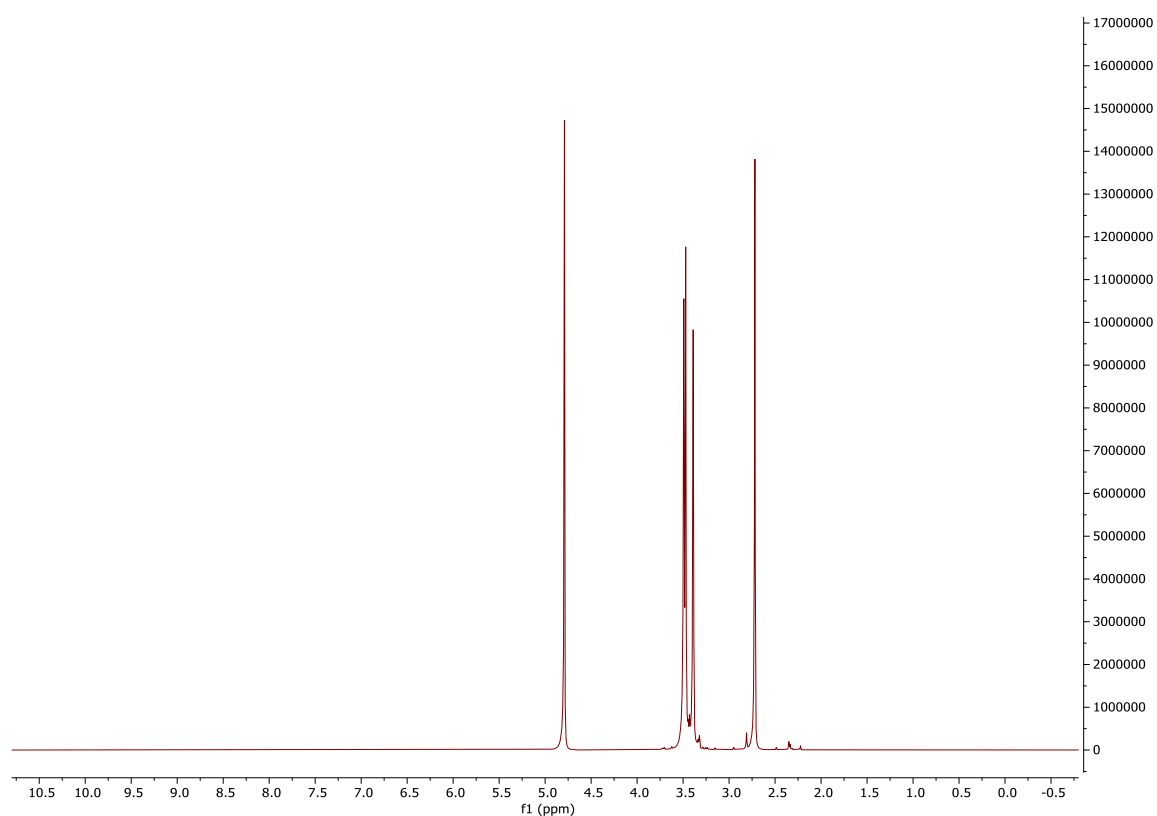


Figure S1. ^1H NMR spectrum of **1** in D_2O .

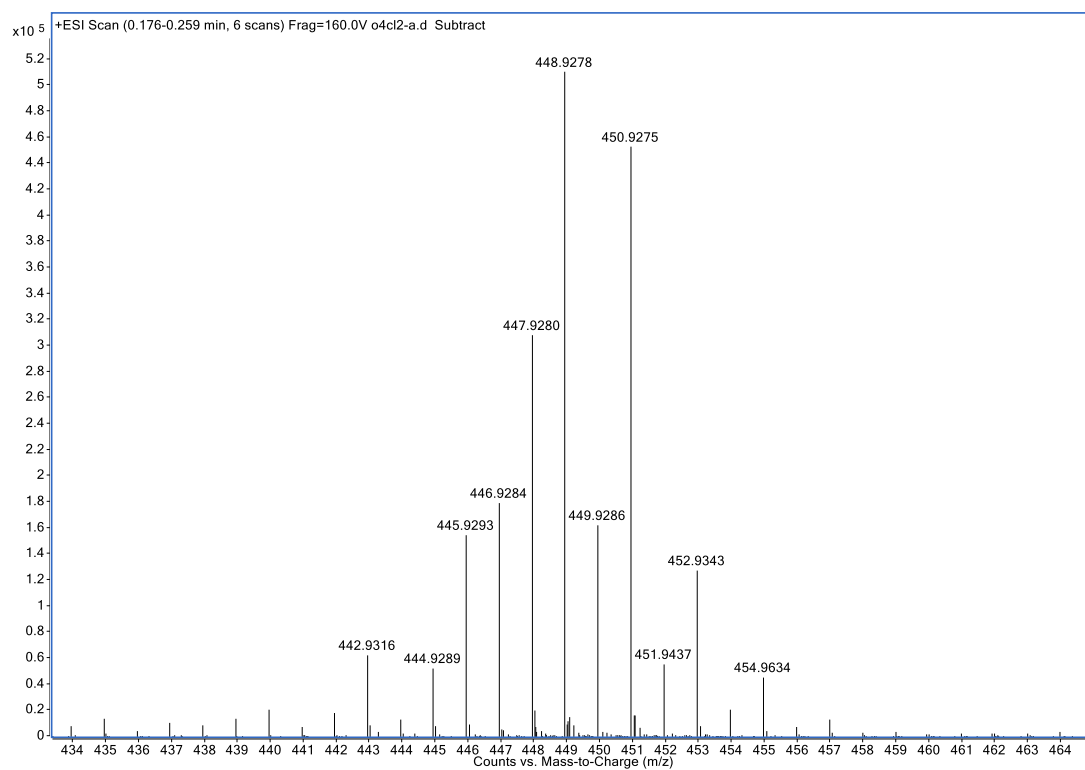


Figure S2. HRMS spectrum of **1**.

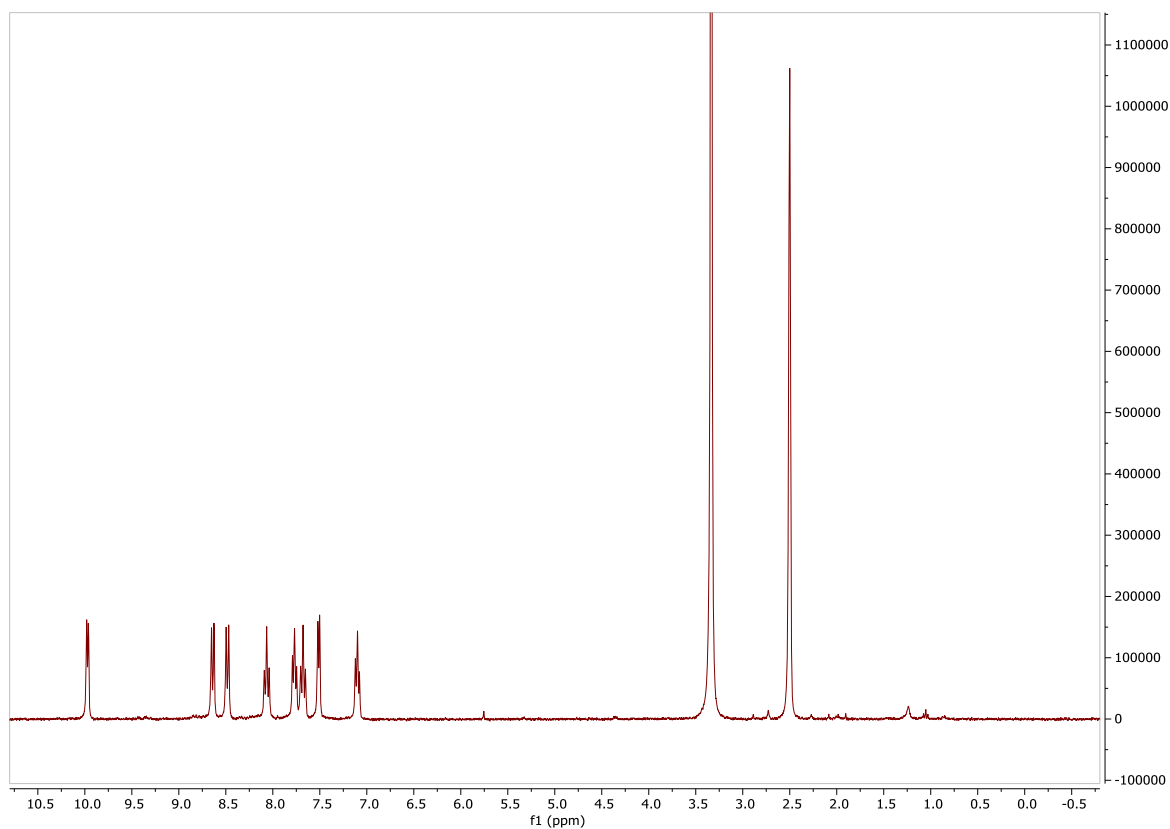


Figure S3. ^1H NMR spectrum of **2** in CD_3SO .

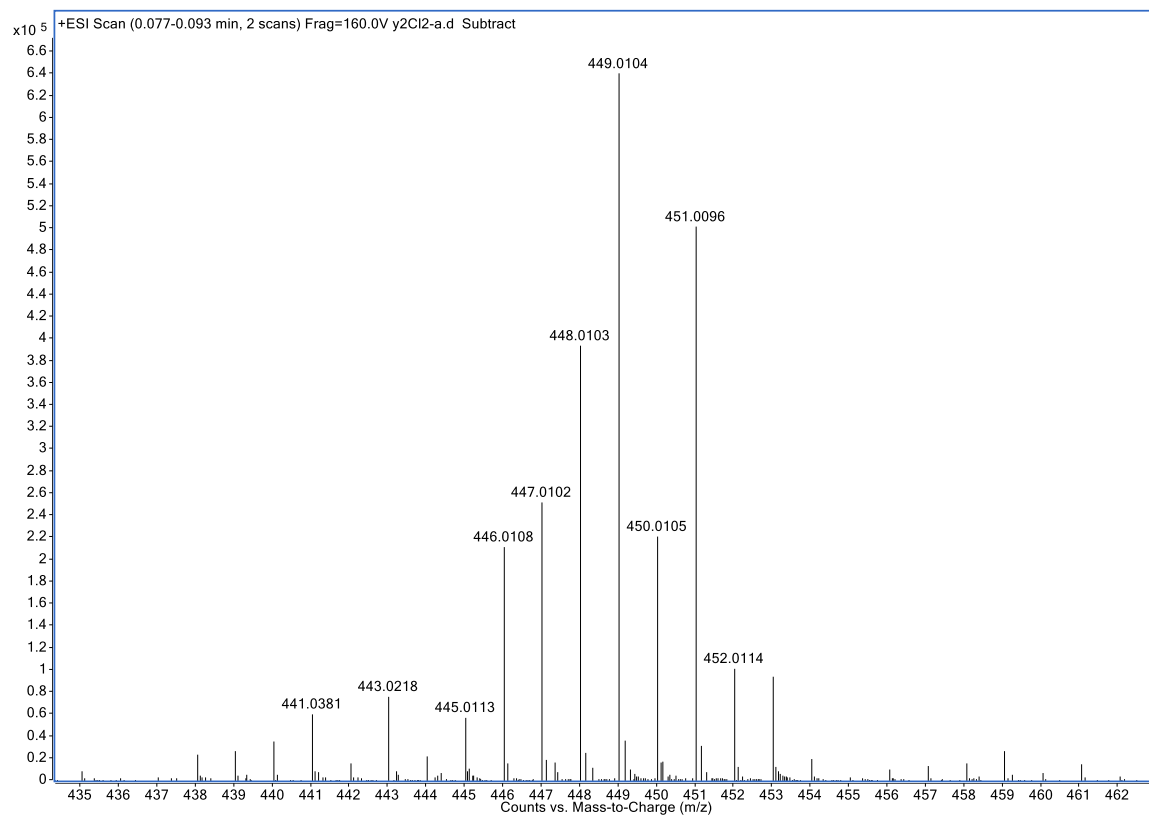


Figure S4. HRMS spectrum of **2**.

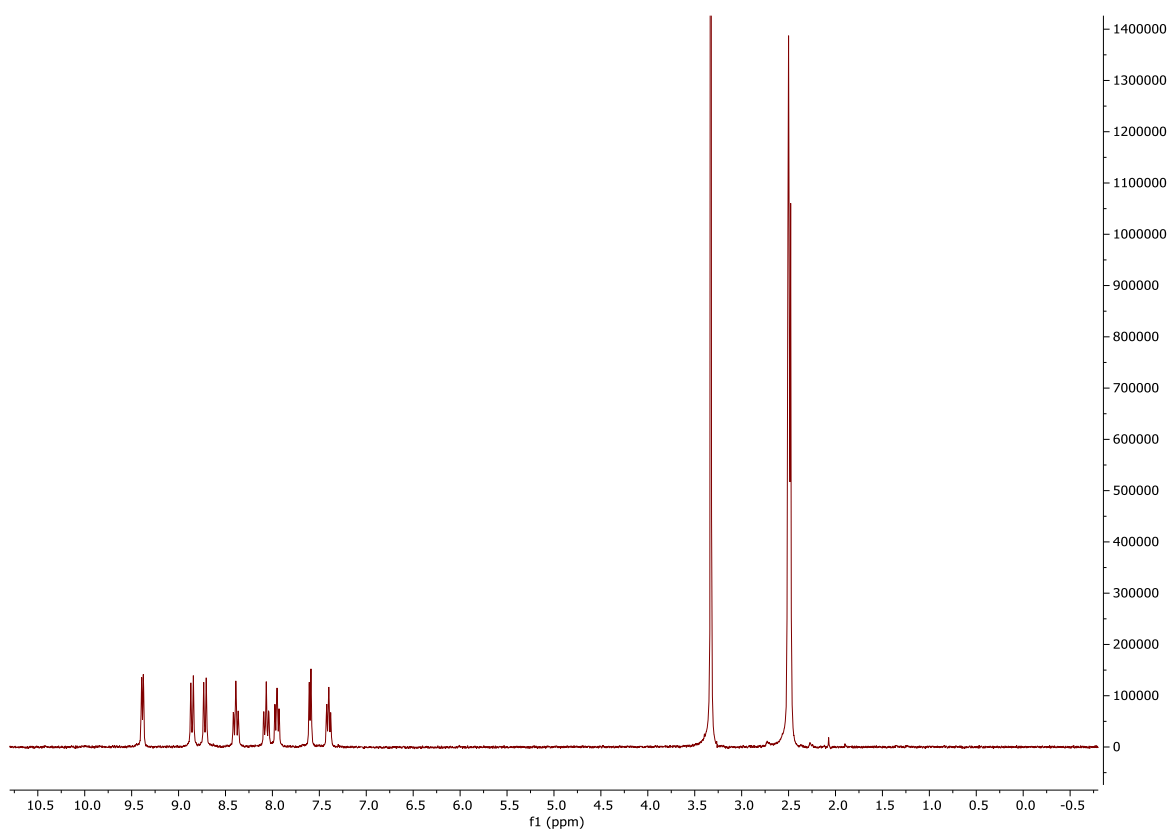


Figure S5. ^1H NMR spectrum of **3** in CD_3SO .

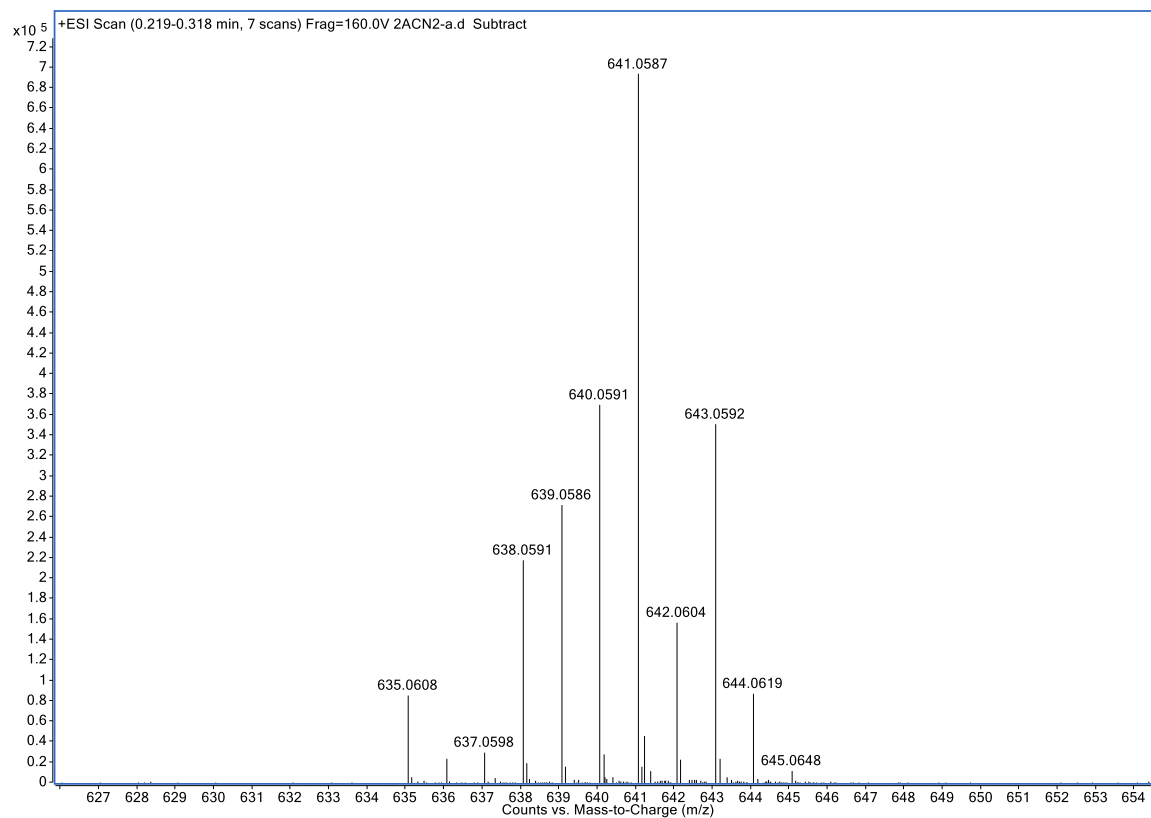


Figure S6. HRMS spectrum of **3**.

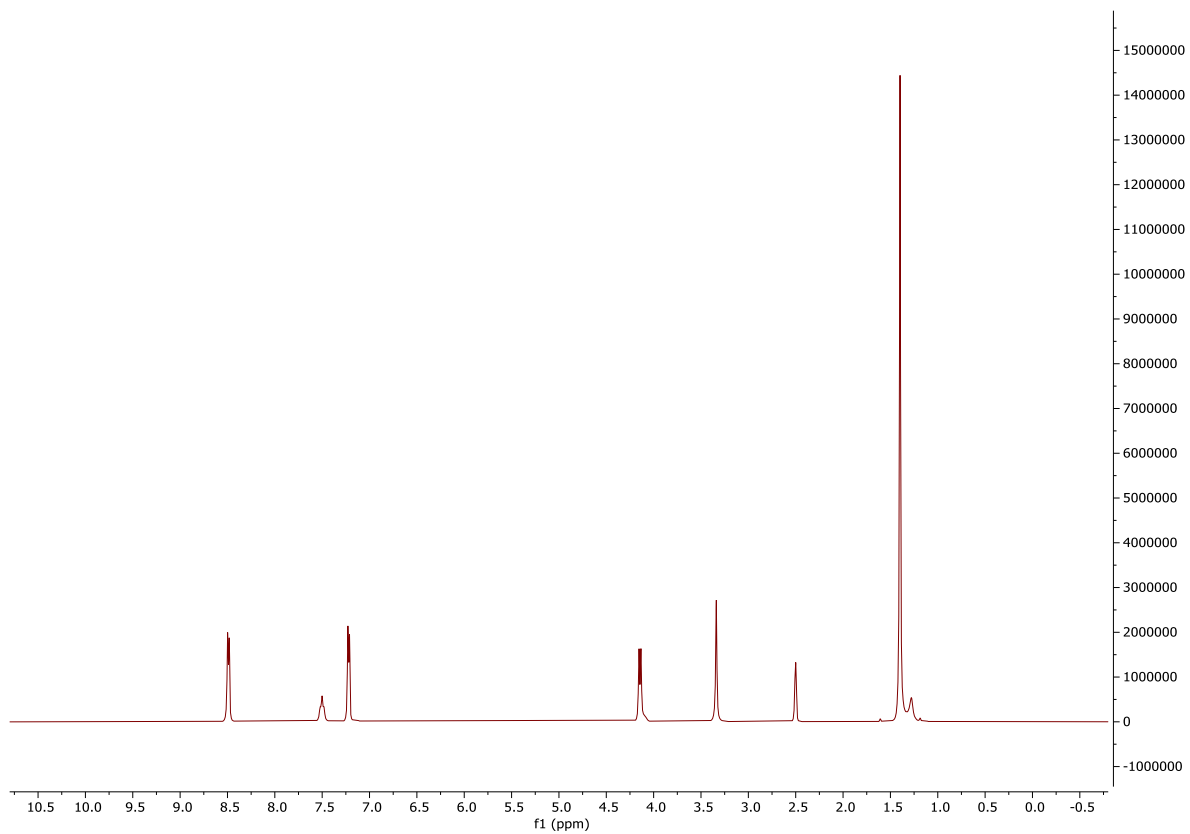


Figure S7. ^1H NMR spectrum of **4** in CD_3SO .

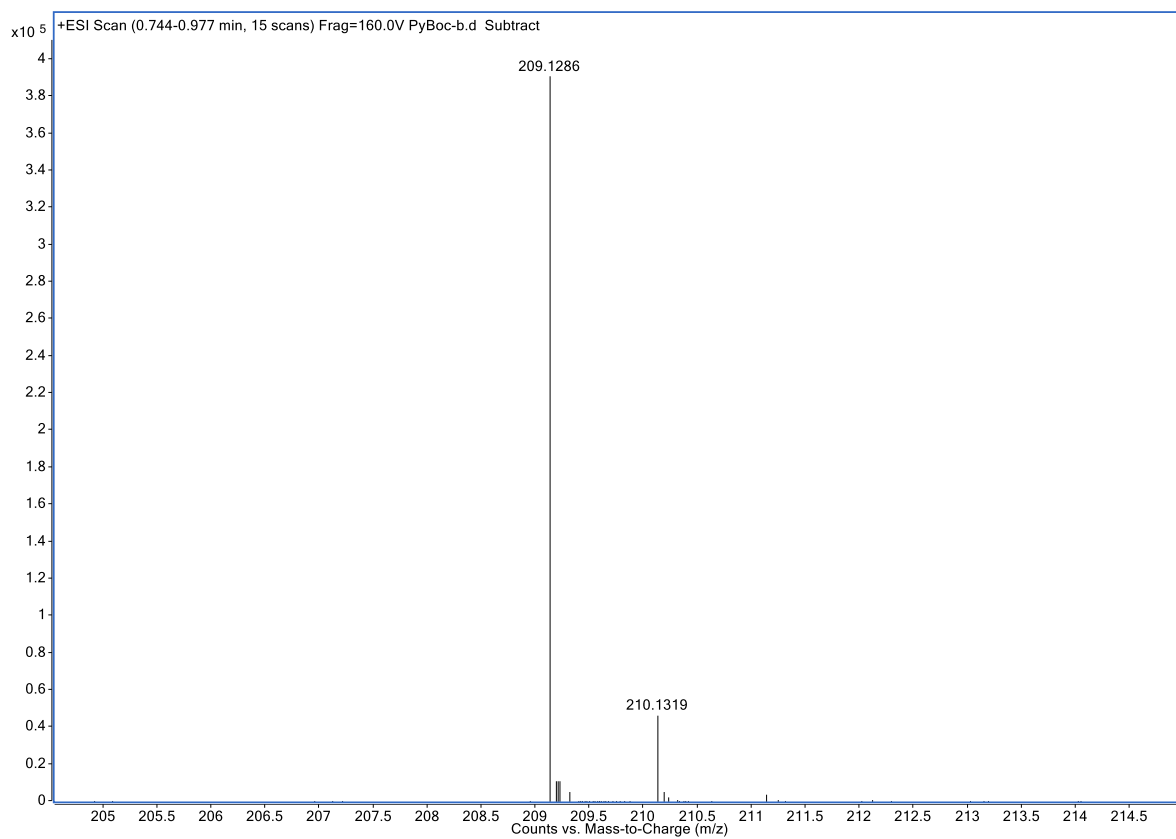


Figure S8. HRMS spectrum of **4**.

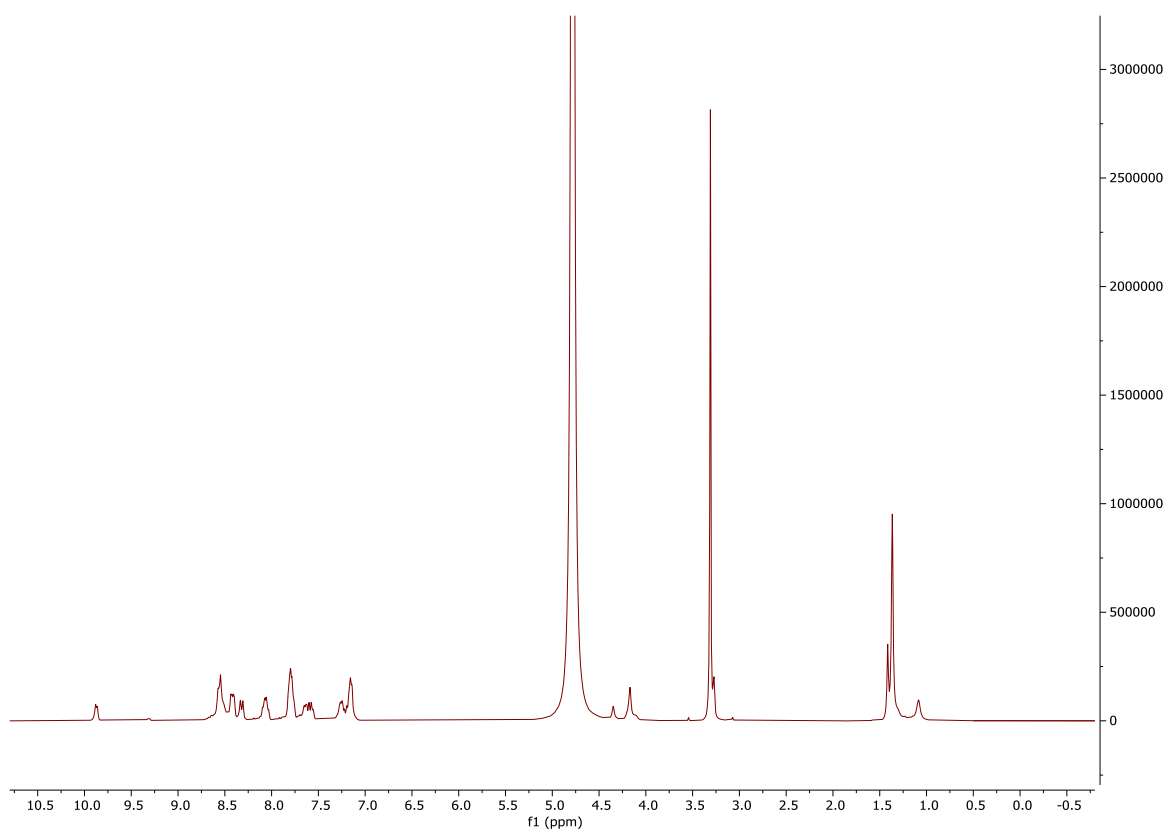


Figure S9. ^1H NMR spectrum of **5** in CD_3OD .

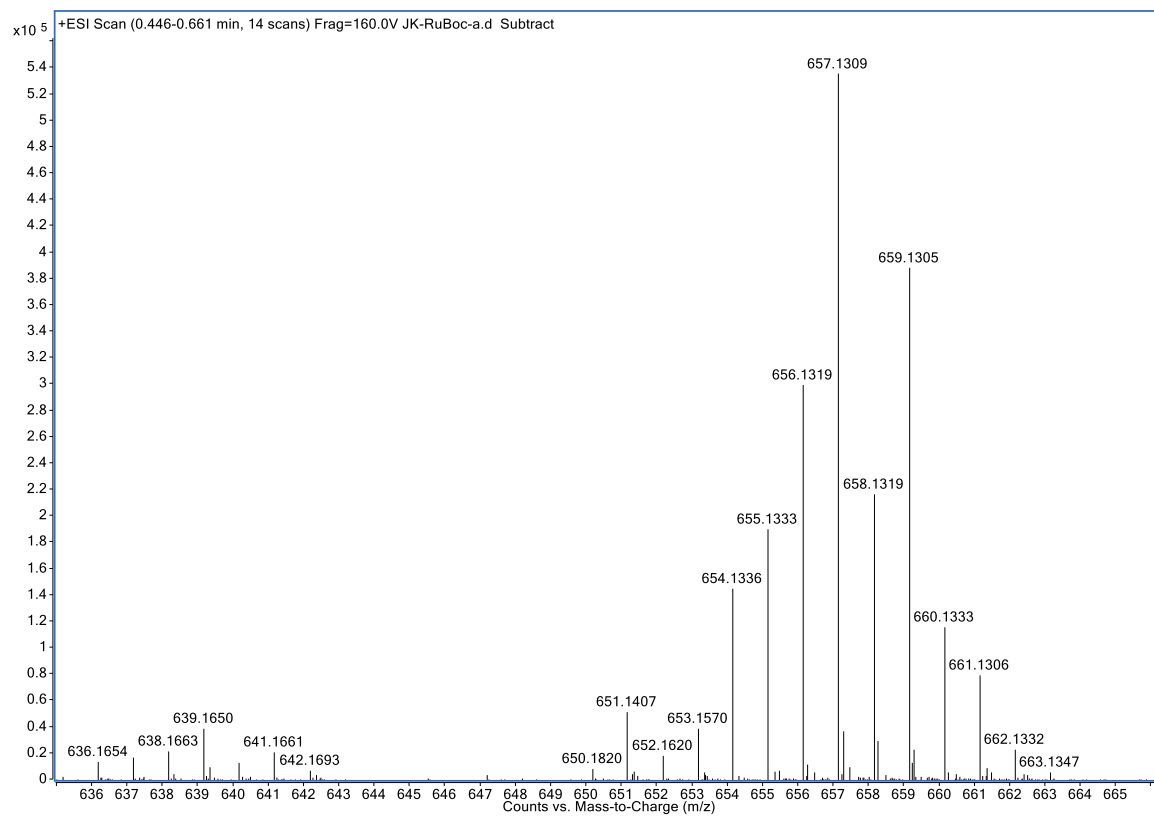


Figure S10. HRMS spectrum of **5**.

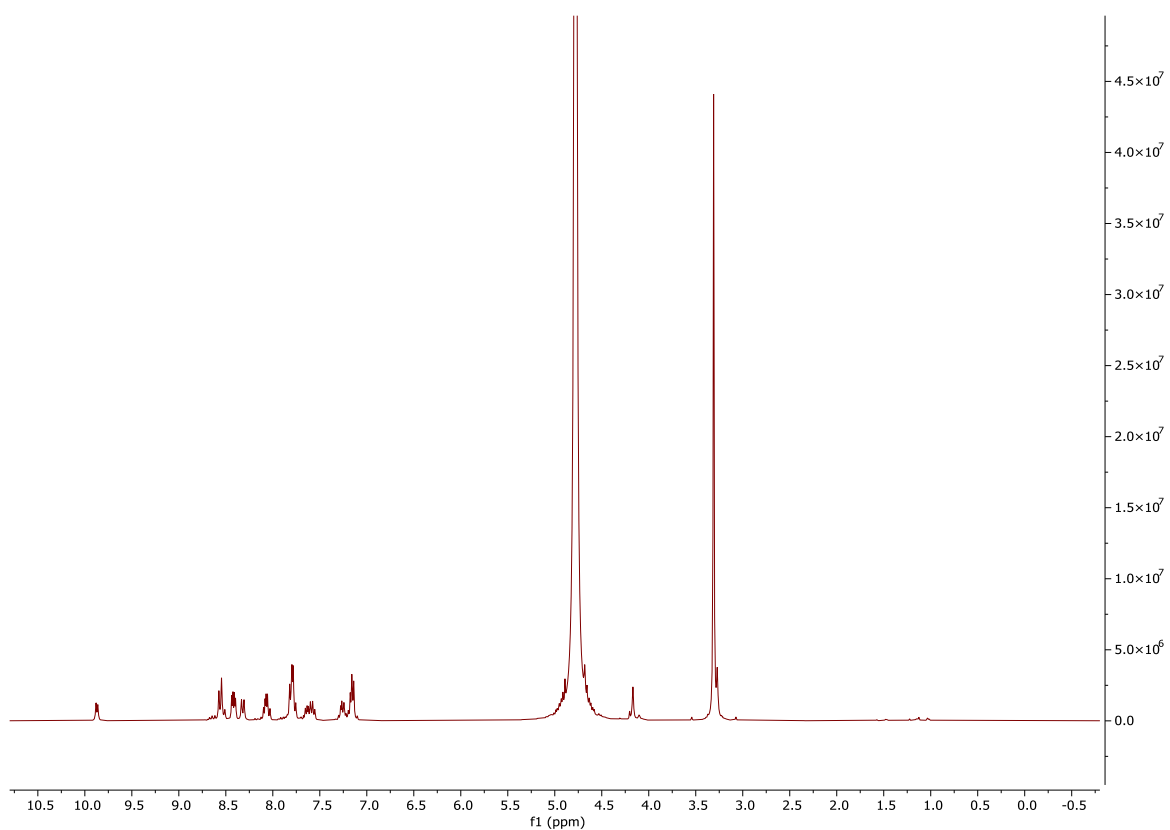


Figure S11. ^1H NMR spectrum of **6** in CD_3OD .

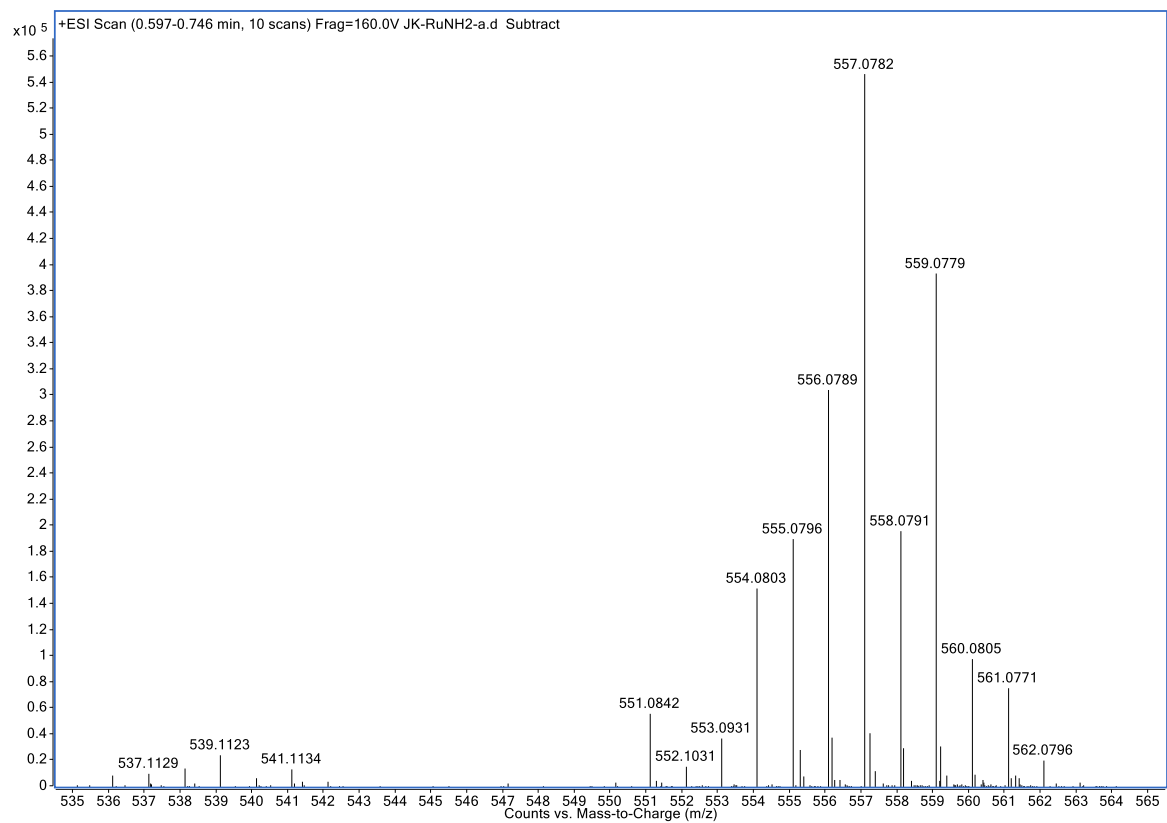


Figure S12. HRMS spectrum of **6**.

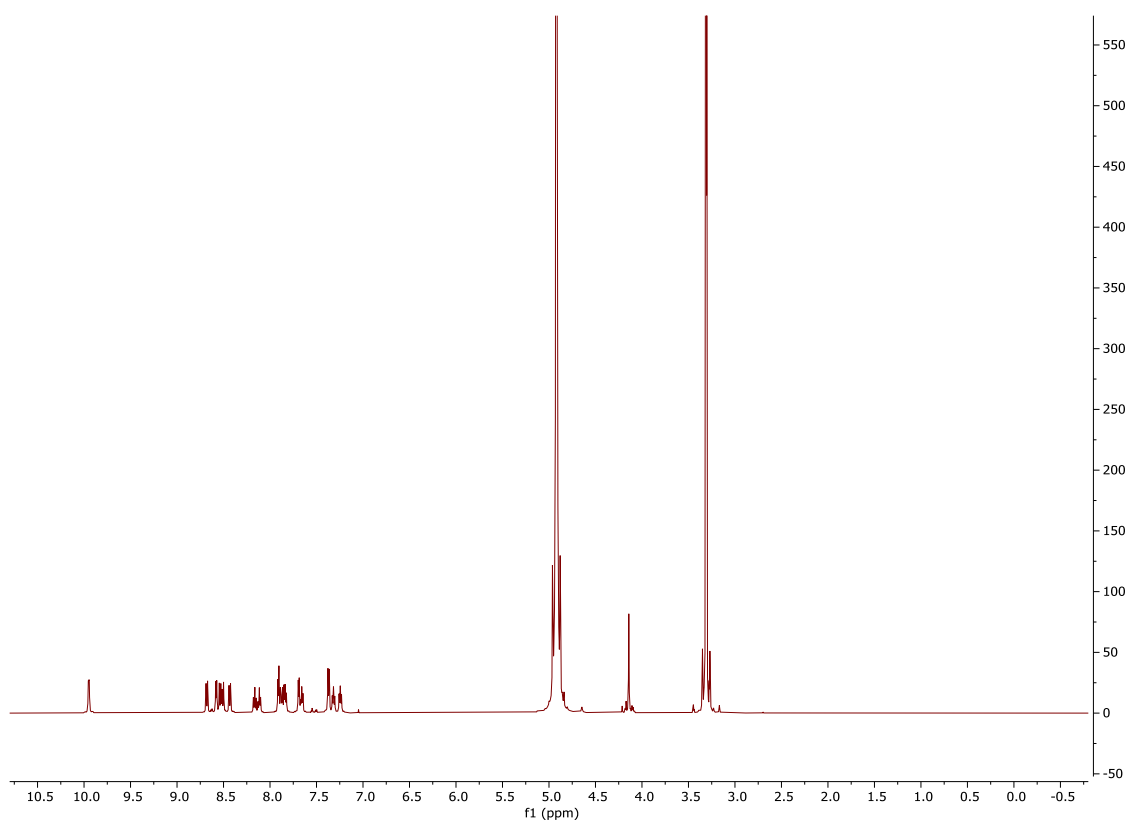


Figure S13. ^1H NMR spectrum of **7** in CD_3OD .

RWS-Ru-a #95-102 RT: 1.75-1.87 AV: 8 SB: 9 0.42-0.56 NL: 9.76E6
T: + c ESI Full ms [200.00-1300.00]

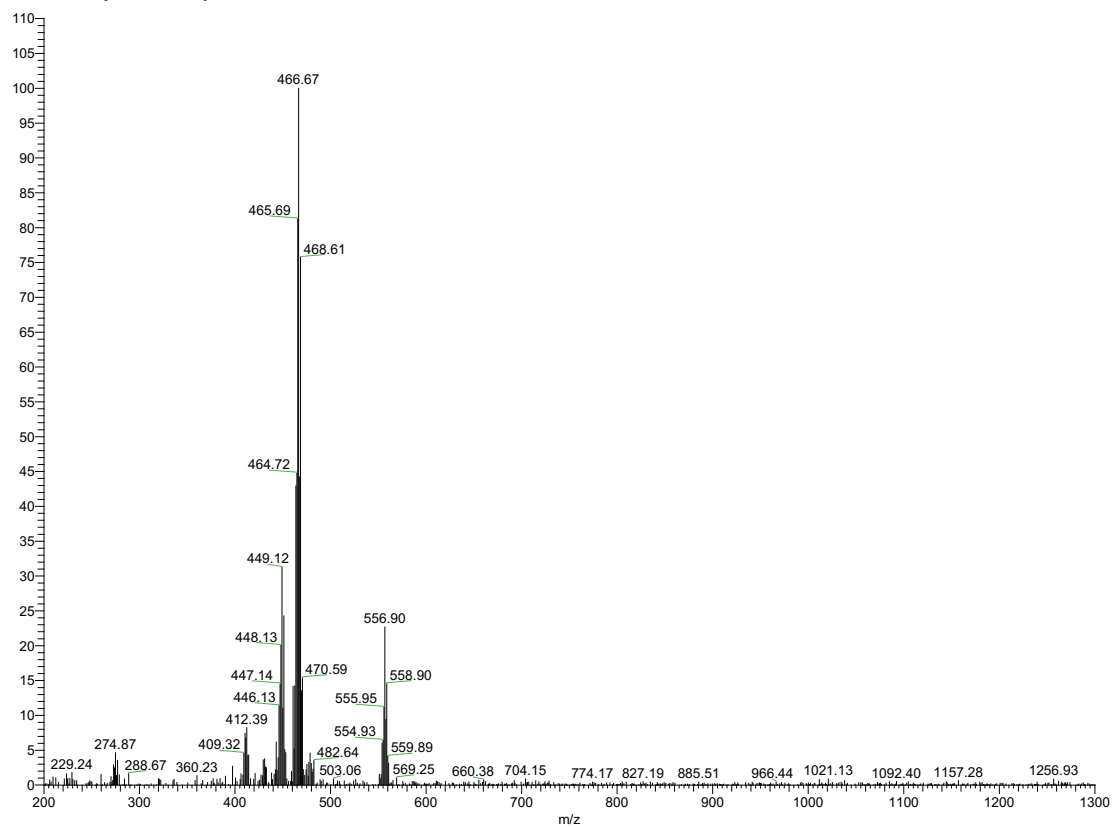


Figure S14. MS spectrum of **7**.

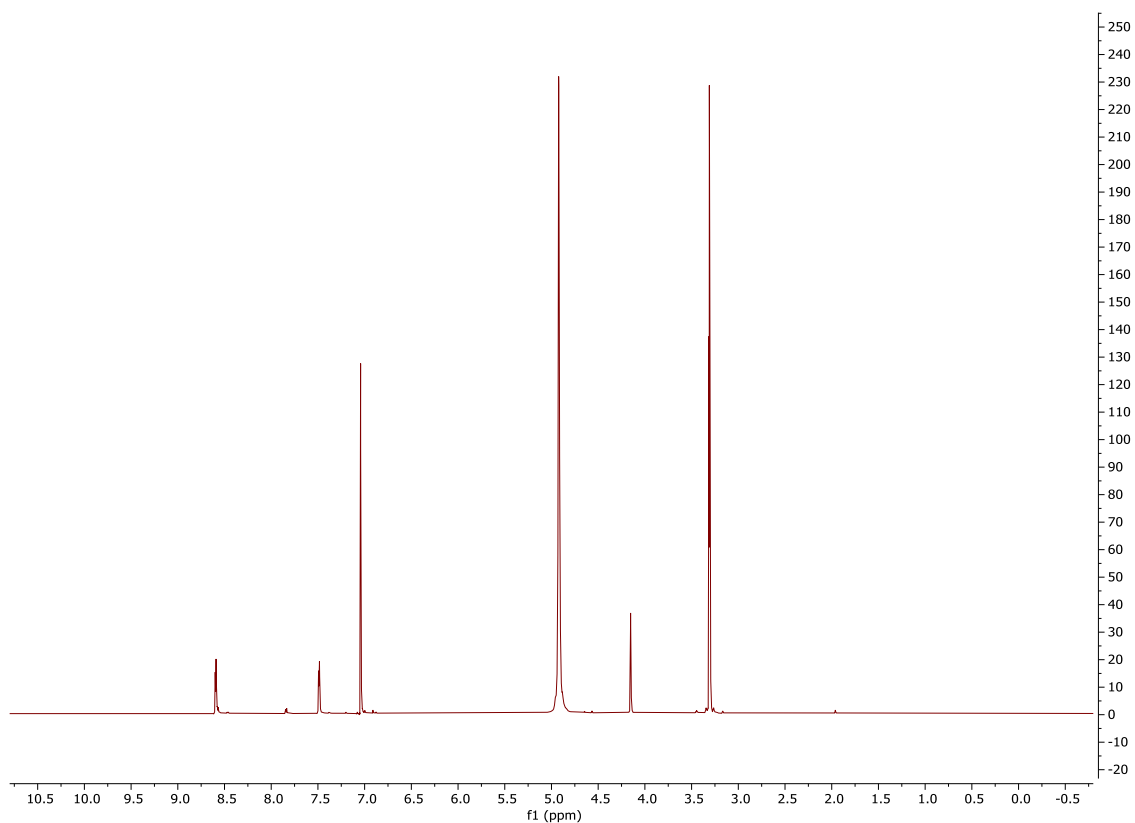


Figure S15. ¹H NMR spectrum of **8** in CD₃OD.

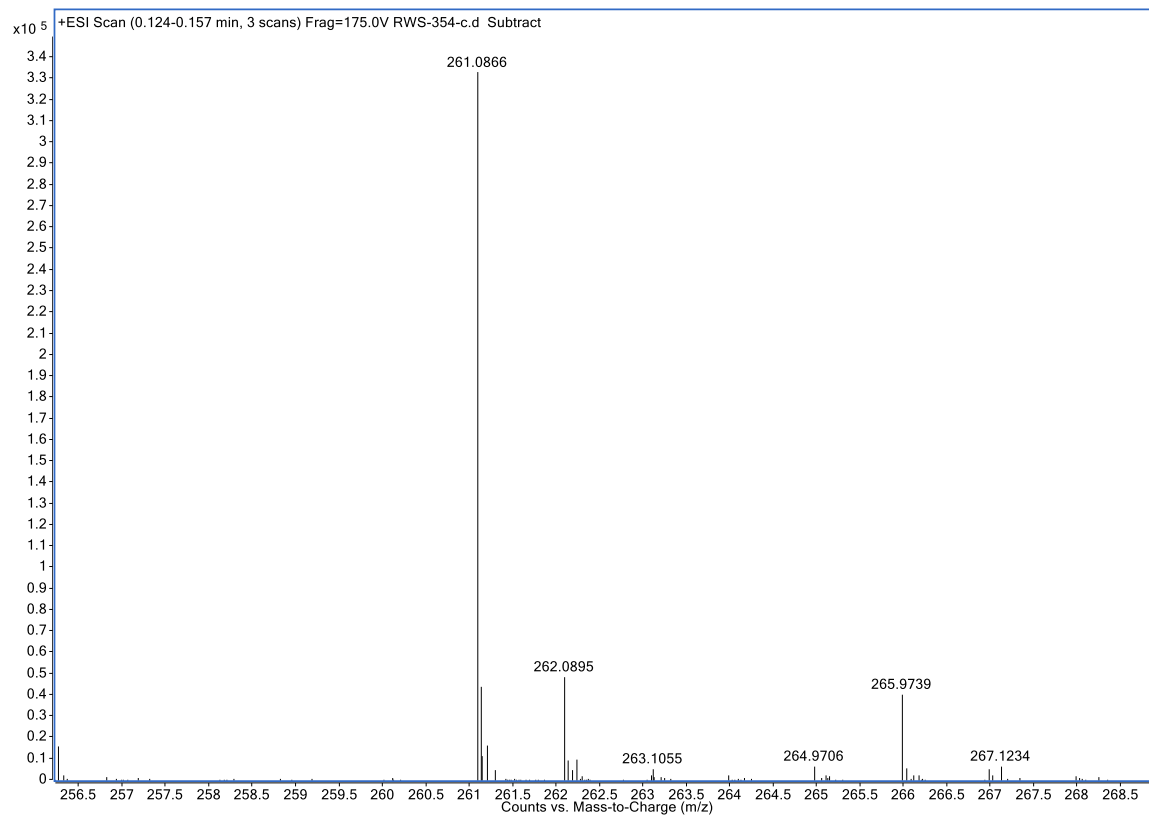


Figure S16. HRMS spectrum of **8**.

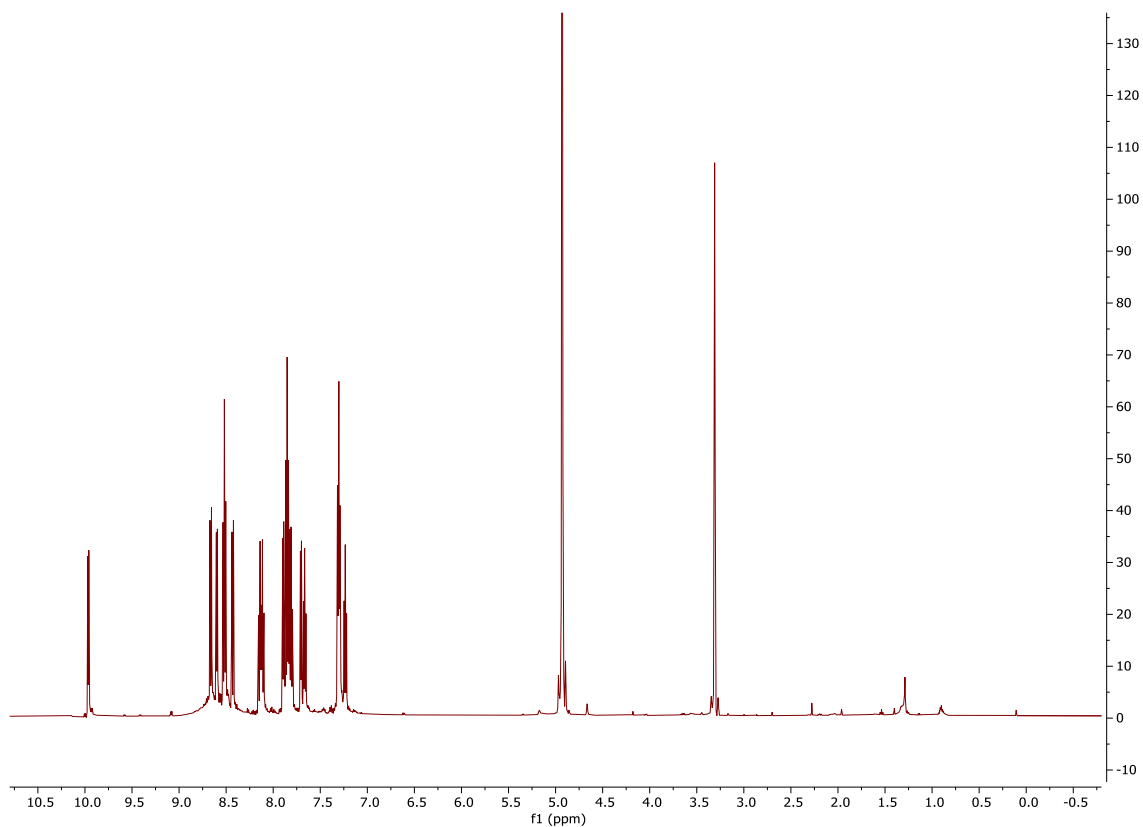


Figure S17. ^1H NMR spectrum of **9** in CD_3OD .

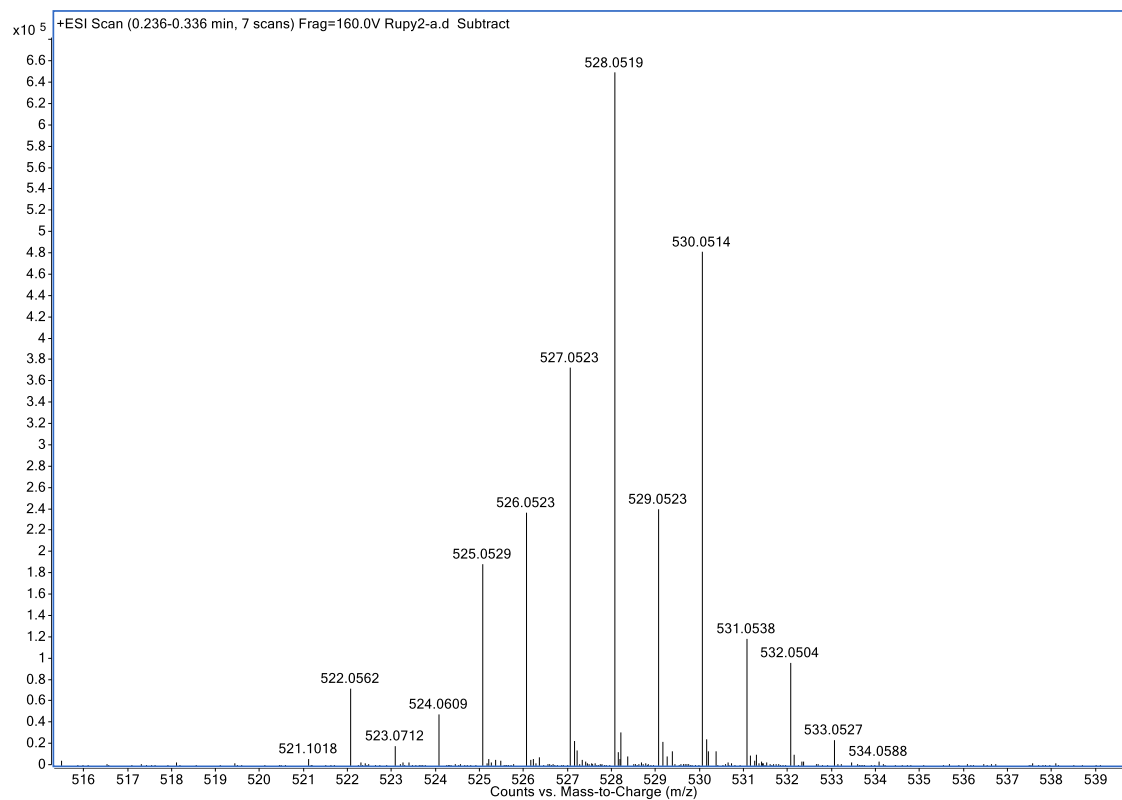


Figure S18. HRMS spectrum of **9**.

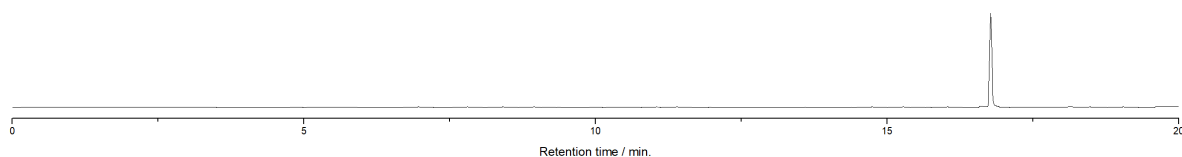


Figure S19. HPLC chromatogram of **2**.

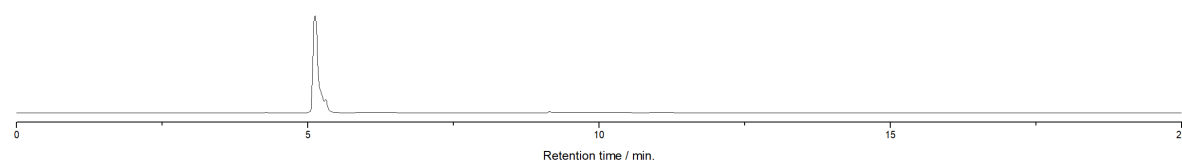


Figure S20. HPLC chromatogram of **7**.

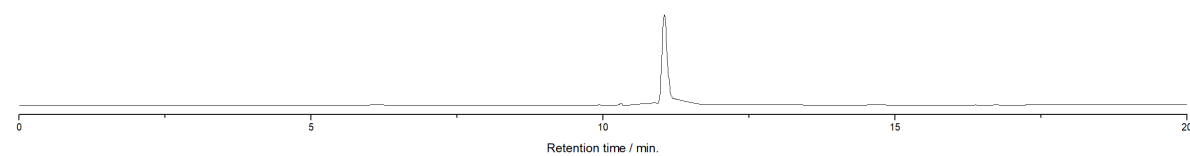


Figure S21. HPLC chromatogram of **8**.

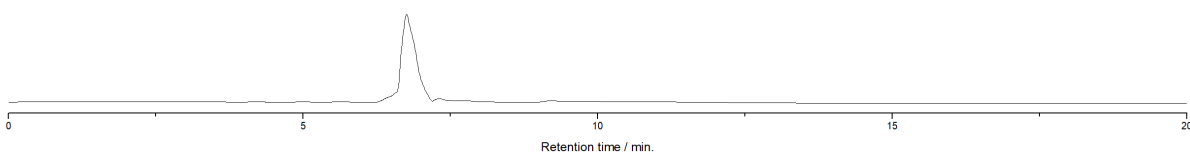


Figure S22. HPLC chromatogram of **9**.

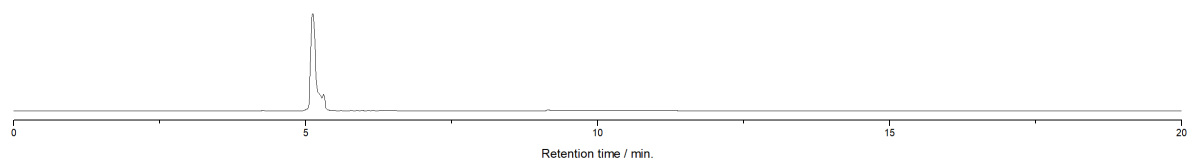
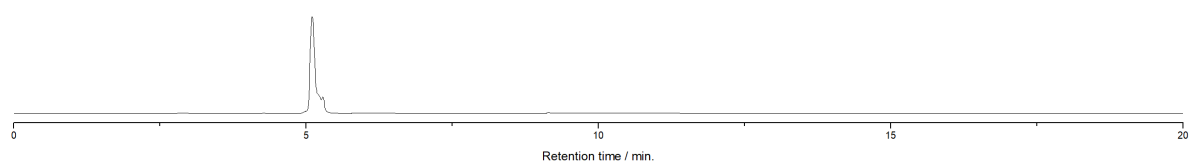


Figure S23. HPLC chromatogram of **7** upon incubation at 0 h (*top*) and after 48 h in H₂O (*bottom*).

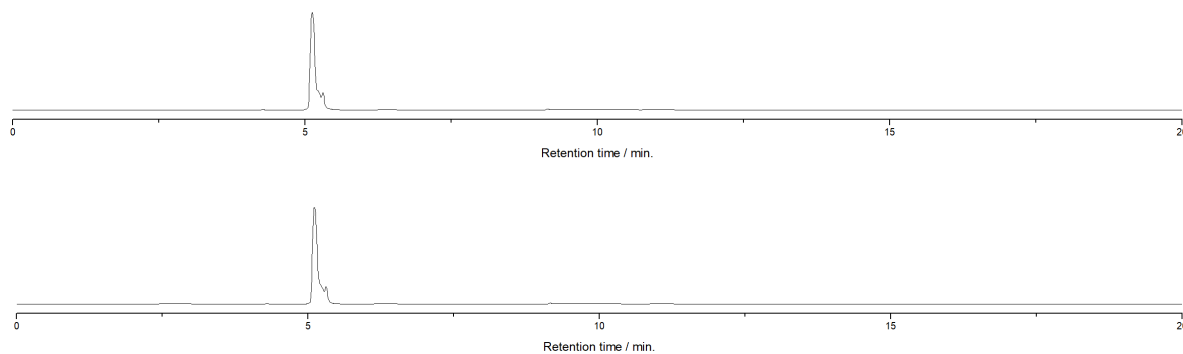


Figure S24. HPLC chromatogram of **7** upon incubation at 0 h (*top*) and after 48 h in PBS (*bottom*).

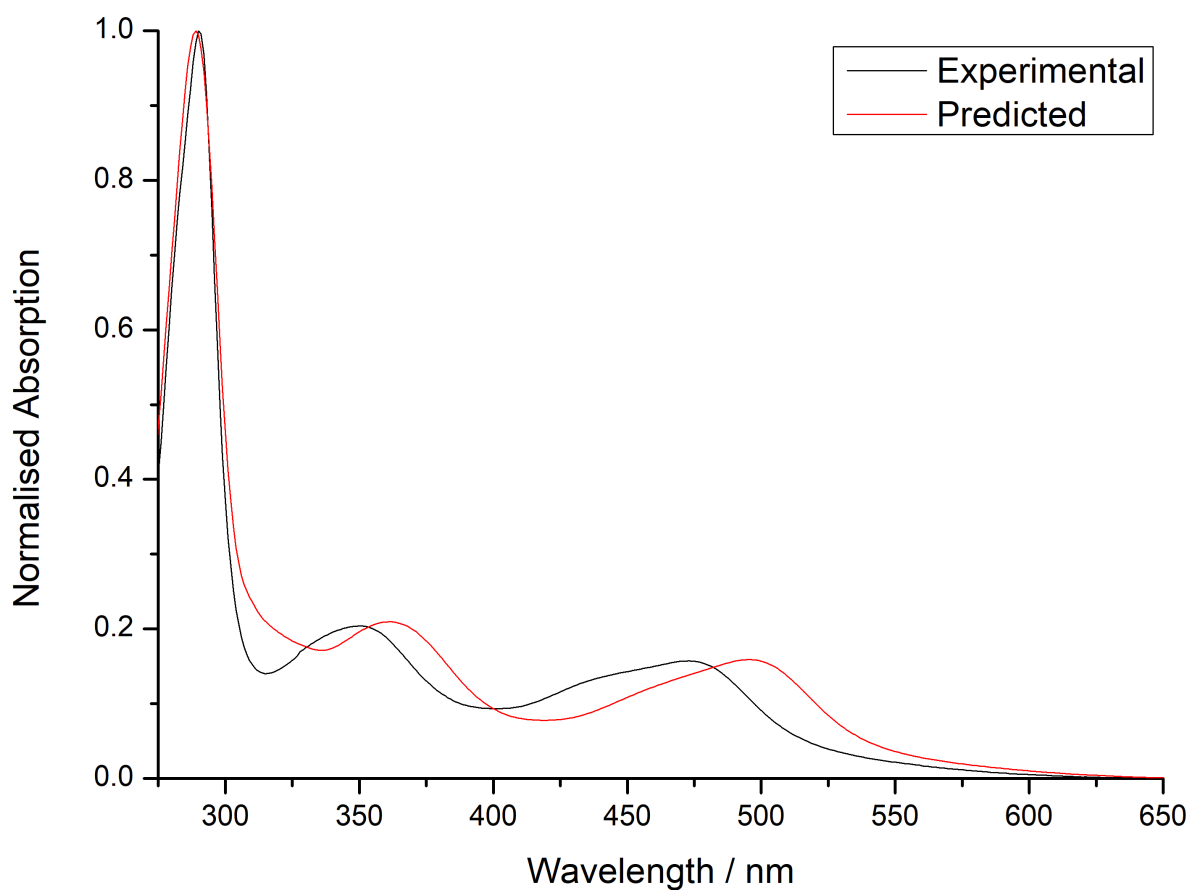


Figure S25. Comparison of the experimentally measured and TD-DFT predicted absorption spectrum of **7** in H₂O.

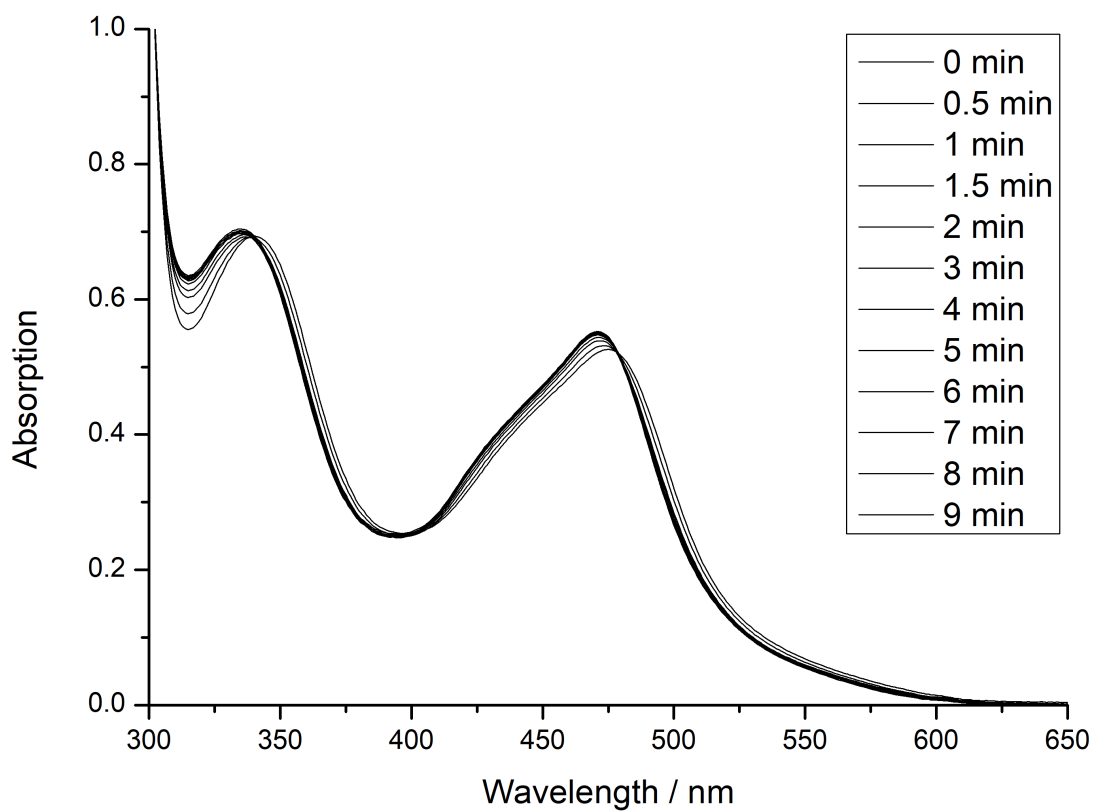


Figure S26. UV-vis spectroscopy showing temporal change of the absorption spectrum of **9** upon incubation in H₂O and upon irradiation at 450 nm.

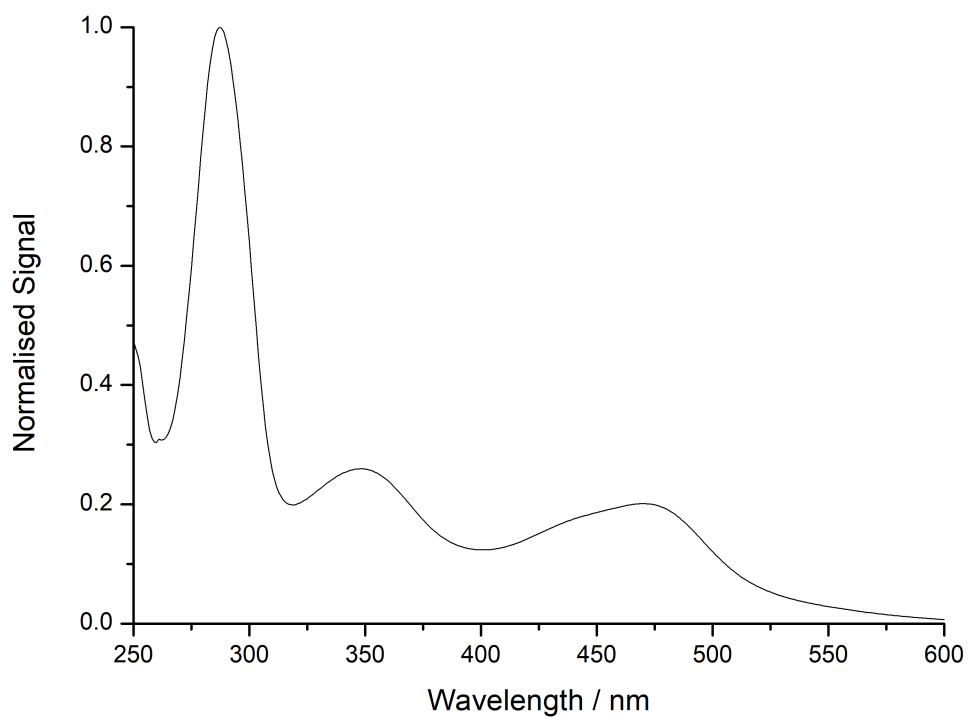


Figure S27. Excitation spectrum of **7** in H₂O.

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