Electronic Supplementary Material (ESI) for ChemComm.

Electronic Supplementary Information

Design and Development of a Photoswitchable DFG-Out Kinase Inhibitor

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1. General Experimental Information

All reagents and solvents were purchased from commercial sources and used as supplied, unless stated otherwise. THF and toluene were distilled from sodium/benzophenone. DCM, DMF and MeCN were purchased as HPLC grade and used as received. Reactions were monitored by Thin Layer Chromatography (TLC, Merck, silica gel 60 F254) and analyzed under UV (254 nm/365 nm) or potassium permanganate solution ($KMnO_4$) staining. Microwave reactions were performed in a Biotage Initiator Reactor using single mode irradiation with temperature and pressure control and with fixed hold time on. Column chromatography was performed by manual flash chromatography (wet-packed silica, 0.04–0.063 mm) or by automated column chromatography on a Biotage SP-X or Isolera instrument using pre-packed silica columns.

^{1}H- and ^{13}C-NMR spectra were obtained at 400 MHz, using a Varian 400/54 spectrometer, or 700 MHz Bruker spectrometer for some ^{19}F-, ^{1}H- and ^{13}C-NMR spectra. The HRMS data was determined by CMSI (Chalmers Mass Spectrometry Infrastructure) at Chalmers University of Technology. The photostationary distributions were determined by 500 MHz ^{1}H-NMR with DMSO-d_6 as the solvent. All reactions involving azocompounds were carried out avoiding direct light, i.e. covering reaction vessels and columns with aluminum foil and working with the fume hood lamp turned off (ceiling lamp was left on).

UV-Vis absorption spectra were recorded using a Varian CaryBio 50 spectrophotometer. All UV-Vis absorption spectra were recorded in 10×10 mm quartz cuvettes. The light sources for isomerization purposes were LEDs (LED Engin) centered at around 365 nm (LZ1-10UV00, fwhm=12 nm), 405 nm (LZ1-10UB00-00U8. fwhm=19 nm), and 460 nm (LZ1-10B200, fwhm=21 nm).

2. Synthesis

2.1. Detailed Synthetic Procedure and Characterization of Compounds

Tert-butyl 1-(3-(methoxycarbonyl)phenyl)hydrazine-1-carboxylate (2)

N-Boc hydrazine (475 mg, 3.60 mmol), Cs_2CO_3 (1.46 g, 4.50 mmol) and CuI (28.5 mg, 0.15 mmol) were transferred to an oven-dried MW vial. The vial was then capped and backfilled with N_2 (x3) before 3-iodobenoic acid ethyl ester **1** (830 mg, 500 µL, 3.00 mmol) and DMSO (3 mL) were added. The mixture was stirred at 50 °C for 5 h. After cooling to r.t., the reaction mixture was diluted with saturated NH_4Cl solution (5 mL) and extracted with EtOAc (3 × 10 mL). The combined organic phase was washed with water (5 × 30 mL), followed by brine (20 mL), dried over Na_2SO_4, filtered, concentrated and purified by flash column chromatography on silica gel (n-pentane-EtOAc, 5% EtOAc) afforded **2** (716 mg, 2.55 mmol, yield 85%) as white crystal. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.16 (t, J = 1.9 Hz, 1H), 7.77 (ddd, J = 7.7, 1.6, 1.1 Hz, 1H), 7.68 (ddd, J = 8.1, 2.3, 1.0 Hz, 1H), 7.35 (t, J = 7.7 Hz, 1H), 4.45 (s, 2H), 4.36 (q, J = 7.1 Hz, 1H), 1.50 (s, 9H), 1.38 (t, J = 7.5 Hz, 1H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 166.3, 154.9, 143.3, 130.5, 128.1, 127.3, 125.4, 124.2, 82.2, 81.0, 28.3, 14.3. Data is consistent with that previously reported (M. Wolter, *et. al*., *Org. Lett*. **2001**, *3*, 23, 3803–3805)

tert-butyl 1-(3-(ethoxycarbonyl)phenyl)-2-(1-tosyl-1H-pyrrolo[2,3-b]pyridin-5-yl)hydrazine-1-carboxylate (4)

Compound **2** (256 mg, 0.92 mmol), **3** (216 mg, 0.60 mmol), $Pd(OAc)_2$ (20 mg, 0.09 mmol), Xantphos (90 mg, 0.15 mmol) and Cs_2CO_3 (390 mg, 1.20 mmol) were transferred to an oven-dried MW vial. The vial was then capped and backfilled with N_2 (x3) before toluene (5 mL) was added. The reaction mixture was then heated using microwave irradiation at 110 °C for 2 h. TLC showed complete reaction. After cooling to r.t., the reaction mixture was filter over celite, washing thoroughly with EtOAc. The solvent was removed by rotary evaporator. The residue was purified by automated column chromatography on silica gel (n-pentane-EtOAc, 40% EtOAc) afforded **4** (286 mg, 0.52 mmol, yield 86%) as light brown solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.26 (t, J = 1.8 Hz, 1H), 8.09 (d, J = 2.8 Hz, 1H), 7.98 (m, 2H), 7.81 (ddd, J = 7.8, 1.5, 1.1 Hz, 1H), 7.78 (ddd, J = 8.2, 2.3, 1.1 Hz, 1H), 7.64 (d, J = 4.0 Hz, 1H), 7.38 (t, J = 8.2 Hz, 1H), 7.23 (m, 2H), 7.20 (d, J = 2.7 Hz, 1H), 6.57 (s, 1H), 6.44 (d, J = 4.0 Hz, 1H), 4.36 (q, J = 7.2 Hz, 2H), 2.34 (s, 3H), 1.37 (s, 9H), 1.37 (t, J = 7.2 Hz, 3H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 166.2, 153.6, 145.0, 143.2, 142.7, 140.9, 135.4, 133.0, 131.0, 129.6, 128.6, 127.8, 127.4, 126.0, 125.8, 123.2, 122.9, 113.2, 105.2, 83.3, 61.1, 28.1, 21.6, 14.3. HRMS (ESI-TOF) *m/z* calcd for $C_{28}H_{31}N_4O_6S^+$ $[M + H]^+$: 551.1959, found: 551.1964.

Ethyl (*E*)-3-((1-tosyl-1H-pyrrolo[2,3-b]pyridin-5-yl)diazenyl)benzoate (5)

Compound **4** (260 mg, 0.47 mmol) in DMF (5 mL) was heated using microwave irradiation at 180 °C for 30 min. After cooling to r.t., pure oxygen was backfilled into the vial (x2), and the reaction was heated at 100 °C 2 h. The reaction mixture was cooled down, concentrated under vacuum, and the residue was purified by automated column chromatography on silica gel (n-pentane-EtOAc, 40% EtOAc) affording **5** (150 mg, 0.33 mmol, yield 71%) as red solid. ^{1}H-NMR (400M Hz, $CDCl_3$): δ 9.09 (d, J = 2.2 Hz, 1H), 8.56 (t, J = 1.8 Hz, 1H), 8.33 (d, J = 2.2 Hz, 1H), 8.16 (dt, J = 7.7, 1.5 Hz, 1H), 8.11 (d, J = 8.5 Hz, 2H), 8.08 (ddd, J = 7.9, 1.9, 1.2 Hz, 1H), 7.80 (d, J = 4.0 Hz, 1H), 7.59 (t, J = 8.0 Hz, 1H), 7.29 (d, J = 8.0 Hz, 2H), 6.69 (d, J = 4.1 Hz, 1H), 4.44 (q, J = 7.2 Hz, 2H), 2.37 (s, 3H), 1.43 (t, J = 7.2 Hz, 3H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 165.9, 152.4, 148.2, 145.5, 144.9, 144.2, 135.1, 131.9, 131.7, 129.7, 129.2, 128.3, 128.2, 126.6, 124.3, 123.2, 120.4, 106.2, 61.4, 21.7, 14.4. HRMS (ESI-TOF) *m/z* calcd for $C_{23}H_{21}N4O_4S^+$ $[M + H]^+$: 449.1278, found: 449.1284.

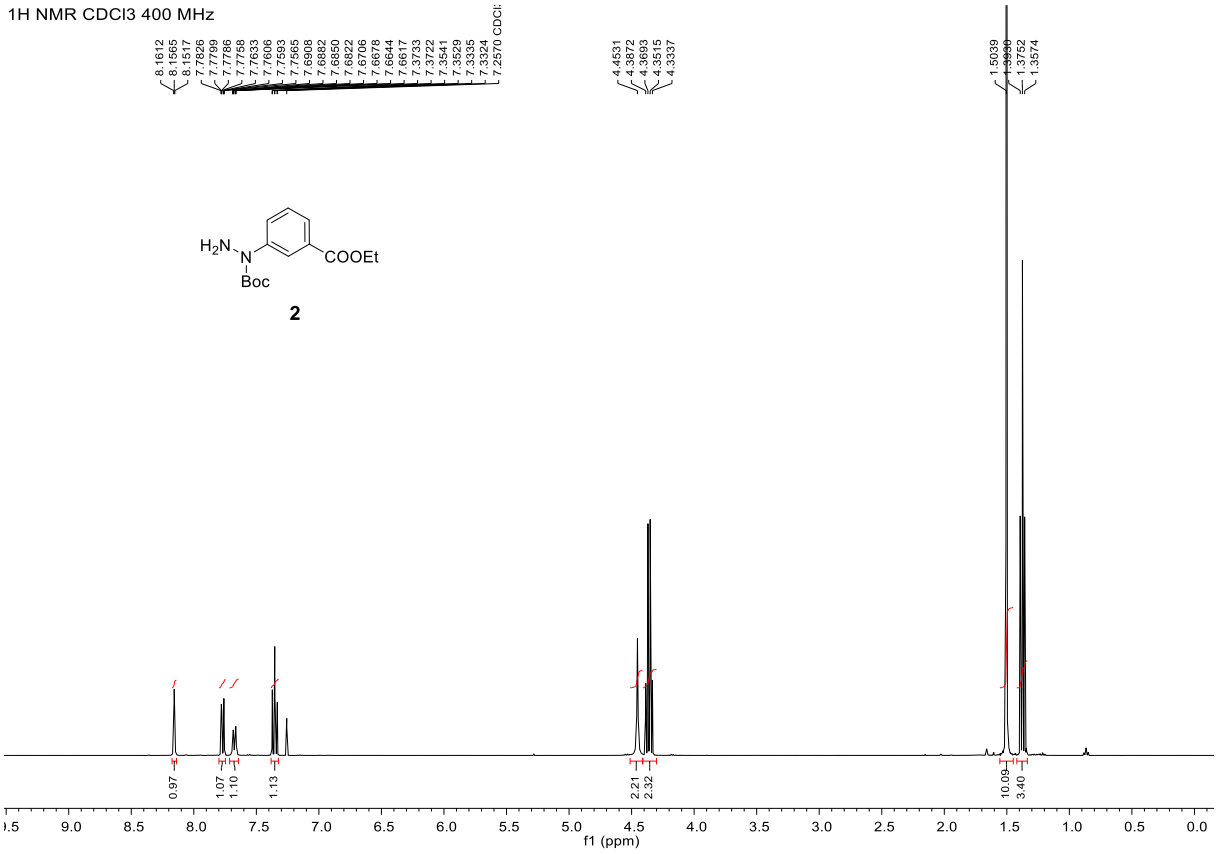
(*E*)-3-((1H-pyrrolo[2,3-b]pyridin-5-yl)diazenyl)benzoic acid (6)

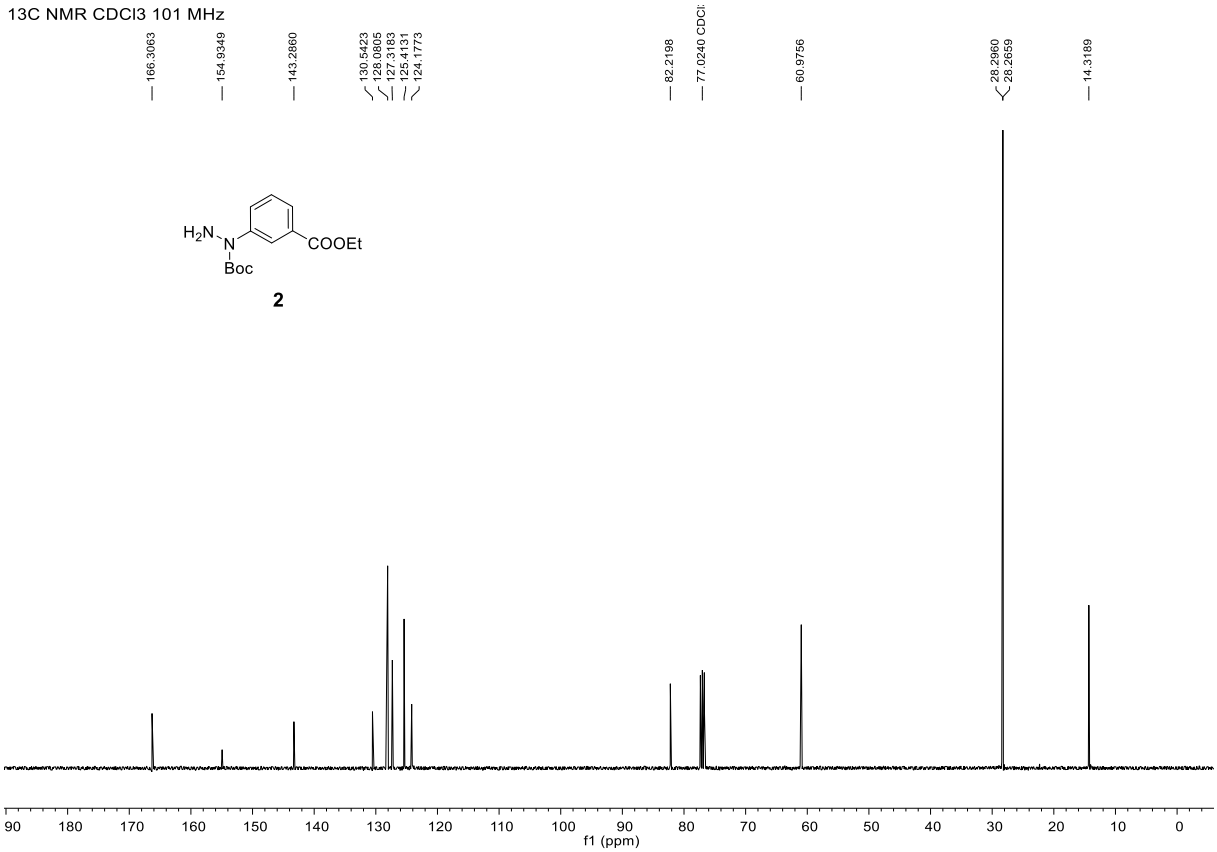
To a solution of **5** (150 mg, 0.33 mmol) in EtOH/H_2O (1:1, 5 ml) NaOH (360 mg, 90 mmol) was added. The reaction mixture was then heated at 90 ^oC for 5 h. The organic solvent was removed by air-blowing. The aqueous phase was diluted with H_2O (4 ml) and washed with Et_2O (3 ml × 2). Afterwards, 2 N HCl was added to adjust the pH to 6.0, before the solution was extracted with EtOAc/*i*-PrOH (3:1, 10 ml × 3). The combined organic phase was washed by brine, dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure affording **6** (81 mg, 0.30 mmol, 90%) as light orange powder. ^{1}H-NMR (400 MHz, DMSO-*d6*) δ 12.07 (s, 1 H), 8.90 (d, *J* = 2.3 Hz, 1 H), 8.46 (d, *J* = 2.2 Hz, 1 H), 8.38 (t, *J* = 1.8 Hz, 1 H), 8.11 (dt, *J* = 7.8, 1.5 Hz, 1 H), 8.07 (dt, *J* = 7.8, 1.5 Hz, 1 H), 7.70 (t, *J* = 7.8 Hz, 1 H), 7.61 (dd, *J* = 3.5, 2.4 Hz, 1 H), 6.65 (dd, *J* = 3.5, 1.8 Hz, 1 H); ^{13}C-NMR (101 MHz, DMSO-*d6*) δ 167.2, 152.5, 150.5, 142.9, 142.3, 132.7, 131.7, 130.3, 129.2, 127.6, 122.4, 120.4, 119.9, 102.6. HRMS (ESI-TOF) *m/z* calcd for $C_{14}H_{11}N_4O_2^+$ [M + H]$^+$: 267.0877, found: 267.0884.

(*E*)-3-((1H-pyrrolo[2,3-b]pyridin-5-yl)diazenyl)-N-(4-((4-methylpiperazin-1-yl)methyl)-3-(trifluoromethyl) phenyl)benzamide (8)

Compound **6** (53 mg, 0.20 mmol), **7** (60 mg, 0.22 mmol), EDC•HCl (154 mg, 0.80 mmol) and HOBt (54 mg, 0.40 mmol) were added to an oven-dried MW vial. The vial was then sealed and backfilled with N_2 (x?), before Et_3N (146 mg, 200 µL, 1.44 mmol) and DCM (3.0 mL) were added. The mixture was then stirred for 36 h at r.t. The solvent and Et_3N were evaporated, and the residue was purified by PLC plate ($CHCl_3$-MeOH, 12:1 affording **8** (53 mg, 0.20 mmol, yield 50%) as orange solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 12.06 (s, 1 H), 10.70 (s, 1 H), 8.90 (d, *J* = 2.3 Hz, 1 H), 8.45 (d, *J* = 2.1 Hz, 2 H), 8.21 (d, *J* = 2.1 Hz, 1 H), 8.13 – 8.01 (m, 3 H), 7.73 (t, *J* = 8.1 Hz, 1 H), 7.68 (d, *J* = 8.6 Hz, 1 H), 7.60 (t, *J* = 3.2 Hz, 1 H), 6.64 (d, *J* = 3.5 Hz, 1 H), 3.54 (s, 2 H), 2.44-2.22 (br, 8 H), 2.15 (s, 3 H); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 165.5, 152.4, 150.5, 143.0, 142.2, 138.6, 136.2, 132.6, 131.7, 130.4, 130.2, 129.2, 128.0 (q, J_{CF} = 30.6 Hz), 126.1(q, J_{CF} = 272.7 Hz), 125.7, 124.0, 122.0, 120.4, 120.0, 117.7 (q, J_{CF} = 6.0 Hz), 102.6, 57.9, 55.1, 53.0, 46.0. M.P. 179 - 186 ^{o}C. HRMS (ESI-TOF) *m/z* calcd for $C_{27}H_{27}F_3N_7O^+$ [M + H]$^+$: 522.2224, found: 522.2228.

2.2. ^{1}H and ^{13}C NMR spectra





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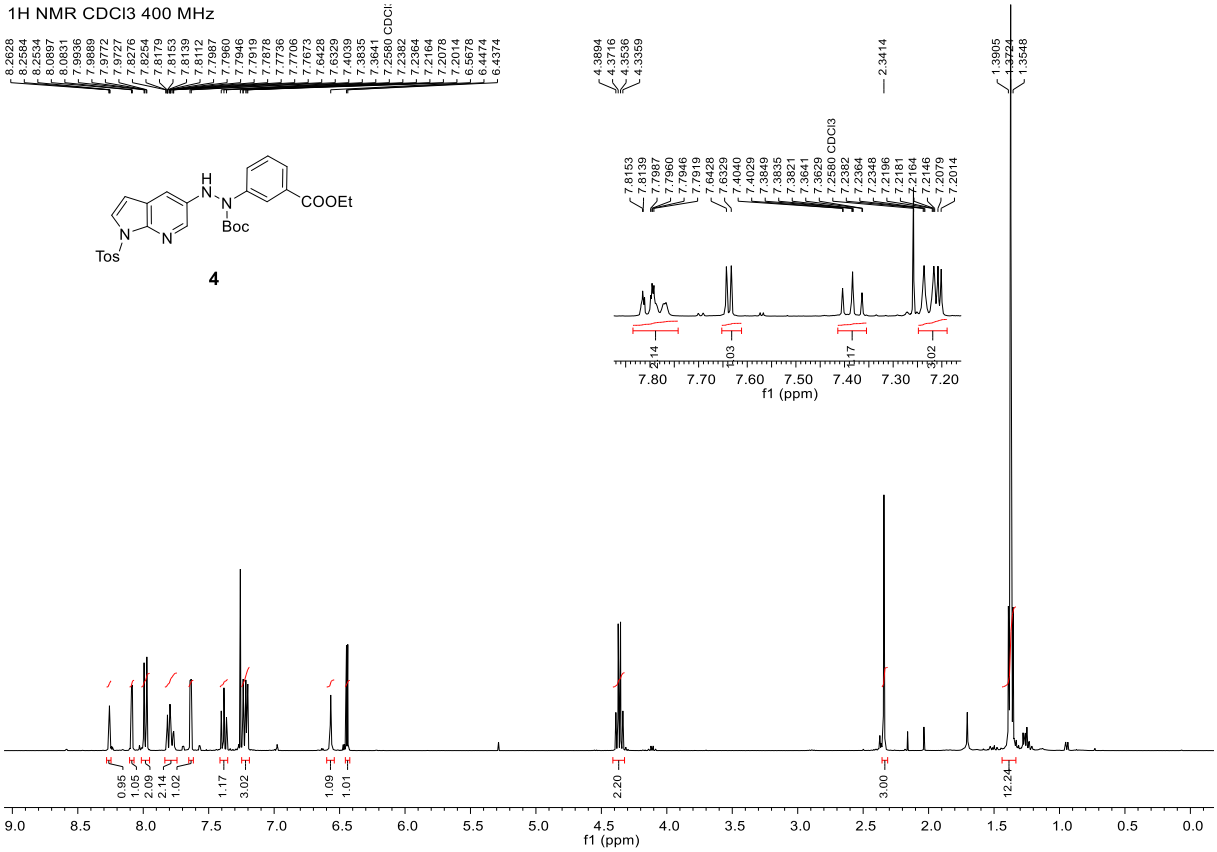

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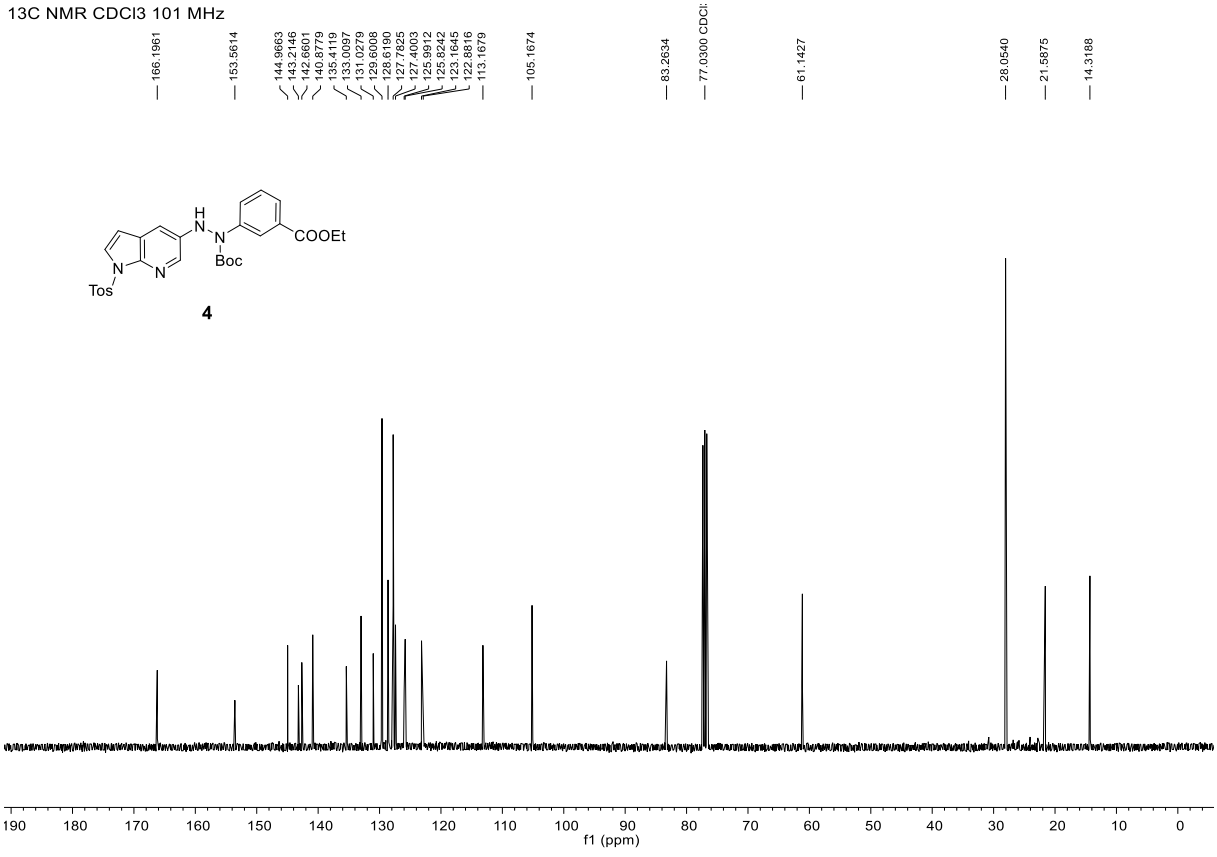

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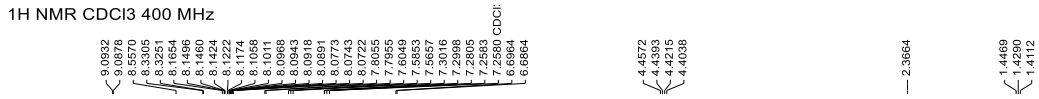

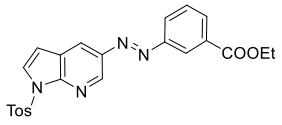

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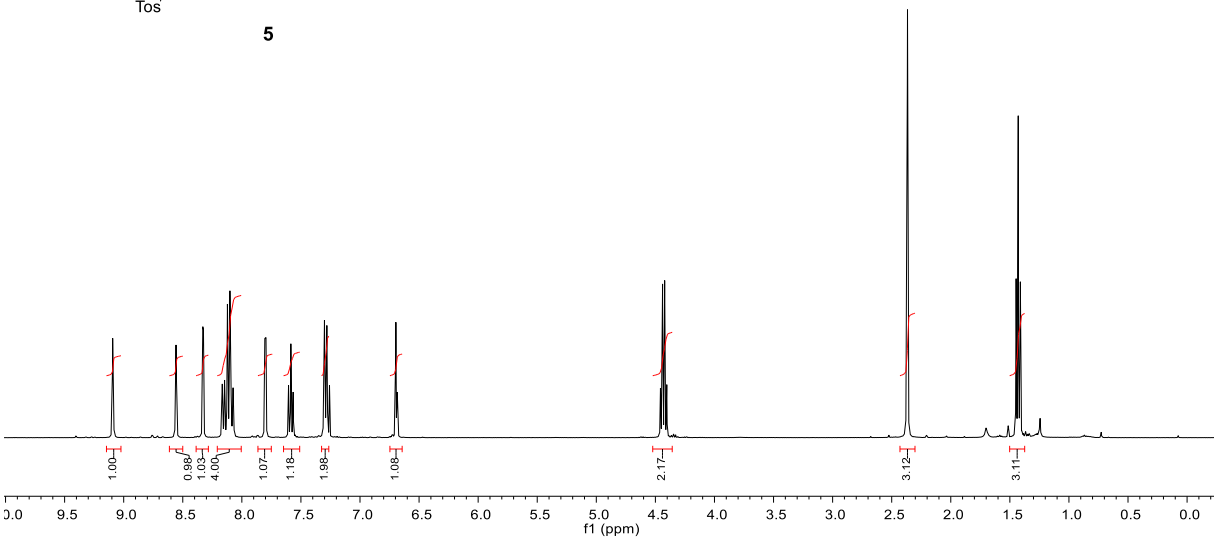

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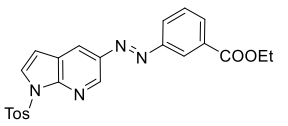

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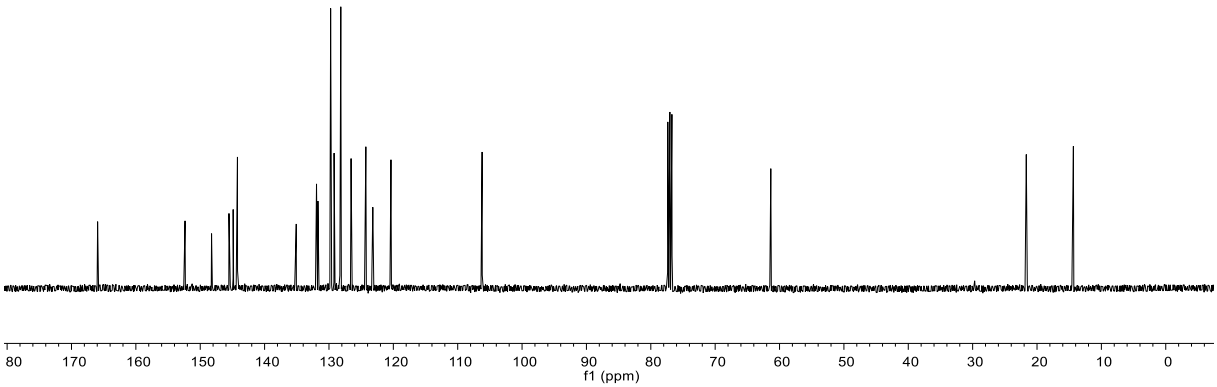

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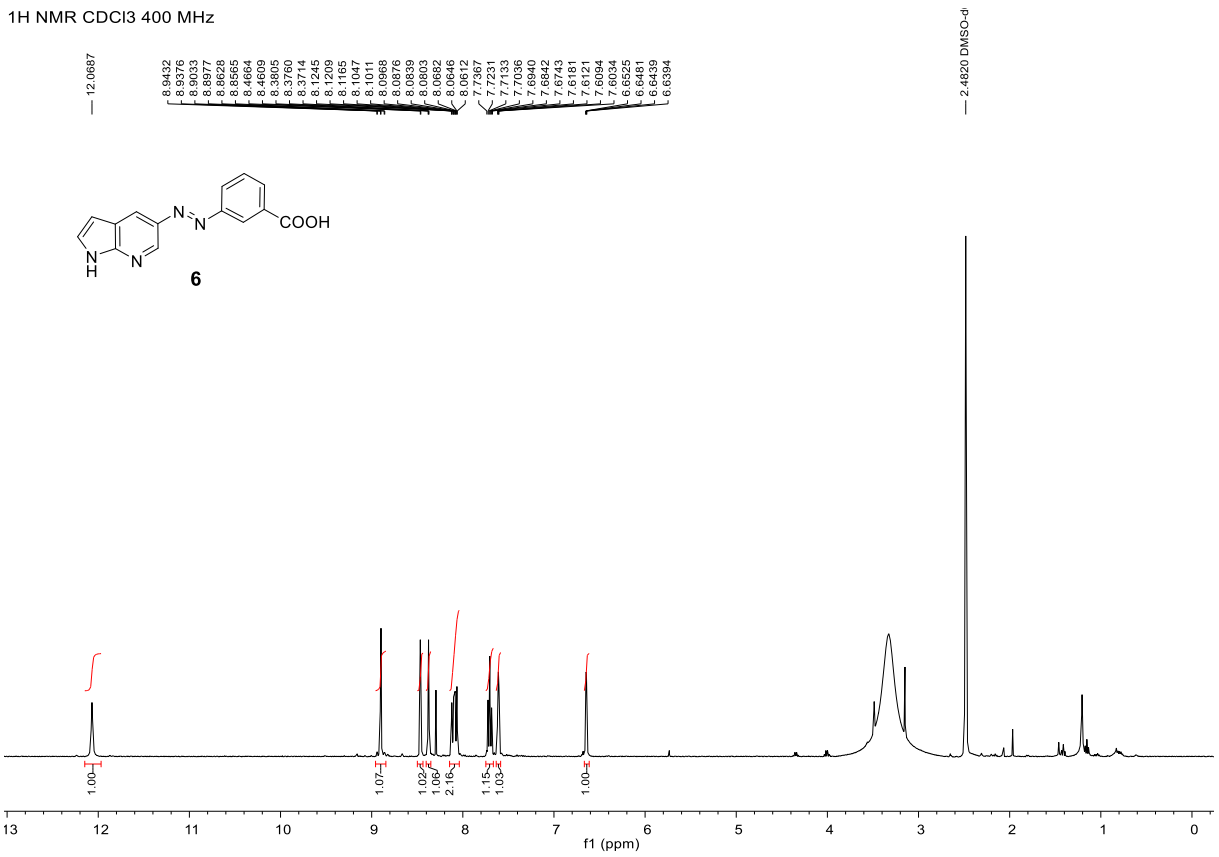

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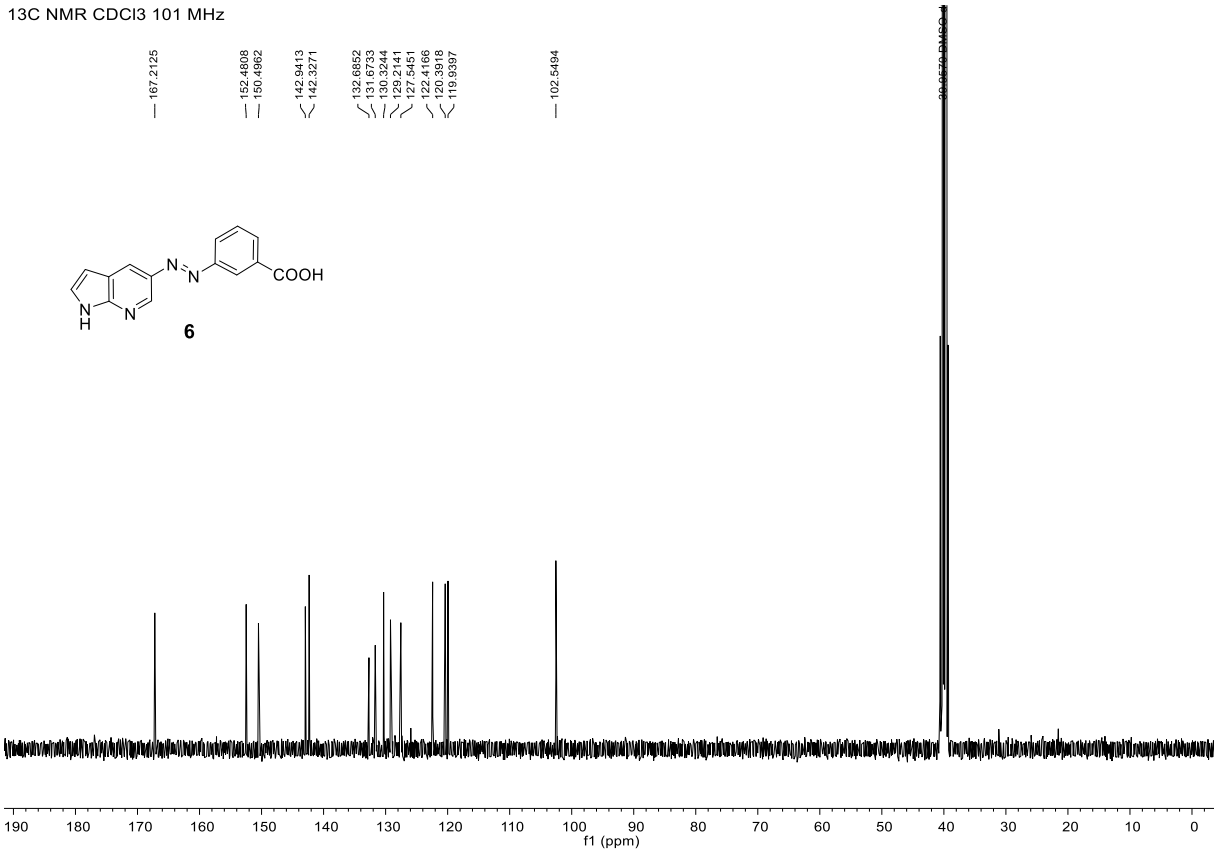

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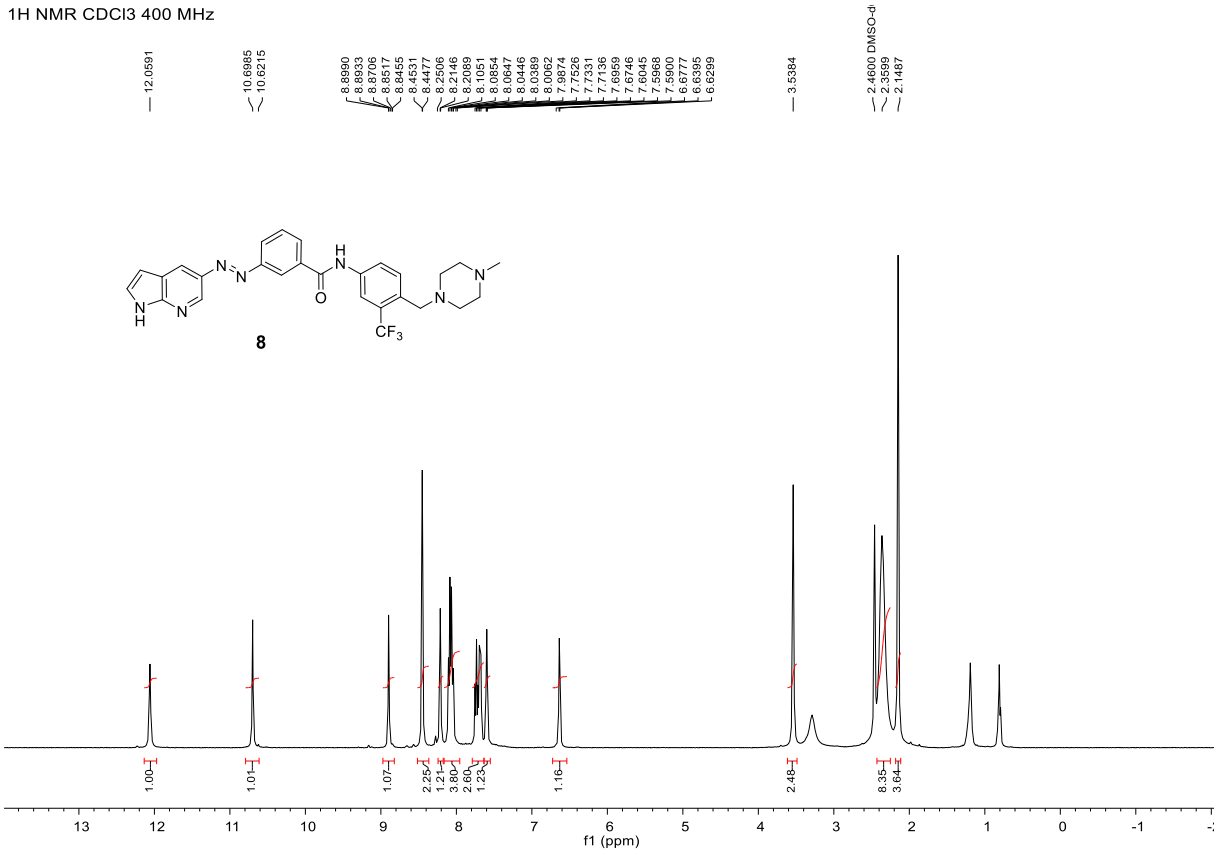

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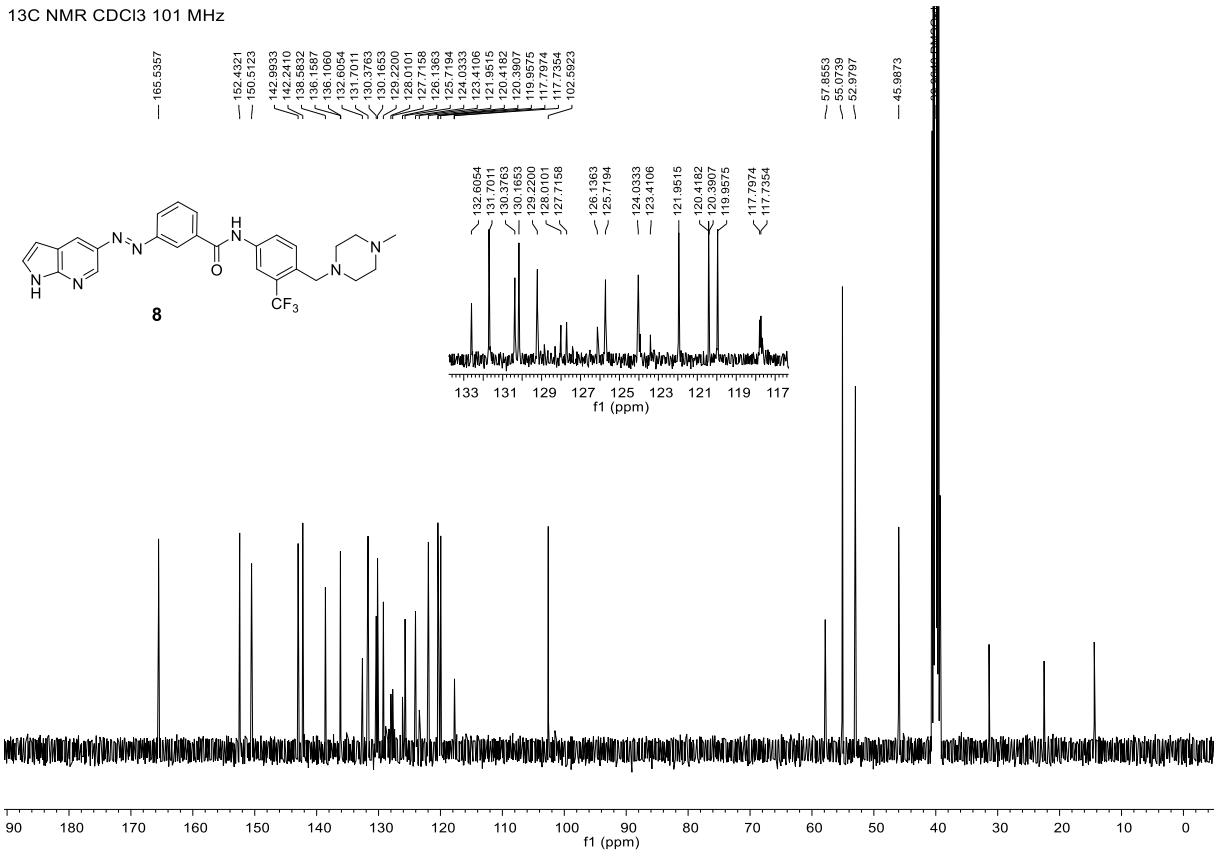
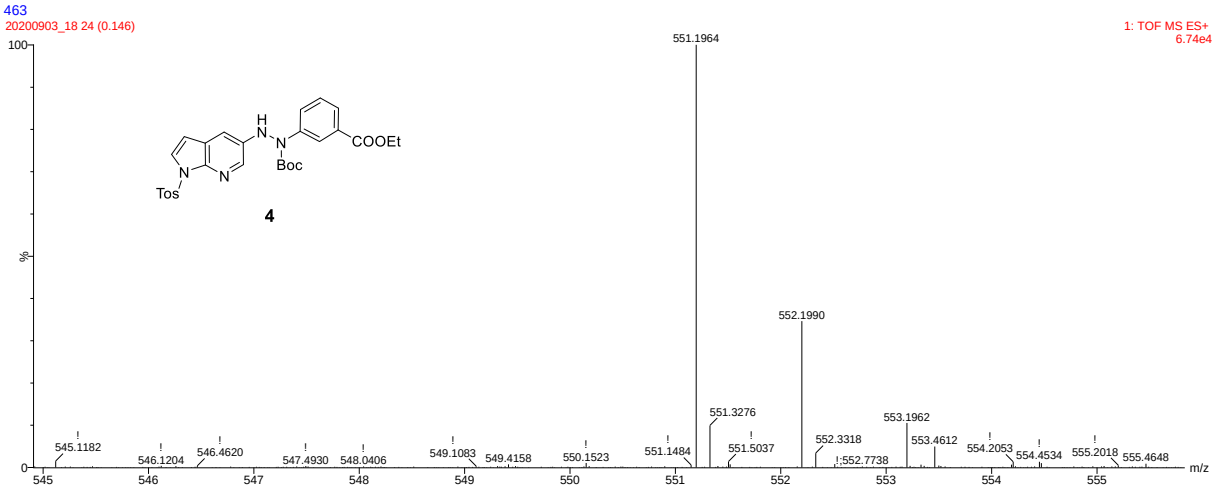
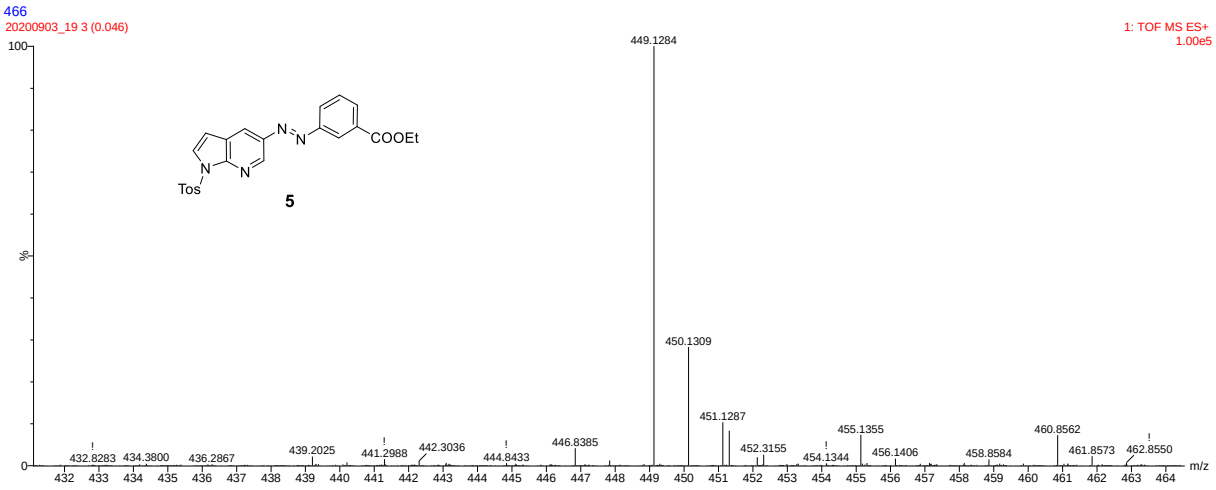
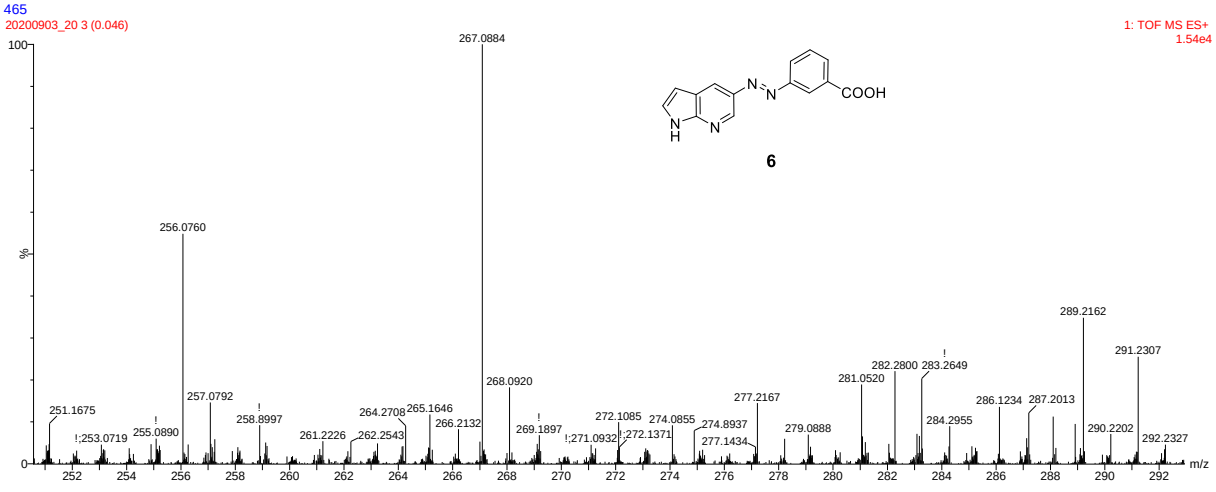
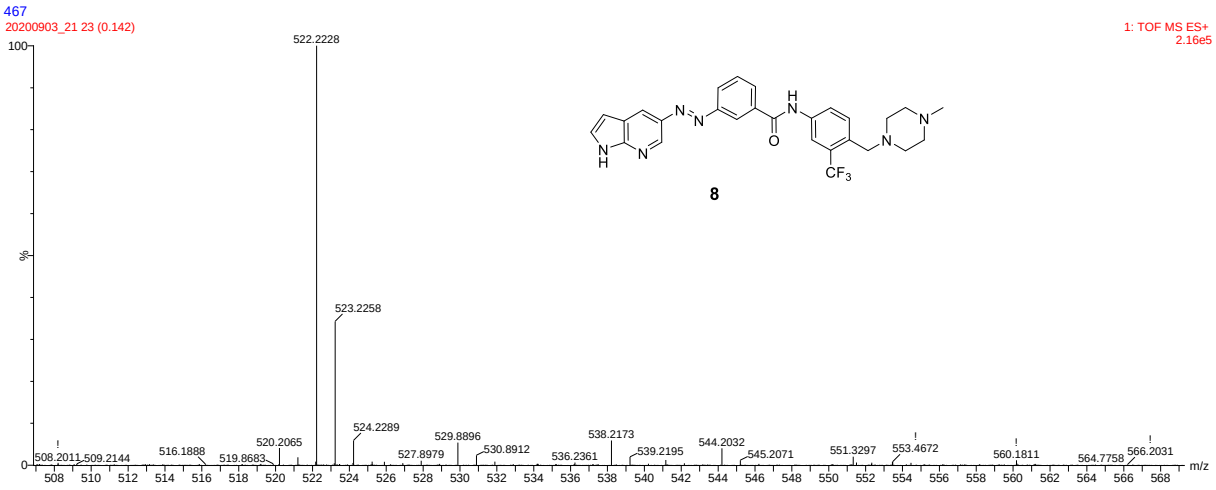


2.3. HRMS spectrum





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8

3. Photophysical characterization

3.1 UV-vis absorption spectra

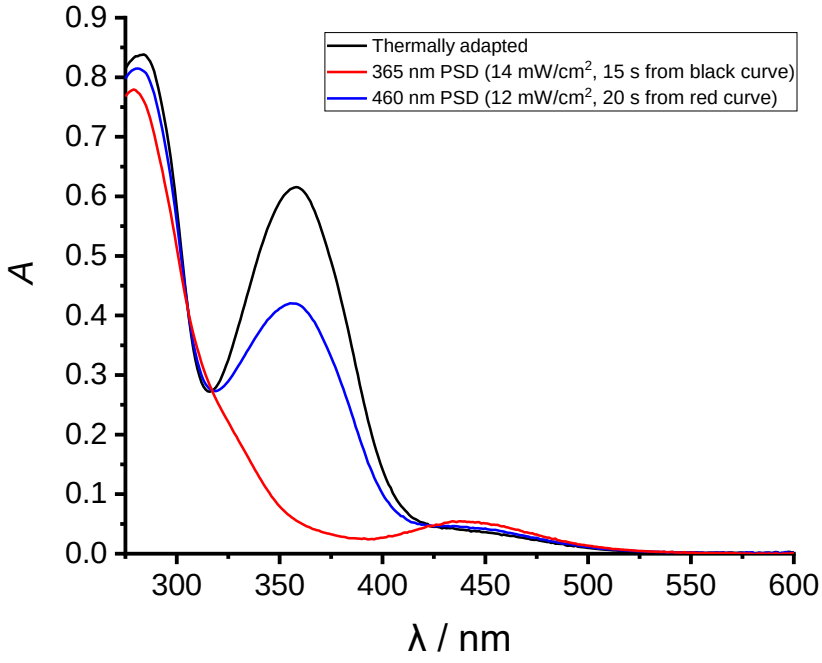

Figure S1. Photoisomerization UV-vis absorption spectrum of compound **8** in DMSO solution (25 μM).

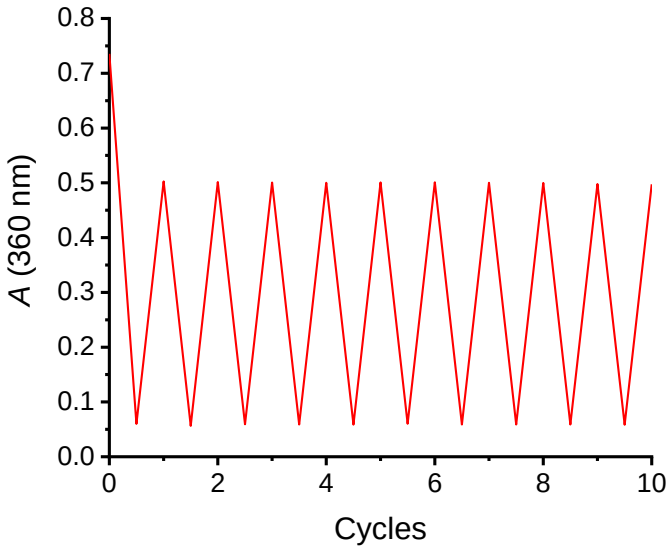

Figure S2. Extended photocycling for compound **8** in DMSO (30 μM). Ten cycles of 365nm/460nm light irradiation were performed without significant photodecomposition.

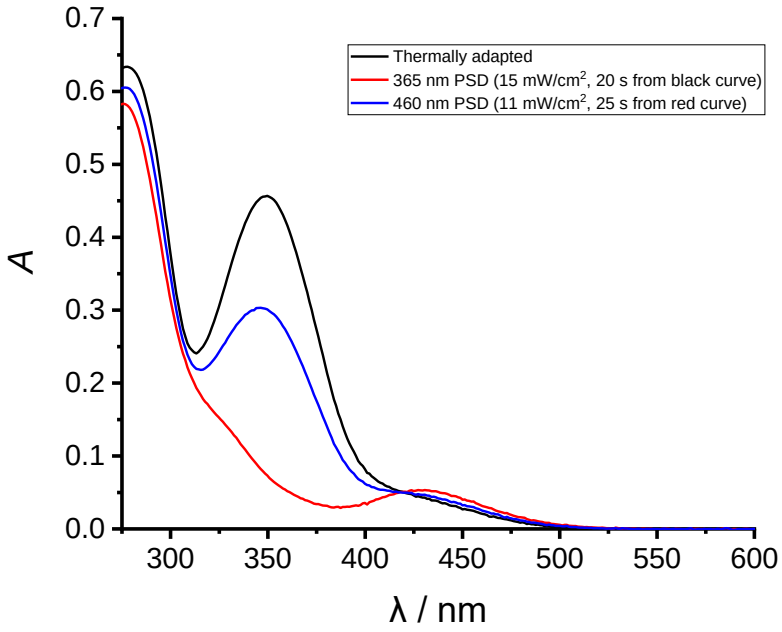

Figure S3. Photoisomerization UV-vis absorption spectrum of compound **8**·HCl in H_2O solution (25 μM).

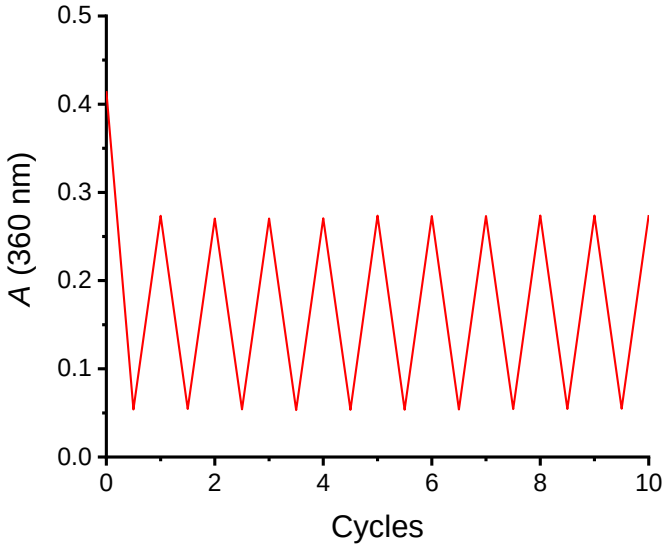

Figure S4. Extended photocycling for compound **8**·HCl in H_2O (25 μM). Ten cycles of 365nm/460nm light irradiation were performed without significant photodecomposition.

3.2 Determination of thermal stability

Prepare samples in cuvette: **8** was dissolved in DMSO at r.t.; **8**·HCl was dissolved in H_2O at r.t.. Firstly, PSD was reached upon 365 nm irradiation, after which the samples was left in dark and its absorption

spectra was recorded every day (19 days in total for DMSO solution, and 30 days in total for aqueous solution). The curve presents the time-lapse absorption at 360 nm for DMSO solution and 350 nm for aqueous solution, and the red line shows the fit using mono-exponential (first order) kinetics.

3.3 PSD determination by ^{1}H-NMR

Azo compounds **8** were dissolved in DMSO-d_6 (5 mM, 25 ^{o}C) and irradiated by LEDs (365nm/ 460nm) to achieve the respective PSD (as judged by monitoring the UV-vis absorption spectra). 0.6 mL of the irradiated samples was submitted to H-NMR measurement (400M HZ, 128 scans).

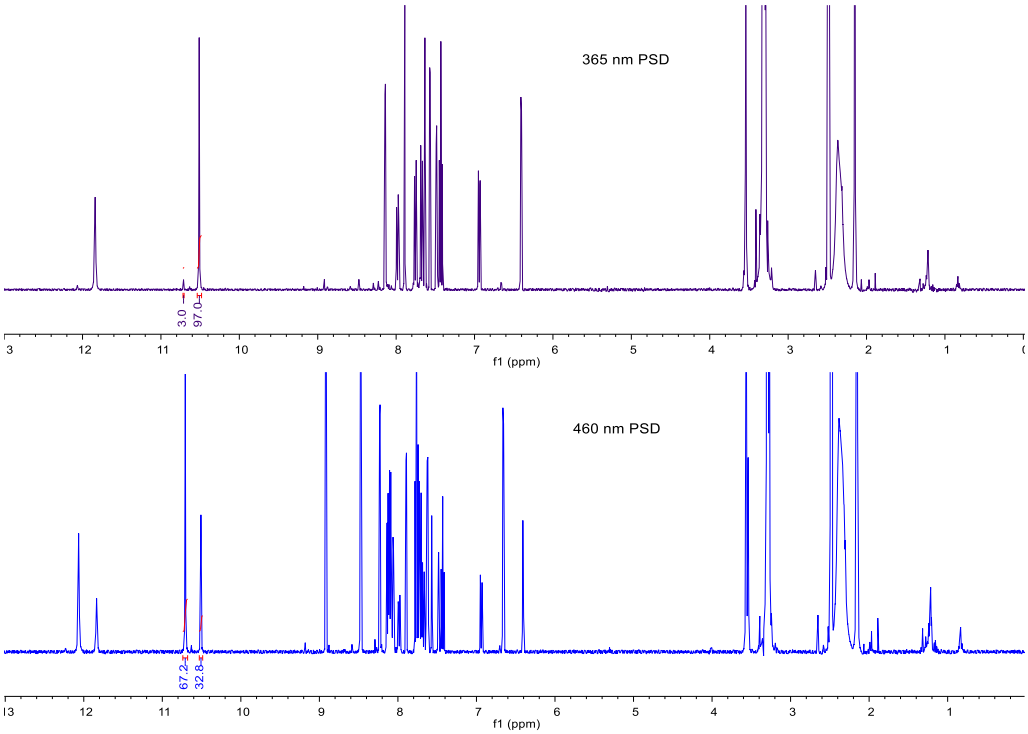

Figure S5. ^{1}H-NMR spectrum used to determine PSD after 365 nm and 460 nm LED irradiation for **8**.

4. Biochemical assay

4.1. GSH reduction assay

Compound **8**·HCl was dissolved in PBS (10 mM, pH 7.4) to obtain the solutions (300 μM, 25 oC) to which reductive GSH in PBS stock solution (1 M) was added to yield a final concentration of 10 mM. The sample was incubated at 25 ^{o}C for 18 h before being submitted to LC-MS. Two panels in each figure: upper panel: UV spectrum; lower panel: Mass spectrum.

Further UV/vis cyclic isomerization GSH reduction assay: diluting DMSO stock solution of **8** (3 mM) with 1 mM GSH solution in H_2O to achieve a final concentration of 30 μM. Five cycles of 365 nm / 460 nm light irradiation were performed, during the six circle, 365 nm UV light enriched Z-isomer sample was incubated at 25 ^{o}C for 2.5 h, then extended five circles of 460 nm / 365 nm light irradiation were performed.

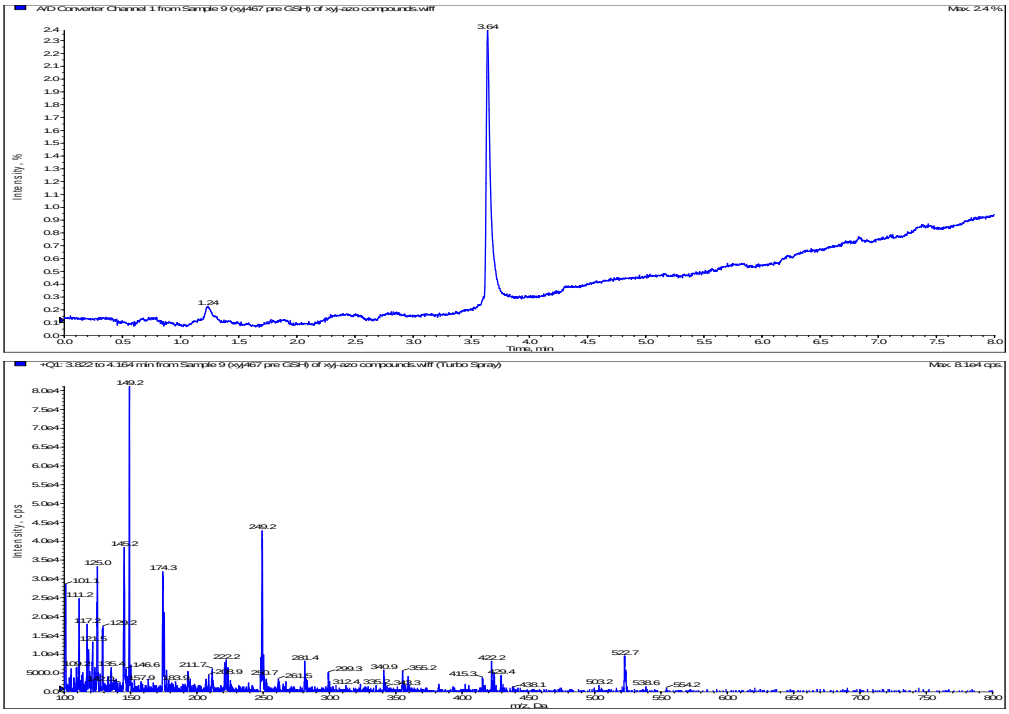

Figure S6. Compound **8**·HCl in PBS.

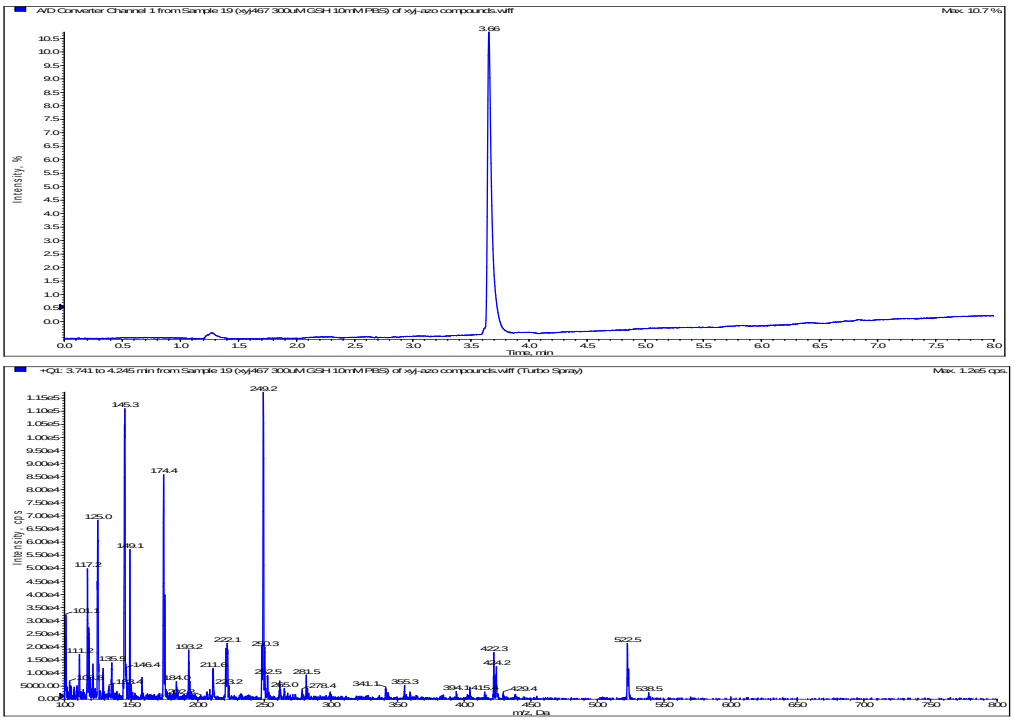

Figure S7. Compound **8**·HCl in PBS incubated with GSH (10 mM) for 18 h at 25°C.

4.2. DTT reduction assay

Compound **8**·HCl was dissolved in H_2O (300 μM, 25 °C) and a DTT solution (100 mM, Promega) to achieve a gradient of three different concentrations (20 μM, 200 μM, 2 mM). These samples were monitored by LC-MS (Upper panel: UV spectrum; Lower panel: Mass spectrum) at 18 h after DTT was

added.

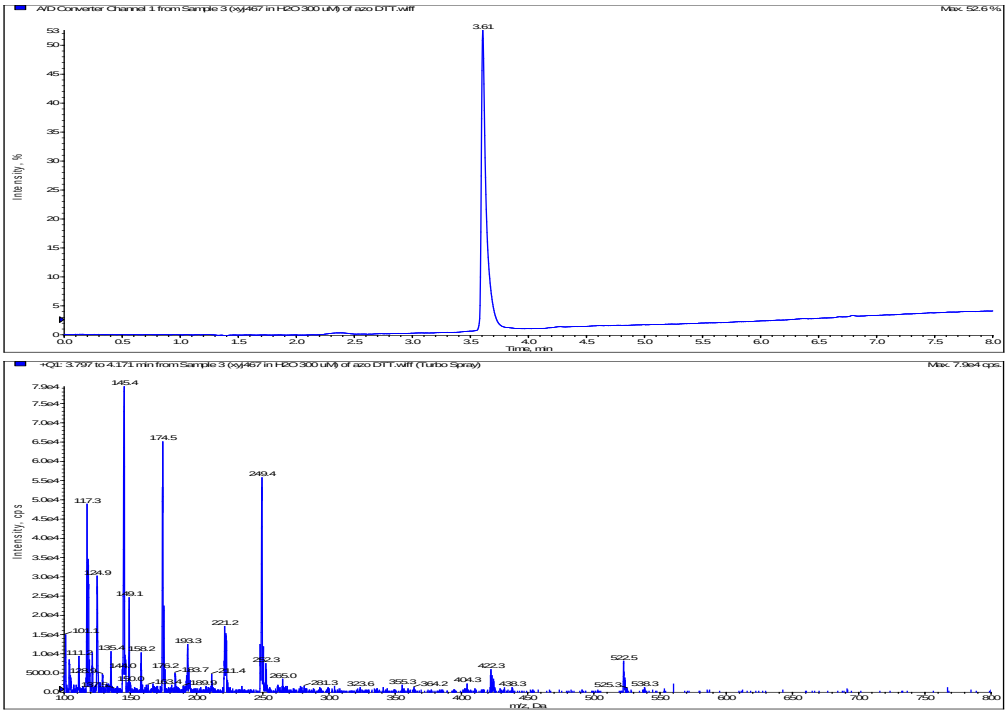

Figure S8. Compound **8**·HCl in H_2O before adding DTT solution

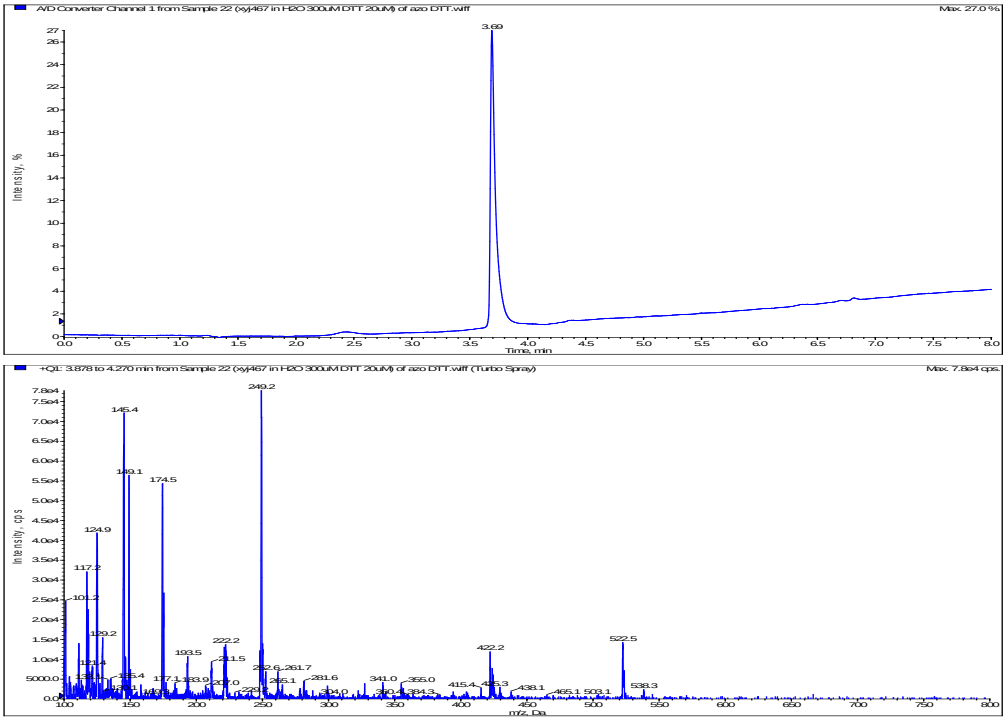

Figure S9. Compound **8**·HCl in H_2O was added DTT (20 μM), incubation for 18 h

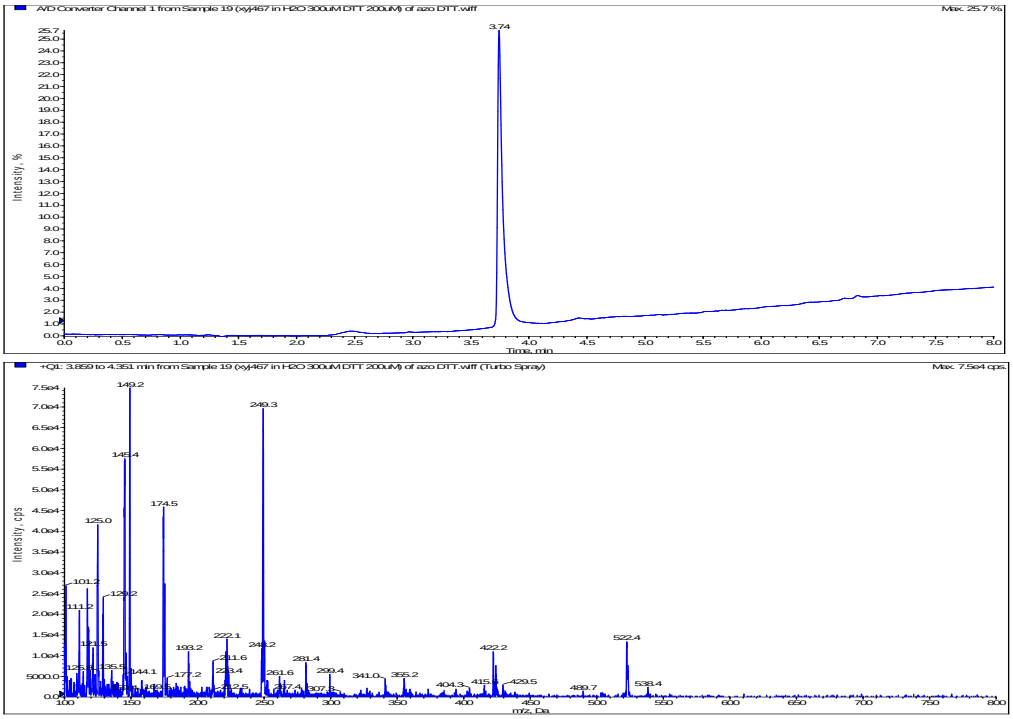

Figure S10. Compound **8**·HCl in H_2O was added DTT (200 μM), incubation for 18 h

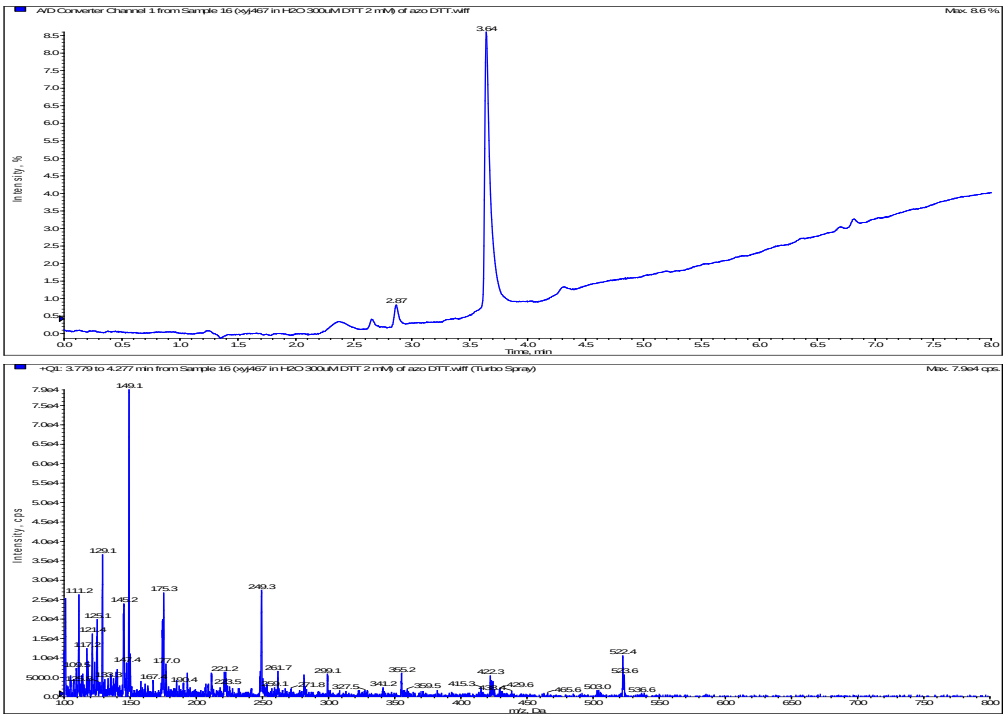

Figure S11. Compound **8**·HCl in H_2O was added DTT (2 mM), incubation for 18 h

4.3. ADP-Glo™ RET kinase assay

Compound **8** was dissolved in DMSO (1 mM) and heated at 60 °C for 20 min prior to use to ensure that it consisted of pure *E*-isomers. Serially diluted **8** (concentration gradients ranging from 1 mM to 16.9 nM) were pipetted into white 96-well V bottom plate. The concentration gradient of the *Z*-isomer were

prepared by irradiating the serially diluted samples of *E*-isomers on a 96-well V bottom plate with 96 array UV365 LED (AMUZA INC, LEDA-365, 11V, 20 min).

The ADP-GloTM Kinase Assay (Promega, V9101) for RET (Promega, V3761) was carried out according to the manufacturer's protocol (Promega protocol TM313), with white 384-well low volume plate (Corning, Cat. # 3824), 5 µl total reaction volume, and in triplicates for each inhibitor. Kinase reactions were run in kinase buffer (40 mM Tris (pH 7.5), 20 mM $MgCl_2$, 0.1 mg/mL BSA, 60 µM DTT) with 10 µM ATP, 4.0 ng RET kinase, and 0.4 mg/mL substrate (IGF1Rtide). All the serially diluted samples (in DMSO) were further diluted (1:10) by kinase buffer to prepare 5× inhibitor stock solutions. Then 1 µl of each 5× inhibitor stock solution (including DMSO control solution for each inhibitor) were added into corresponding wells of 384-well plate, followed by addition of RET kinase and the mixture was incubated at r.t. for 40 min. The kinase reaction was initiated by adding the RET substrate working solution and the mixture was incubated at r.t. for 60 min. The reaction was terminated with 5µL of ADP-Glo™ Reagent and incubated at r.t. for 40 minutes, before 10µL Promega Kinase Detection reagent was added to each well and the mixture incubated at r.t. for 40 min. The luminescence signal was recorded by a plate reader (Molecular Devices SpectraMax iD5, whole wavelength, integration time: 1 second, read from 1 mm above plate). Kinase activities were calculated in percent of DMSO control and plotted against the logarithm of inhibitor concentration. Data points were means of triplicates with standard deviations as error bars. Sigmoidal dose-response fitting (log_{10}(inhibitor) vs. Activity–variable slope) were performed by GraphPad Prism7.0 to give the IC_{50} values.

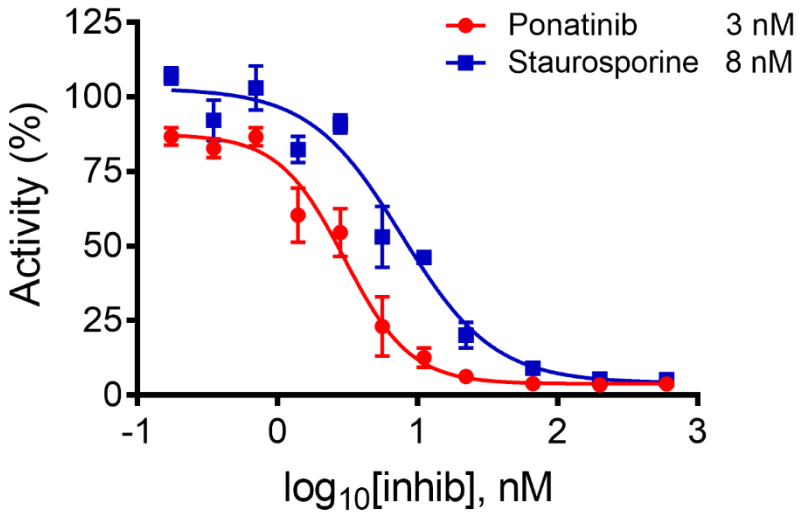

Figure S12. IC_{50} of ponatinib and staurosporine determined in the parallel experiment with **8.**

5. NanoBRET™ TE Intracellular Kinase Assays

Full-length RET ORF (Promega, V804M) cloned in frame with a C-terminal NanoLuc-fusion were transfected into HEK293 cells following the protocol from the supplier (TM589, Promega). Serially diluted inhibitor (both E and Z-isomers of **8**, as well as staurosporine and ponantinib as reference compounds) and NanoBRET Kinase Tracer K10 (Promega, N2642) were pipetted into white 96-well plates (Corning

3610) to achieve a working concentration of 0.13 μM. The RET-transfected cells were added and reseeded at a density of 2×10^5 cells/mL after trypsinization and resuspending in Opti-MEM without phenol red. The system was allowed to equilibrate for 90 min at 37°C/5% CO_2. Prior to BRET measurements, some set of cells were exposed to UV-radiation at 365 nm using a UV365 LED array (12.0 V) to convert **8***E* to **8***Z*. The UV-irradiation was carried out in in two rounds: First for 15 sec, then 10 min in the incubator, followed by a 10 sec irradiation, before the cells was left for 15 min at r.t. To measure BRET, a stock solution containing Extracellular NanoLuc Inhibitor (Promega, N2161) at 60 μM and NanoBRET NanoGlo Substrate was added according to the manufacturer's protocol, and filtered luminescence was measured on a SpectraMax iD5 (Molecular Devices) equipped with a 447 nm filter (donor) and a 610 nm filter (acceptor). Competitive displacement data were then graphed using GraphPad Prism 7 software applying sigmoidal dose-response fitting (log10(inhibitor) vs. Activity–variable slope).

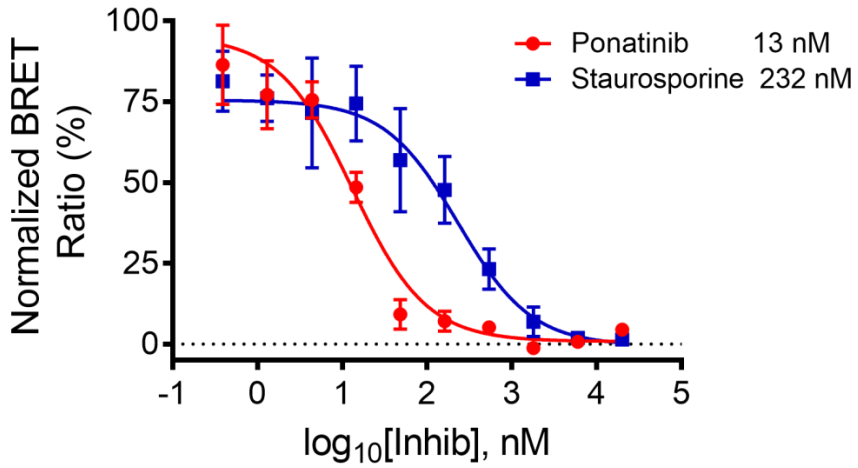

Figure S13. IC_{50} of ponatinib and staurosporine determined in the parallel experiment with **8**.

6. Docking study

The structure of RET DFG-out conformation was obtained by homology modeling. The protein sequences of RET were obtained from NCBI database (GeneID: 547807). The crystal structure of human KIT (PDB code 4U0I) was used as the template. The sequence similarity between these two proteins is 46%. The homology model was built using the Structure Prediction Wizard in the Schrödinger Suite (Schrödinger, LLC, New York, NY). The energy-based model-building method is used. The ClustalW method was used to align the target and template sequences in Prime.

With the kinase structure obtained above, the docking simulation was then performed by MOE (2016.0802 win64) in forcefield MMFF94X. The Site Finder tool was used to define the binding site, using a method of Triangle Matcher Placement and Rigid Receptor Refinement.