Targeting multidrug resistant *Staphylococcus* infections with bacterial histidine kinase inhibitors

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Contents

General methods and information.

Materials. Starting material 2-phenylacetamide was purchased from Fisher Scientific (cat # 501448864), 2-(4-chlorophenyl)acetamide, diethyl oxalate and oxalyl chloride from Millipore-Sigma (cat# EN300-69217, cat# 135364, and cat# 310670). 4-Hydroxyphenylacetamide and potassium *tert*-butoxide 1 M THF were purchased from Acros Organics (cat# 254270050 and cat# 371221000), 2-(4-fluorophenyl)acetamide from Oakwood (cat# 475850). Indoline (cat# I5605) and 2-methylindoline (cat# M51601) were purchased from Millipore-Sigma, 5-chloroindoline from Santa Cruz Biotechnology (cat# sc-284611), 6-methoxyindoline from Matrix Scientific (cat# 074899). *N*-Methylaniline (cat# M29304), 4-fluoro-*N*-methylaniline (cat# 223069), 4-chloro-*N*-methylaniline (cat# 210358) were purchased from Sigma-Aldrich. 4-Pyridineacetamide was bought from Alfa Aesar (cat# B24734). SYPRO™ Orange was purchased from ThermoFisher Scientific (cat# S6650). NH125 was purchased from Tocris (cat# 3439). GSK3β Kinase Enzyme System (cat# V1991) and ADP-Glo™ assay (cat# V6930) were purchased from Promega. The Pierce™ Silver Stain Kit (cat# 24612) and BODIPY-FL-ATPγS (cat# A22184) were bought from ThermoFisher Scientific. All other reagents were purchased from commercial sources, unless otherwise indicated, and used as directed.

HK853 Protein expression and purification. Total gene synthesis of HK853, protein production and expression were performed according to Wilke *et al.* and Chase *et al*.[1, 2] In summary, the gene corresponding to the cytoplasmic portion of HK853 from *Thermotoga maritima* was ligated into the pHis-parallel 1 vector with a His-tag at the *N*-terminus. The recombinant plasmids were transformed into *E. coli* DH5α then into *E. coli* BL21(DE3)Rosetta/pLysS overexpression cells and plated on LB agar containing 34 μg/mL chloramphenicol and 100 μg/mL ampicillin. Plates were incubated at 37 °C overnight. A single colony was transferred to 10 mL of LB media supplemented with antibiotics as noted above. The cells were grown at 37 °C at 220 RPM overnight. To 1 L of LB media containing the antibiotics noted above, 10 mL of the previous culture were added. The cells were grown at 37 °C and 220 RPM until the OD_{600} of the solution was about 0.6. The culture was allowed to cool to room temperature. The cultures were induced with 220 μL of 1 M IPTG and incubated at 20 °C and 220 RPM for 20 h. Subsequently, the cells were spun down at 8,000 x *g* for 40 min. The supernatant was discarded. The pellets were kept in the storage buffer (10 mM Tris, 0.1 mM EDTA, 0.5 M NaCl, 12% glycerol, 2 mM DTT, pH = 7.6) at -80 °C. Then, the lysis buffer (50 mL, Tris 25mM, NaCl 1 M, glycerol 10%, imidazole 5 mM, DTT 2 mM, pH = 8), 1 tablet of mini EDTA free Roche and DNAse (10 μg/1 L) were added. The suspension was homogenized with a hand glass homogenizer (10 times). The cells were lysed by sonication on ice (2 min sonication, every 5 minutes for 30 min, power 3) and spun down. The supernatant was passed through on a 0.22 μm filter. The protein was purified via nickel affinity column (Ni-NTA) on GE ÄKTA FPLC using buffer A (Tris 25 mM, NaCl 1 M, glycerol 10%, imidazole 5 mM, β-ME 2 mM, pH = 8) and buffer B (Tris 25 mM, NaCl 1 M, glycerol 10%, imidazole 1 M, β-ME 2 mM, pH= 8) with a gradient of 5 mM to 1 M of imidazole. Protein detection was performed at 210 nm. HK853 was further purified via size exclusion using HiLoad 16/60 Superdex 200 pg column using the storage buffer (10 mM Tris, 0.1 mM EDTA, 0.5 M NaCl, 12% glycerol, 2 mM DTT, pH = 7.6) as the eluent.

AirS Protein expression and purification. AirS plasmid in BL21 star (DE3) was a gift from Prof. Taeok Bae. The protein was expressed and purified according to Sun *et al*. procedure.[3] Briefly, the BL21 cells were grown in LB supplemented with ampicillin (100 µg/mL) at 37 °C and 220 RPM until the OD_{600} of the solution was about 0.6. The culture was allowed to cool to room temperature. The cultures were induced with 1 mM of IPTG and incubated at 16 °C and 220 RPM for 20 h. Subsequently, the cells were spun down at 8,000 x *g* for 40 min. The supernatant was discarded. The pellets were kept in the storage buffer (10 mM Tris, 0.1 mM EDTA, 0.5 M NaCl, 12% glycerol, 2 mM DTT, pH = 7.6) at -80 °C. Then, the lysis buffer (50 mL, Tris 25mM, NaCl 1 M, glycerol 10%, imidazole 5 mM, DTT 2 mM, pH = 8), 1 tablet of mini EDTA free Roche and DNAse (10 µg/1 L) were added. The suspension was homogenized with a hand glass homogenizer (10 times). The cells were lysed by sonication on ice (2 min sonication, every 5 minutes for 30 min, power 3) and spun down. The supernatant was passed through on a 0.22 µm filter. The protein was purified via nickel affinity column (Ni-NTA) on GE ÄKTA FPLC using buffer A (Tris 25 mM, NaCl 1 M, glycerol 10%, imidazole 5 mM, β-ME 2 mM, pH = 8) and buffer B (Tris 25 mM, NaCl 1 M, glycerol 10%, imidazole 1 M, β-ME 2 mM, pH= 8) with a gradient of 5 mM to 1 M of imidazole.

Fluorescence polarization assay. Performed according to a previously published procedure.[4]

Protein concentration determination. Protein concentration was determined using an Implen's Nanophotometer spectrophotometer (Thermo Fisher Scientific) at 280 nm. HK853 $\varepsilon = 27390\ M^{-1}.cm^{-1}$, Mw = 32468 g. mol^{-1}; AirS $\varepsilon = 31400\ M^{-1}.cm^{-1}$, Mw = 41880 g. mol^{-1}.

Reaction buffer for IC_{50} determination and thermal shift assays. The reaction buffer was composed of 50 mM Tris-HCl, pH 7.8, 200 mM KCl, 5 mM $MgCl_2$.

SDS-PAGE *(Sodium dodecyl sulfate-polyacrylamide gel electrophoresis).* The 10% separating gel was prepared using MilliQ water (4.2 mL), 40% acrylamide/bisacrylamide (2.1 mL) 1.5 M Tris.HCl pH = 8.8 (2.1 mL), 10% ammonium persulfate (28 µL), TEMED (3 µL). The resolving gel was added to the cassette and 250 µL of ethanol was added to allow for polymerization (1 h). The ethanol was removed, and the gel was washed with MilliQ water. The 4.5% stacking gel was prepared with MilliQ water (1.6 mL), 0.5 M Tris.HCl pH = 6.8 (0.63 mL), 40% acrylamide/bisacrylamide (29:1, 0.28 mL), 10% ammonium persulfate (7.5 µL), TEMED (2.5 µL). Running parameters were 180 V, 400 mA, and 60 W for 1 h. SDS-PAGE running buffer was diluted ten-fold from a 10X Tris-Glycine SDS Running buffer and chilled during electrophoresis (for 1 L, 30 g of Tris, 144 g of glycine, 10 g of SDS).

Native Page gel. The 7.5% polyacrylamide gel was prepared using MilliQ water (4.8 mL), 1.5 M Tris.HCl pH = 8.8 (2.1 mL), 40% acrylamide/bisacrylamide (1.58 mL), 10% ammonium persulfate (28 µL), TEMED (3 µL). Running parameters were 180 V, 400 mA, and 60 W for 1 h 30. The native running buffer was prepared with Tris and glycine in MilliQ water (for 1 L, 30 g of Tris, 144 g of glycine).

SDS-PAGE loading buffer. 4X SDS-PAGE sample loading buffer containing 200 mM Tris, pH 6.8, 40% glycerol, 8% SDS (w/v), 4% β-mercaptoethanol, and 0.8% bromophenol blue (w/v) was used.

Native-PAGE loading buffer. 4X native sample loading buffer containing 40 mM Tris, pH 7.5, 8% glycerol, and 0.08% Bromophenol blue (w/v) was used.

In-gel fluorescence detection. After SDS-PAGE, the gels were washed three times with MilliQ water and scanned using a Typhoon FLA 9500 scanner (GE Healthcare) at 526-nm (short-pass filter) detection for BODIPY-FL (λex: 503 nm, λem: 512 nm). Integrated density measurements were determined using ImageJ (NIH).

Inhibition of HK853 and AirS activity. BODIPY-FL-ATPγS competition screening was performed at inhibitor concentrations that did not cause protein aggregation. Triton X-100 was premixed with reaction buffer to yield 0.1% (v/v) in final 25-μL reactions. In reaction buffer, HK853 or AirS (0.48 μM) was preincubated with test compounds (final concentration, 0.01−1250 μM for HK853, 0.01-1000 or 1250 μM for AirS) in 25 μL for 30 min. BODIPY-FL ATPγS (1 μL) was added to bring the final 26-μL reactions to HK853 (0.46 μM) and BODIPY-FL-ATPγS (2 μM) in the presence of competitors and 5% DMSO. Samples were mixed and incubated in the dark at RT for 1 h before quenching with 4× SDS-PAGE sample loading buffer (8.6 μL) and loading 15 μL on a 10% resolving gel. After SDS-PAGE, in-gel fluorescence detection elucidated HK853 activity, and coomassie staining of the gels ensured even protein loading. Integrated density values of the fluorescent gel bands were normalized as "% Inhibition" with respect to a control that contained no inhibitor. Data were plotted in GraphPad Prism with relation to the log of molar inhibitor to determine IC_{50} values (Equation 1).

Data analysis. Integrated density measurements of in-gel fluorescence were performed in ImageJ. Data were prepared and analyzed in GraphPad Prism. For all DRCs (control FP competition and activity assays), data were fit to a four-parameter logistic equation,

$$y = Bottom + \frac{(Top - Bottom)}{1 + 10^{\left((LogIC_{50} - x) * HillSlope\right)}} \qquad \text{(Equation 1)}$$

where *y* is the response, *Bottom* and *Top* are plateaus in the units of the y-axis, *x* is the log of the molar concentration of inhibitor, *HillSlope* is the slope of the curve, and IC_{50} is the concentration of compound required for 50% inhibition of the enzyme (a response half way between *Bottom* and *Top*).

Aggregation assay. The lead compounds were tested for aggregation of HK853. HK853 (0.48 μM) was incubated with the compounds (1-1250 μM) dissolved in DMSO for 30 min in 20 mM HEPES (25 μL final volume containing 5% v/v DMSO). NH125 (1250 μM), a known histidine kinase aggregator, was used as a positive control. In its presence, the dimer bands disappear. The reactions were quenched with 4X native loading buffer (8.6 μL) and 15 μL was loaded onto a 7.5% Native gel for PAGE protein separation. Protein aggregation was evaluated using Silver staining.

Silver staining. The Pierce™ Silver Stain Kit was used according to the manufacturer instructions with the following modifications. All the steps were performed using a nutator. The gel was washed with MilliQ water (2 x 5 min), fixed in 30% ethanol, 10% acetic acid (2 x 15 min; 2 times), washed in 10% ethanol (2 x 5 min) then in MilliQ water (2 x 5 min). The gel was incubated with the sensitizer working solution (50 μL of sensitizer in 25 mL MilliQ water) for exactly 1 min, then washed with water (2 x 1 min). The gel was stained in the staining working solution (500 μL of enhancer in 25 mL silver stain) for 1 h, then washed with MilliQ water (2 x 20 s). The developer

working solution (500 µL of enhancer in 25 mL developer) and the gel was incubated for 3-5 min or until the bands appeared. When the desired band intensity was reached, the developer solution was replaced with the stop solution (5% acetic acid in MilliQ water). The gel was washed briefly. Next, the stop solution was replaced and the gel incubated for 10 min. After staining/destaining, the gels were scanned on a Typhoon Variable Mode Imager 9500(GE) using silver stain settings.

AirS sequence and modeling. The sequence of AirS from *Staphylococcus aureus* strain Newman was determined based on the primers used to design the protein.[3] No crystal structure of AirS was available. Only the CA domain highlighted in yellow was modeled using the online protein structure homology-modelling server SWISS-MODEL. The CA domain was modeled based the structure of VraS (PDB 4gt8). Nevertheless, the homology was low [sequence identity (23.58%) and sequence similarity (0.34)] generating a poor model.

MEQRTRLALLKEIAEFLNEETEMYSMTQGALKYLIEGSNFTTGWIFFINSVGEHELVSHV
ALPQSLTADHCHYIKDGSCWCVKAFNQRRLMKASNIVNCSRINLASKAFPSQNDNITHH
ATVPLKSGQEQFGILNVASPNTEIYSDEDLELLESVAFQLGSAIKRIYLTDREKEAAKINER
NRLARDLHDSVNQMLFSVKLTAHAAYGMSNESIAKQAFKTIEETSQNAVNEMRALIWQ
LKPVGLEQGLIHALTAYSKLMHIQLNVNVEGLIDLSNEIEENIYRALQECINNVKKHADT
NKMDLTLKQMNDILYIDVIDYGQGFEIDNVQIASSHGINNIKQRVKLLRGKVTFHSQPTK
GTQIQFTIPIK

Molecular docking of PMI compounds with HK853. Maestro from Schrödinger was utilized to dock the PMI compounds. HK853 (PDB 3DGE) was prepared with the protein preparation wizard by optimizing the H-bond assignment, removing all waters, and using OPLS_2005 force field for restrained minimization. Only one histidine kinase (A) and ADP were left for preparation. All the compounds were prepared with the LigPrep function using the defaults settings and OPLS_2005 force field. The receptor grid was generated by selecting ADP in the workspace and by defining the center of the enclosing box as the centroid of workspace ligand. The rotatable groups were defined as Tyr384, Tyr429, Thr442, and Ser385. The prepared ligands were docked using the generated receptor grid function with the default settings.

AutoQSAR of PMI compounds. All the compounds were prepared with the LigPrep function using the defaults settings and OPLS_2005 force field. The IC_{50} values for the compounds were added to the project table, transformed into the pIC_{50} values by the software (pIC50 = -log IC50), and used as the prediction property for building the QSAR model in the AutoQSAR menu. The traditional method was used with a random training set of 75% (15 compounds for training and 5 for test). The model was imported and analyzed in Canvas using the Kernel-Based PLS Regression function to determine the important features of the compounds for potency. The model was built according to Schrödinger tutorial "Machine Learning for Materials Science".[5]

Inhibition of GSK3β activity using ADP-Glo assay.[6] All the steps of the assays were done at room temperature and the plate was incubated on a plate shaker. The protocol was developed according to the manufacturer's instructions. The kinase buffer contains 40 mM Tris, pH = 7.5, 20 mM $MgCl_2$, 0.1 mg/mL BSA and 50 µM DTT. Luminescence measurements were conducted on a TECAN Spark® Multimode Microplate Reader with an integration time of 1.5 s.

A serial dilution of each test compound (stock concentrations 50 nM to 5 mM, final concentrations 10 nM to 1 mM) was done using 25% DMSO/75% kinase buffer supplied by the manufacturer to achieve a maximum final DMSO concentration of 5%. To a white flat-bottomed 384 µL-well plate, each test compound (1 µL) was dispensed in triplicate followed by GSK3β (1 ng, 2 µL) dissolved in the kinase buffer and the solutions were incubated for 10 min at room temperature. A pre-mixed solution (2 µL) of the kinase substrate (final concentration 0.2 µg/µL) and ATP (final concentration 25 µM) dissolved in kinase buffer was added. The reactions were incubated for 60 min. ADP-Glo™ Reagent (5 µl) was added and the reactions were incubated for 40 min. The Kinase Detection Reagent (10 µl) was added and the reactions were incubated for 30 min. Luminescence was recorded. A reference sample without any inhibitor was used for normalization. Background reactions without GSK3β and any inhibitor were included as well as control reactions without ATP/substrate and any inhibitor. The IC_{50} values were determined using GraphPad Prism.

Calculations

Background luminescence of the ATP/substrate mix: [background]
GSK3β + ATP/substrate mix + inhibitor: [sample]
GSK3β + ATP/substrate mix = [maximum]
% Normalized luminescence signal in presence of the inhibitors = [% Luminescence]. Value plotted on the y-axis.

$$[\%\ \text{Luminescence}] = \frac{[\text{sample}]-[\text{background}]}{[\text{maximum}]-[\text{background}]}$$

Thermal shift assay. The assays were conducted as previously published with modifications.[7] All the reagents were kept on ice. On ice, in a 96-PCR-well plate, HK853 (5 µM) was mixed with PMI-3, 5, 11, 12, 15, 18 (negative control), 20 or ADP (positive control) (500 µM final concentration from 25 mM stock, final DMSO concentration 2%) and Sypro Orange (12x from a 5000x stock solution) in reaction buffer to reach a 25 µL reaction size. Changes in fluorescence were recorded every 0.5 °C from 25 to 95 °C with a 10 s hold at each temperature. DMSO was used as a control to determine HK853 melting temperature.

Mouse model of skin infection. The 8-week-old B6 mice (N=7) were anesthetized with isoflurane gas; the skin of mice was shaved and disinfected with chlorhexidine. Every mouse was inoculated subcutaneously with the mid logarithmic growth phase of *S. aureus* (2.5×10^8 CFUs) in 50 µL PBS with 1 mM **PMI-5** or vehicle control. Total lesion size was measured with a millimeter ruler as a reference after infection. The infected mice were sacrificed 3 days after infection, and the lesional skin, liver and spleen were isolated from infected mice and homogenized for viable CFU. The results were statistically analyzed using a two-tailed T-Test. All experiments were performed in compliance with the University of Minnesota's policy on animal use and ethics as determined by the UMN Institutional Animal Care and Use Committee (IACUC).

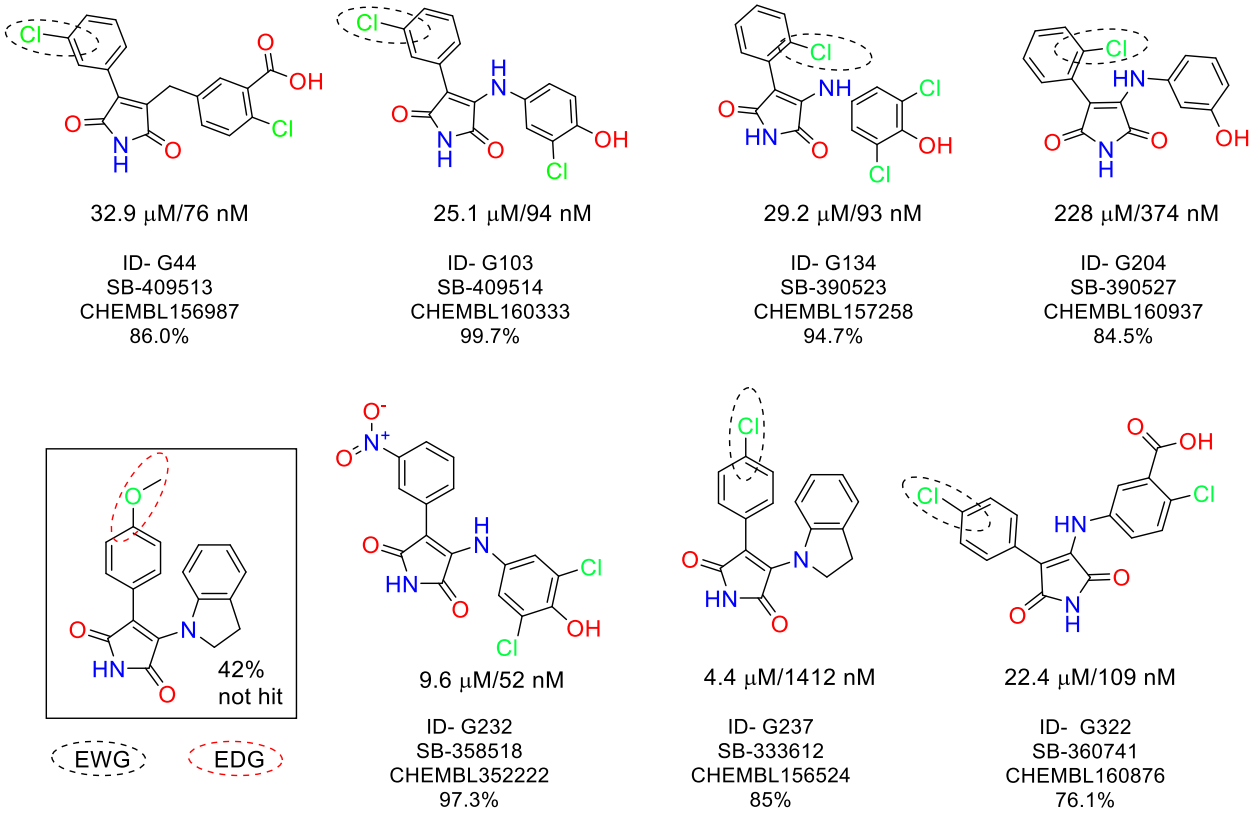
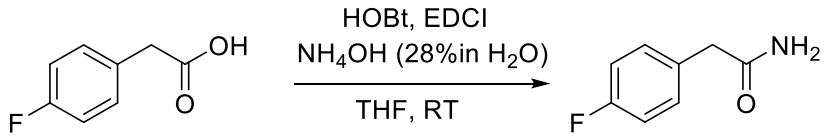
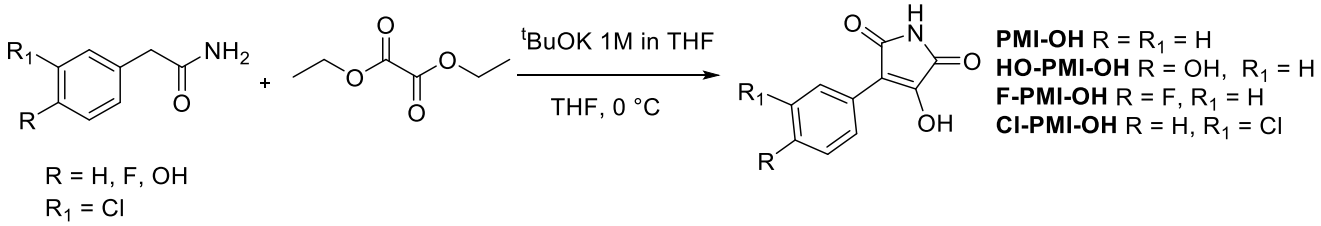


Figure S1. Maleimide hits identified in the high-throughput screening using a fluorescence polarization assay. The reported percentage corresponds to the percent inhibition of the compounds tested (100 μM) against HK853 (25 μM) obtained using a fluorescence polarization assay with BODIPY-FL-ADPγS (10 nM). Structure and IC_{50} values of the maleimide hits are reported for HK853 (left, this work) and for hGSK-3α (right, previously published results[5]). Electron withdrawing group (EWG), Electron donating group (EDG).

Synthesis. The PMI compounds were synthesized as described in published protocols with modifications.[8-11] The final compounds were above 95% pure. The purity was assessed by HPLC (Agilent HPLC1200 series, Agilent Eclipse XDB-C18, 5 μm, 9.4x250 mm, 20 min gradient with 95% water to 95% acetonitrile in presence of 0.1% ammonium acetate). ESI-MS was performed on an Agilent UPLC-QTOF instrument in positive or negative ionization mode.



To a 250 mL round-bottom flask and under nitrogen atmosphere, 4-fluorophenylacetic acid (1.0 g, 6.49 mmol) and THF (30 mL) were added followed by hydroxybenzotriazole (3.51 g, 26 mmol). The reaction mixture was stirred for 20 min until complete dissolution. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (2.5 g, 13 mmol) was added and the reaction mixture was stirred until dissolution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, which resulted in the formation of a sticky solid in solution. After addition of aqueous ammonium hydroxide solution (30 mL, 215 mmol, 28%), the reaction mixture was stirred for 16 h. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (2.5 g, 13 mmol) was added and the reaction mixture was allowed to stir for 24 h. A 1 M HCl solution (30 mL) was added and the product was extracted with EtOAc (3 x 50 mL). The combined organic layers were successively washed with a saturated solution of $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The crude solid was recrystallized from EtOAc to afford 2-(4-fluorophenyl)acetamide as a white solid (0.30 g, 30%). ^{1}H NMR (500 MHz, $(CD_3)_2SO$) δ: 7.45 (bs, 1H, NH), 7.26-7.29 (nfom, 2H, H1, H3), 7.09-7.13 (nfom, 2H, H4, H6), 6.88 (bs, 1H, NH), 3.56 (s, 2H, C**H$_2$**). ^{13}C NMR (125 MHz, $(CD_3)_2SO$) δ 172.11, 160.97 (d, J_{CF} = 240 Hz), 132.65 (d, J_{CF} = 3.8 Hz), 130.86 (d, J_{CF} = 7.5 Hz), 114.82 (d, J_{CF} = 29 Hz), 42.21 (**C**H_2). ESI-MS: expected (M+H) = 154.0663, found = 154.0671.

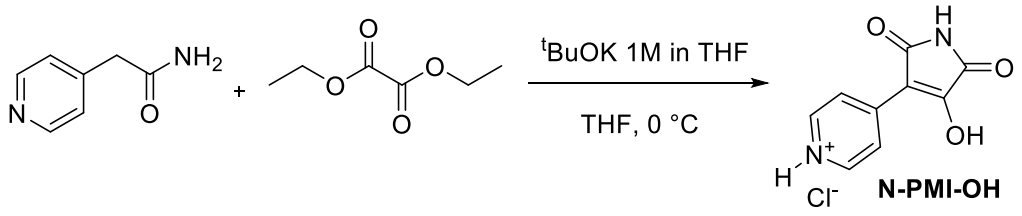


To a 100 mL oven-dried round bottom flask and under nitrogen atmosphere, 2-phenylacetamide, 2-(4-chlorophenyl)acetamide, 2-(4-hydroxyphenyl)acetamide, or 2-(4-fluorophenyl)acetamide and diethyloxalate (2 eq) were added followed by anhydrous THF (30 V). The solution was cooled to 0 ºC. A solution of ^{t}BuOK (2.5 eq, 1 M in THF) was added dropwise over 10 min. The reaction was stirred at 0 ºC for 2 h and allowed to warm up to RT. A solution of 1 M HCl (25 V) was slowly added and the aqueous layer was extracted with EtOAc (3 x 25 V). The organic layer was successively washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo* to afford **PMI-OH**, **Cl-PMI-OH**, or **F-PMI-OH**. Compound **HO-PMI-OH** was directly used in the chlorination step without purification.

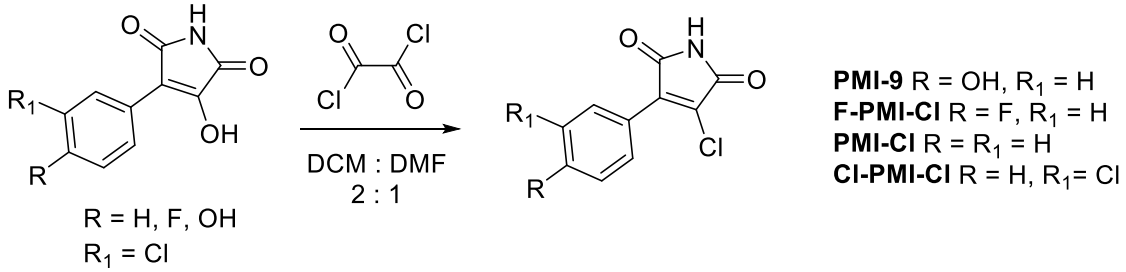
PMI-OH (yellow solid, 2.71 g, 97%) ^{1}H NMR (500 Hz, CD_3CN) δ: 8.31 (bs, 1H, NH), 7.91-7.94 (m, 2H), 7.41-7.45 (m, 2H), 7.33 (tt, 2H, *J* = 2.0, 9.0 Hz). ^{13}C NMR (125 MHz, $(CD_3)_2SO$) δ 172.31, 167.86 (2C carbonyl), 153.25, 129.84, 128.23, 127.58, 127.28, 106.34 (8C, sp^2). ESI-MS: expected (M-H) = 189.0353, found = 188.0343.

Cl-PMI-OH (yellow solid, 0.66 g, 61%) ^{1}H NMR (500 Hz, CD_3CN) δ: 8.41 (bs, 1H, NH), 7.97 (t, 1H, *J* = 2.0 Hz), 7.88-7.90 (m, 1H), 7.42 (t, 1H, *J* = 10 Hz), 7.34 (dq, 1H, *J* = 2.0, 10.0 Hz). ^{13}C NMR (125 MHz, $(CD_3)_2SO$) δ 172.10, 167.52 (2C carbonyl), 154.85, 132.95, 132.16, 130.16, 126.82, 126.60, 125.79, 104.31 (8C, sp^2). ESI-MS: expected (M-H) = 221.9963, found = 221.9961.

F-PMI-OH (yellow solid 0.91 g, 100%) ^{1}H NMR (500 Hz, $(CD_3)_2SO$) δ: 10.67 (s, 1H, NH), 7.97-8.01 (m, 2H), 7.23-7.27 (m, 2H). ^{13}C NMR (125 MHz, $(CD_3)_2SO$) δ 172.29, 167.81 (2C carbonyl), 160.95 (d, J_{CF} = 245 Hz), 153.15, 129.55 (d, J_{CF} = 7.5 Hz), 126.43 (d, J_{CF} = 3.8 Hz), 115.24 (d, J_{CF} = 21 Hz), 105.34 (8C, sp^2). ESI-MS: expected (M-H) = 206.0259, found = 206.0257.



To a 100 mL oven-dried round bottom flask and under nitrogen atmosphere, 4-pyridineacetaldehyde (300 mg, 2.2 mmol), diethyl oxalate (449 µL, 3.3 mmol) were added followed by anhydrous THF (12 mL). The solution was cooled to 0 ºC. Solid ^{t}BuOK (0.62 g, 5.5 mmol) was added over 5 min. The reaction was stirred at 0 ºC for 30 min, allowed to warm up to RT and stirred for another 30 min. A solution of 1 M HCl (3 mL) was slowly added to precipitate the product, which was subsequently filtered, washed with diethyl ether, and dried under vacuum to afford **N-PMI-OH** as the HCl salt (325 mg, 65%, yellow solid).[4] ^{1}H NMR (500 Hz, $(CD_3)_2SO$) δ: 8.41-8.47 (m, 2H), 7.31-7.37 (m, 2H). ^{13}C NMR (125 MHz, $(CD_3)_2SO$) δ 179.03, 173.25, 168.42, 148.95, 148.80, 148.67, 148.52, 147.65, 145.32. ESI-MS: expected (M-H) = 191.0451, found = 191.0466.



Procedure for PMI-Cl, Cl-PMI-Cl and F-PMI-Cl. To a 100 mL oven-dried round bottom flask and under nitrogen atmosphere, PMI-OH, Cl-PMI-OH, or F-PMI-OH was added followed by a mixture of DCM: DMF (1: 1, 15 V). The solution was cooled down to 0 ºC. Oxalyl chloride (3 equiv.) dissolved in DCM (5 V) was slowly added and the reaction proceeded for 1 h. The reaction

mixture was added to cold water (50 V). The aqueous layer was extracted with DCM (3 x 20 V). The organic layer was successively washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The product dissolved in a minimal amount of DMF precipitated upon addition of water. The products were filtered and dried under vacuum to afford **PMI-Cl**, **Cl-PMI-Cl**, or **F-PMI-Cl**.

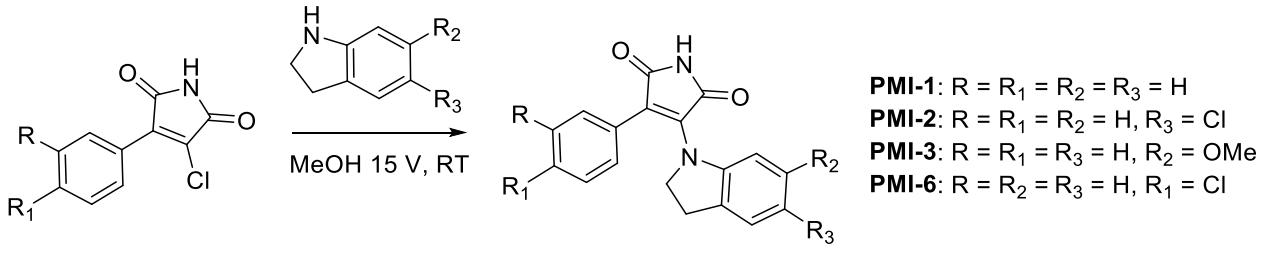
PMI-Cl (flaky white solid, 0.38 g, 70%) ^{1}H NMR (500 Hz, CD_3CN) δ: 8.89 (bs, 1H, NH), 7.81-7.85 (m, 2H), 7.52-7.55 (m, 2H). ^{13}C NMR (125 MHz, $(CD_3)_2SO$) δ 169.25, 169.04 (2C carbonyl), 135.39, 129.84, 130.98, 130.50, 129.41, 128.56, 127.09 (8C, sp^2). ESI-MS: expected (M-H) = 206.0014, found = 206.0010.

Cl-PMI-Cl (off-white solid, 0.38 g, 93%) ^{1}H NMR (500 Hz, CD_3CN) δ: 9.02 (bs, 1H, NH), 7.82 (s, 1H), 7.76-7.81 (m, 1H), 7.50-7.54 (m, 2H). ^{13}C NMR (125 MHz, $(CD_3)_2SO$) δ 168.96, 165.81 (2C carbonyl), 160.95, 134.11, 133.16, 132.26, 130.57, 130.28, 129.04, 128.90, 128.03 (8C, sp^2). ESI-MS: expected (M-H) = 239.9625, found = 239.9620.

F-PMI-Cl (light yellow solid, 0.75 g, 76%) ^{1}H NMR (500 Hz, $(CD_3)_2SO$) δ: 11.54 (bs, 1H, NH), 7.87-7.91 (m, 2H), 7.39-7.43 (m, 2H). ^{13}C NMR (125 MHz, $(CD_3)_2SO$) δ 169.22, 166.00 (2C carbonyl), 163.03 (d, J_{CF} = 248 Hz), 134.43, 131.99 (d, J_{CF} = 7.5 Hz), 130.72, 123.61 (d, J_{CF} = 2.5 Hz), 115.82 (d, J_{CF} = 21 Hz) (8C, sp^2). ESI-MS: expected (M-H) = 223.9920, found = 239.9625.

Procedure for PMI-9 or HO-PMI-Cl. To a 100 mL oven-dried round bottom flask and under nitrogen atmosphere, HO-PMI-OH was added followed by a mixture of anhydrous DCM: DMF (1: 1, 15 V). The solution was cooled down to 0 ºC. Oxalyl chloride (3 equiv.) dissolved in anhydrous DCM (5 V) was slowly added and the reaction proceeded for 2 h. The reaction mixture was added to cold water (10 V). The aqueous layer was extracted with DCM (20 V). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel using hexanes: EtOAc gradient (90:10 to 70:30) to afford **PMI-9** or **HO-PMI-Cl** (33 mg, 3.5%, yellow solid). ^{1}H NMR (500 Hz, $(CD_3)_2SO$) δ: 11.37 (s, 1H, NH), 10.22 (s, 1H, OH), 7.78-7.81 (m, 2H), 6.90-6.93 (m, 2H). ^{13}C NMR (125 MHz, $(CD_3)_2SO$) δ 169.68, 166.38 (2C carbonyl), 159.79, 134.85, 131.42, 127.00, 117.99, 115.60 (8C, sp^2). ESI-MS: expected (M-H) = 221.9963, found = 221.9961

General procedure for the syntheses of PMI-1, PMI-2, PMI-3, and PMI-6



PMI-1: R = R_1 = R_2 = R_3 = H
PMI-2: R = R_1 = R_2 = H, R_3 = Cl
PMI-3: R = R_1 = R_3 = H, R_2 = OMe
PMI-6: R = R_2 = R_3 = H, R_1 = Cl

PMI-Cl or **Cl-PMI-Cl** was dissolved in methanol (15 V) followed by the desired indoline (2-3 eq). The reaction was stirred at 25 °C for at least 16 h. The methanol was evaporated *in vacuo*, and EtOAc was added. The organic layer was successively washed with 1M HCl, water and brine, dried

over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel using DCM: MeOH (20:1) followed by HPLC purification (H_2O: ACN gradient 0.1% formic acid) to afford **PMI-1** or hexanes: EtOAc (80:20) to afford **PMI-2**, **PMI-3** or **PMI-6**.

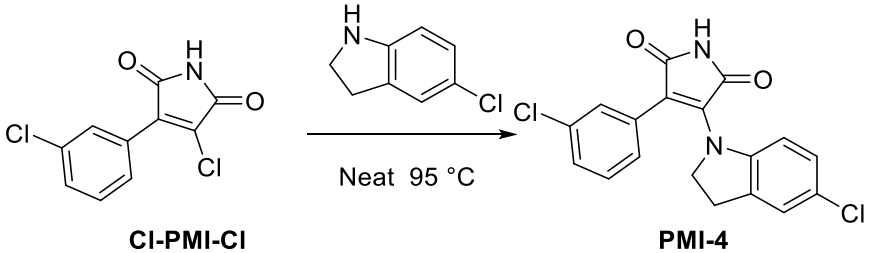
PMI-1 (orange solid, 7%). ^{1}H NMR (500 Hz, $CDCl_3$) δ: 7.28 (bs, 1H, NH), [7.18-7.24 (m, 6H) 5H phenyl ring Hs and 1H indoline ring H], 6.85 (t, 1H, *J* = 7.5 Hz, H21), 6.72 (t, 1H, *J* = 8.0 Hz, H20), 6.01 (d, 1H, *J* = 8.5 Hz, H19), 4.34 (t, 2H, *J* = 8.0 Hz, N-C**H₂**-CH_2), 3.19 (t, 2H, *J* = 8.0 Hz, N-CH_2-C**H₂**). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.46, 167.90 (2C carbonyl), 141.94, 137.57, 132.74, 129.92, 129.77, 128.03, 127.77, 126.15, 124.73, 122.87, 115.25, 110.79 (14 C, sp^2), 54.04 (1C, N-**C**H_2-CH_2), 29.55 (1C, N-CH_2-**C**H_2). ESI-MS: expected (M-H) = 289.0983, found = 289.0963.

PMI-2 (bright red solid, 5%). ^{1}H NMR (500 Hz, $CDCl_3$) δ: 7.19-7.24 (m, 3H), 7.12-7.14 (m, 2H) phenyl ring Hs], 7.01 (d, 1H *J* = 8.0 Hz, H22), 6.90 (t, 1H, *J* = 8.0 Hz, H21), 6.78 (t, 1H, *J* = 8.0 Hz, H20), 5.98 (d, 1H, *J* = 8.0 Hz, H19), 4.37 (t, 2H, *J* = 8.0 Hz, N-C**H₂**-CH_2), 3.22 (t, 2H, *J* = 8.0 Hz, N-CH_2-C**H₂**). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 169.72, 167.25 (2C carbonyl), 141.34, 133.66, 132.90, 131.45, 129.59, 128.67, 127.75, 126.02, 124.81, 123.36, 115.44, 108.27 (14 C, sp^2), 54.19 (1C, N-**C**H_2-CH_2), 29.46 (1C, N-CH_2-**C**H_2). ESI-MS: expected (M-H) = 323.0587, found = 323.0599.

PMI-3 (red solid, 9%). ^{1}H NMR (500 Hz, $CDCl_3$) δ: 7.20-7.26 (m, 5H, phenyl ring Hs, 7.13 (bs, 1H, NH) 7.07 (d, 1H, *J* = 8.0 Hz, indoline ring H), 6.41 (d, 1H, *J* = 8.0 Hz, indoline ring H), 5.51 (s, 1H, H22), 4.48 (t, 2H, *J* = 8.0 Hz, H15), 3.15 (s, 3H, C**H₃**), 3.12 (t, 2H, *J* = 8.0 Hz, H16), ^{13}C NMR (125 MHz, $CDCl_3$) δ: 170.30, 167.86 (2C carbonyl), 158.38 (**C**-OMe), 142.71, 137.03, 130.06, 129.81, 127.92, 127.67, 124.75, 124.51, 110.23, 110.07, 101.04 (14 C, sp^2), 55.01 (1C, CH_3), 54.72 (1C, N-**C**H_2-CH_2), 28.62 (1C, N-CH_2-**C**H_2). ESI-MS: expected (M-H) = 319.1083, found = 319.1089.

PMI-6 (red solid, 6%). ^{1}H NMR (500 Hz, $CDCl_3$) δ: [7.25-7.26 (m, 3H), 7.16-7.19 (m, 2H), 7.16 phenyl ring Hs], [7.16 (bs, 1H), 6.68 (d, 1H, *J* = 8.5 Hz), 5.89 (d, 1H, *J* = 8.5 Hz) indoline ring Hs], 4.36 (t, 2H, *J* = 8.0 Hz, N-C**H₂**), 3.18 (t, 2H, *J* = 8.0 Hz, N-C**H₂**-C**H₂**). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 170.02, 167.58 (2C, carbonyl), 140.58, 137.01, 134.34, 129.61, 128.25, 127.82, 127.71, 125.99, 124.86, 115.56, 111.40 (14C, sp^2), 53.99 (1C, N-**C**H_2-CH_2), 29.20 (1C, N-CH_2-**C**H_2). ESI-MS: expected (M-H) = 323.0587, found = 323.0606.

Procedure for the synthesis of PMI-4

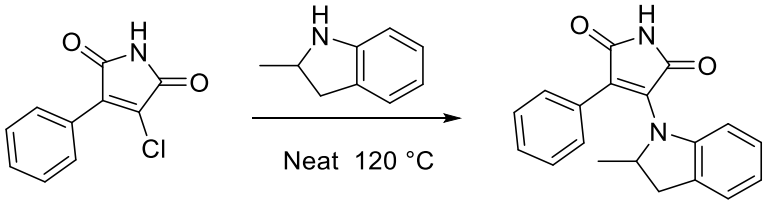


Cl-PMI-Cl **PMI-4**

Cl-PMI-Cl (250 mg, 1.03 mmol) was dissolved in 5-chloroindoline (3.75 mL, 29.5 mmol). The reaction was stirred at 95 °C for at least 6 h. The reaction mixture was diluted with EtOAc. The reaction mixture was successively washed with 3 x 1M HCl, water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel using DCM: methanol (99:1) to afford **PMI-4** (red solid, 226 mg, 61%)

PMI-4. ^{1}H NMR (500 Hz, $CDCl_3$) δ: [7.15-7.26 (m, 4H), 6.98 (m, 1H), 6.75 (m, 1H), 5.89 (d, 1H, J = 8.5 Hz) aromatic Hs], 4.38 (t, 2H, J = 8.0 Hz, N-C**H$_2$**CH_2), 3.20 (t, 2H, J = 8.0 Hz, N-CH_2-C**H$_2$**). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 169.57, 167.13 (2C carbonyl), 140.15, 137.58, 134.64, 133.87, 131.15, 129.53, 128.87, 128.37, 128.14, 127.75, 126.04, 125.09, 115.90, 109.08 (14C, sp^2), 54.30 (1C, N-CH_2**C**H_2), 29.25 (1C, N-**C**H_2CH_2). ESI-MS: expected (M-H) = 357.0198, found = 357.0226.

Procedure for the synthesis of PMI-5

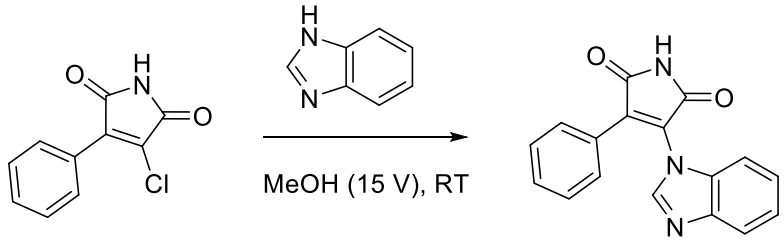


PMI-Cl **PMI-5**

PMI-Cl (141 mg, 0.68 mmol) was dissolved in 2-methylindoline (2.2 mL, 16.7 mmol). The reaction was stirred at 120 °C for at least 12 h. The reaction mixture was diluted with EtOAc. The organic layer was successively washed with 3 x 1M HCl, water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel using hexanes: EtOAc (80:20) to afford **PMI-5** (orange solid, 58 mg, 28%).

PMI-5. ^{1}H NMR (500 Hz, $CDCl_3$) δ: 7.21 (m, 5H, phenyl ring Hs), 7.15 (d, 1H, J = 7.0 Hz, H22), 7.13 (bs, 1H, NH), 6.80 (t, 1H, J = 7.0 Hz, H21), 6.68 (t, 1H, J = 7.0 Hz, H20), 5.89 (d, 1H, J = 7.0 Hz, H19), 5.20 (m, 1H, CH), 3.42 (dd, 1H, J = 9.0, 15.5 Hz, CH_2), 2.87 (dd, 1H, J = 5.5, 15.5 Hz, CH_2), 1.43 (d, 3H, J = 6.0 Hz, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) 170.17, 167.90 (2C carbonyls), 141.91, 137.08, 131.21, 129.73, 129.32, 128.19, 127.75, 126.15, 124.71, 122.22, 114.43, 114.39, (14C, aromatic CH and C), 59.48 (1C, aliphatic CH), 37.53 (1C, CH_2), 22.02 (1C, CH_3). ESI-MS: expected (M-H) = 303.1134, found = 303.1149.

Procedure for the synthesis of PMI-8



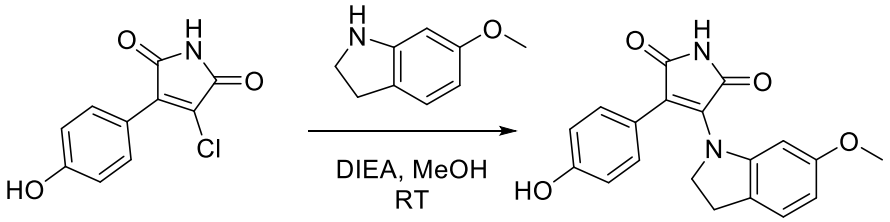
PMI-Cl **PMI-8**

PMI-Cl (200 mg, 0.96 mmol) was dissolved in methanol (3 mL) followed by benzimidazole (341 mg, 2.9 mmol). The reaction mixture was stirred at 25 °C for at least 16 h. The methanol was

evaporated *in vacuo*, and EtOAc was added. The organic layer was successively washed with 1M HCl, water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel using hexanes: EtOAc (50:50) to afford **PMI-8** (orange solid, 9.5 mg, 3.4%).

PMI-8. ^{1}H NMR (500 Hz, $CDCl_3$) δ: 8.33 (s, N-C**H**=N), 8.03 (bs, 1H, NH), 7.85 (d, 1 H, J = 8.0 Hz, benzimidazole ring H), 7.26-7.43 (m, 7H, phenyl ring Hs and benzimidazole ring H), 7.03-7.07 (m, 1 H, benzimidazole ring H), 6.59 (d, 1H, J = 8.0 Hz, benzimidazole ring H). ^{13}C NMR (125 MHz, $CDCl_3$) 168.38, 166.17 (2C carbonyls), 143.77, 142.20, 131.24, 130.72, 130.13, 129.90, 129.04, 127.96, 126.76, 124.28, 124.18, 120.99, 112.86 (15C, sp^2). ESI-MS: expected (M-H) = 288.0779, found = 288.0792.

Procedure for the synthesis of PMI-7

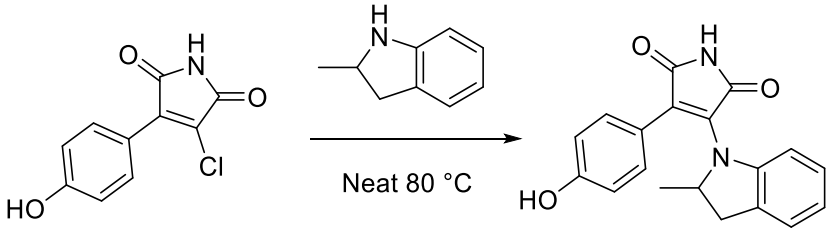


PMI-9 or HO-PMI-Cl **PMI-7**

PMI-9 or HO-PMI-Cl (10 mg, 0.045 mmol) and 2-methoxyindoline (8 mg, 0.054 mmol) were dissolved in MeOH (500 µL). N, N-diisopropylethylamine (7.8 µL, 0.045 mmol) was added. The reaction mixture was stirred at RT for 16 h. The methanol was evaporated *in vacuo*, and EtOAc was added. The organic layer was successively washed with 1M HCl, water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel using DCM: MeOH (9.5:0.5) to afford **PMI-7** (orange solid, 0.8 mg, 5.3%).

PMI-7. ^{1}H NMR (400 Hz, $CDCl_3$) δ: 7.16 (s, 1H, OH), 7.11 (d, 2H, J = 8.5 Hz, H1, H3), 7.06 (d, 1H, J = 8 Hz, H22), 6.72 (d, 2H, J = 8.5 Hz, H4, H6), 6.40 (dd, 1H, J = 8 Hz, 2.3 Hz, H21), 5.54 (d, 1H, J = 2.3 Hz, H19), 4.43 (t, 2H, J = 8 Hz, CH_2), 3.24 (s, 1H, CH_3), 3.13 (t, J = 8 Hz, 1H, CH_2). ^{13}C NMR (500 MHz, $CDCl_3$) 170.38, 168.02 (2C carbonyls), 158.49, 155.51, 142.89, 136.47, 131.36, 124.72, 124.34, 122.55, 114.69, 110.71, 109.70, 101.02 (14C, sp^2), 55.07 (1C, OCH_3), 54.45, 28.59 (2C, CH_2). ESI-MS: expected (M-H) = 335.1037, found = 335.1045.

Procedure for the synthesis of PMI-10



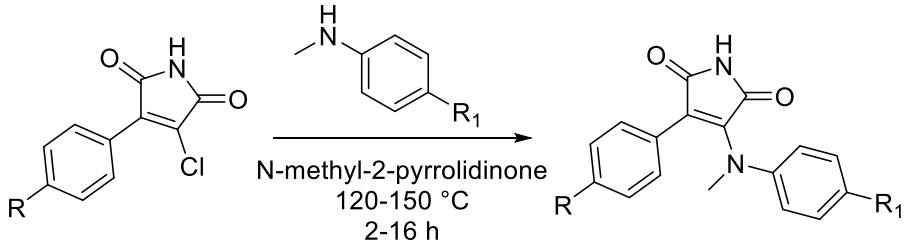
PMI-9 or HO-PMI-Cl **PMI-10**

PMI-9 or HO-PMI-Cl (22 mg, 0.098 mmol) was dissolved in 2-methylindoline (394 µL, 1.5 mmol). The reaction was stirred at 80 °C for at least 12 h. The reaction mixture was diluted with

EtOAc. The organic layer was successively washed with 2 x 1M HCl, water and brine, dried over Na_2SO4, and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel using a gradient of hexanes: EtOAc (90:10 to 80:20) followed by RP-HPLC using an Agilent 1200 series equipped with Agilent Zorbax C3, 5 µm, 9.4 x 250 mm and a gradient of 0.1 % ammonium acetate in water (Buffer A) and 0.1% ammonium acetate in acetonitrile (Buffer B) (gradient 0-3 min 95% Buffer A; 3-10 min 95 to 5% Buffer A; 10-12 min 5% Buffer A) to afford **PMI-10** (orange solid, 4.2 mg, 13%).

PMI-10. ^{1}H NMR (500 Hz, $CDCl_3$) δ: 7.16-7.18 (m, 2H, -C**H**=C-OH, phenol ring), 7.14 (m, 1H, H20), 7.07 (bs, NH), 6.79 (t, 1H, *J* = 7.4 Hz, H22), 6.74 (t, 1H, *J* = 7.4 Hz, H21), 6.69-6.71 (m, 2H, -C**H**-CH=C-OH, phenol ring), 5.91 (d, 1H, *J* = 8 Hz, H23), 5.18 (m, 1H, CH), 4.81 (bs, 1H, OH), 3.41 (dd, J = 9, 15.5 Hz, 1H, C**H$_2$**), 2.88 (dd, J = 6 Hz, 15.5 Hz, 1H, C**H$_2$**), 1.42 (d, 6 Hz, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) 170.29, 167.97 (2C carbonyls), 155.51, 142.07, 133.61, 130.87, 126.24, 124.56, 122.24, 121.79, 115.26, 114.73, 113.94, (14C, sp^2), 59.05 (1C, aliphatic CH), 37.40 (1C, CH_2), 21.97 (1C, CH_3). ESI-MS: expected (M-H) = 319.1088, found = 319.1095.

General procedure for the synthesis of N-methylaniline analogues (PMI-11, PMI-12, PMI-14, PMI-16, PMI-17, PMI-18, and PMI-19)



PMI-11 R = R_1 = H
PMI-12 R = H, R_1 = OMe
PMI-14 R = H, R_1 = Cl
PMI-16 R = F, R_1 = OMe
PMI-17 R = R_1 = F
PMI-18 R = H, R_1 = F
PMI-19 R = F, R_1 = H

4-phenyl-1H-pyrrole-2,5-dione (1 eq) and N-methyl aniline (2.5 eq) were dissolved in *N*-methyl-2-pyrrolidinone (15 V). The reaction was stirred for 2 h to 16 h at 120-150 °C. The reaction mixture was diluted with EtOAc, successively washed with 1M HCl, water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel using either isocratic or gradient solvent system of hexanes: EtOAc (90: 10 to 70:30) to obtain the desired products.

PMI-11 (yellow solid, 42%) ^{1}H NMR (400 Hz, $CDCl_3$) δ: 7.11-7.19 (m, 8H, NH, phenyl ring Hs), 6.94-7.00 (m, 3H, phenyl ring Hs), 3.51 (s, 3H, CH_3). ^{13}C NMR (125 Hz, $CDCl_3$) 170.06, 167.53 (2C carbonyls), 161.13, 144.61, 129.34, 129.29, 128.77, 127.92, 127.59, 124.58, 122.10, 113.81, (14C sp^2), 40.78 (1C CH_3). ESI-MS: expected (M-H) = 277.0977, found = 277.1014.

PMI-12 (orange solid, 63%) ^{1}H NMR (400 Hz, $CDCl_3$) δ: 7.14-7.19 (m, 3H, phenyl Hs), 7.08-7.11 (m, 2H, C**H**-C=C**H** phenyl Hs), 6.86-6.89 (m, 2H, N-C-C**H**), 6.59-6.66 (m, 2H, HO-C-C**H**), 3.71 (s, 3H, OCH_3), 3.48 (s, 3H, NCH_3). ^{13}C NMR (100 Hz, $CDCl_3$) 170.38, 167.64 (2C carbonyls), 157.38, 142.71, 138.16, 129.78, 127.60, 127.57, 124.50, 114.22, 110.79 (14C, sp^2), 55.66 (1C, OCH_3), 42.00 (1C, NCH_3). ESI-MS: expected (M-H) = 307.1088, found = 307.1129.

PMI-14 (orange solid, 69%) ^{1}H NMR (400 Hz, $CDCl_3$) δ: 7.17-7.21 (m, 3H, phenyl Hs), 7.11-7.14 (m, 2H, C**H**-C=C**H** phenyl Hs), 7.06-710 (m, 2H, 2 Cl-C-CH), 6.83-6.87 (m, 2H, 2 N-C-CH), 3.46 (s, 3H, CH_3). ^{13}C NMR (100 Hz, $CDCl_3$) 169.65, 167.41 (2C carbonyls), 164.45, 129.86, 129.34,

128.80, 128.35, 127.80, 122.87 (14C, sp^2), 40.55 (1C, CH_3). ESI-MS: expected (M-H) = 311.0593, found = 311.0631.

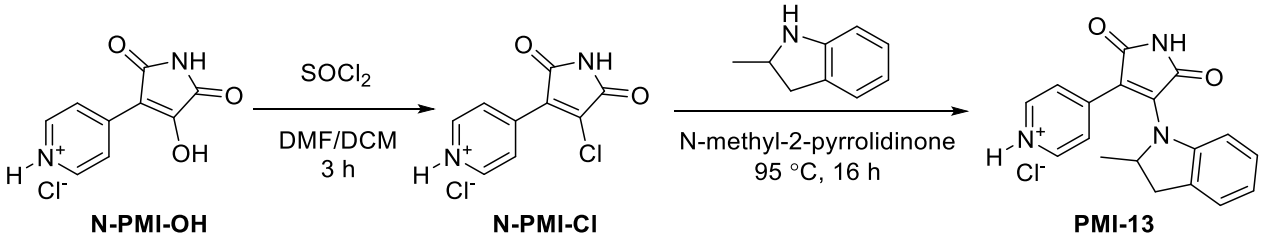
PMI-16 (orange solid, 44%) ^{1}H NMR (400 Hz, $CDCl_3$) δ: 7.12 (bs, 1H, NH), 6.96-7.01 (nfom, 2H, H1, H3), 6.77-6.86 (nfom, 4H, aniline Hs and H4, H6), 6.60-6.64 (m, 2H, aniline Hs), 3.71 (s, 3H, OCH_3), 3.56 (s, 3H, NCH_3). ^{13}C NMR (100 Hz, $CDCl_3$) 170.24, 167.52 (2C carbonyls), 160.76, 157.38, 142.41, 137.67, 131.23 (d, J_{CF} = 10 Hz), 124.45, 114.38 (d, J_{CF} = 28 Hz), 114.06, 108.92 (14C, sp^2), 55.57 (1C, OCH_3), 41.68 (1C, NCH_3). ESI-MS: expected (M-H) = 325.0994, found = 325.1026.

PMI-17 (yellow solid, 31%) ^{1}H NMR (500 Hz, $CDCl_3$) δ: 7.32 (bs, 1H, NH), 7.01-7.05 (nfom, 2H, H1, H3), 6.78-6.90 (nfom, 6H, H4, H6, aniline Hs), 3.55 (s, 3H, NCH_3). ^{13}C NMR (125 Hz, $CDCl_3$) 170.21, 167.59 (2C carbonyls), 162.38 (d, J_{CF} = 253 Hz), 160.00 (d, J_{CF} = 244 Hz), 142.18, 140.75 (d, J_{CF} = 3.8 Hz), 131.32 (d, J_{CF} = 6.7 Hz), 125.40 (d, J_{CF} = 2.5 Hz), 124.33 (d, J_{CF} = 6.7 Hz), 115.76 (d, J_{CF} = 18 Hz), 114.82 (d, J_{CF} = 17.1 Hz), 111.25 (14C aromatic sp^2), 41.34 (1C, CH_3). ESI-MS: expected (M-H) = 313.0794, found = 313.0829.

PMI-18 (yellow solid, 62%) ^{1}H NMR (400 Hz, $CDCl_3$) δ: 7.13-7.20 (nfom, 4H, NH and phenyl Hs, H4, H5, H6), 7.05-7.10 (nfom, 2H, H1,H3), 6.87-6.92 (m, 2H, H4, H6), 6.76-6.82 (m, 2H, 2 N-C-CH), 3.50 (s, 3H, CH_3). ^{13}C NMR (100 Hz, $CDCl_3$) 170.07, 167.50 (2C carbonyls), 161.04, 158.60, 142.20, 140.86, 129.48, 129.22, 127.95, 127.61, 124.12 (d, J_{CF} = 7.9 Hz), 115.55 (d, J_{CF} = 23 Hz), 112.59 (14C, sp^2), 41.32 (1C, CH_3). ESI-MS: expected (M-H) = 295.0888, found = 295.0923.

PMI-19 (yellow solid, 43%) ^{1}H NMR (400 Hz, $CDCl_3$) δ: 7.15 (bs, 1H, NH), 7.08-7.12 (nfom, 2H, aniline Hs), 7.02-7.07 (nfom, 2H, H1, H3), 6.95-6.99 (nfom, 1H, aniline Hs), 6.88-6.91 (nfom, 2H, aniline Hs), 6.78-6.83 (nfom, 2H, H4, H6), 3.58 (s, 3H, CH_3). ^{13}C NMR (100 Hz, $CDCl_3$) 170.91, 167.56 (2C carbonyls), 160.97, 144.29, 141.72, 130.99 (d, J_{CF} = 8.3 Hz), 128.78, 125.30, 124.83, 122.25, 114.59 (d, J_{CF} = 21.8 Hz), 111.73 (14C, sp^2), 40.64 (1C, CH_3). ESI-MS: expected (M-H) = 295.0888, found = 295.0918.

Procedure for the synthesis of PMI-13

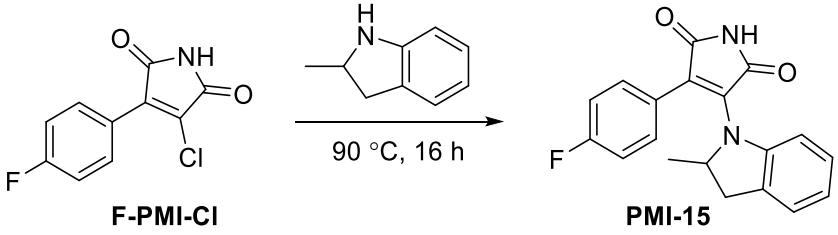


To a dried vial and under nitrogen atmosphere, **N-PMI-OH** (50 mg, 0.22 mmol) was dissolved in anhydrous DMF (100 µL). The reaction mixture was cooled down to 0 ºC. A solution of thionyl chloride (321 µL, 4.4 mmol) dissolved in DCM (1 mL) was added dropwise over 5 min. The reaction mixture was stirred at 0 ºC for 3 h and allowed to warm up to RT before concentration in *vacuo* to afford N-PMI-Cl as a HCl salt. The product was used in the next step without further purification. **N-PMI-Cl** and 2-methylindoline (517 µL, 4.0 mmol) were dissolved in *N*-methyl-2-

pyrrolidinone (1 mL). The reaction mixture was stirred at 95 °C for 16 h. A solution of EtOAc/H_2O (80:20) was added to the reaction mixture. The organic layer was successively washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel using a gradient of hexanes: EtOAc (60: 40) with 0.1% 1M HCl to obtain **PMI-13** (orange solid, 25%).

PMI-13. ^{1}H NMR (400 Hz, $CDCl_3$) δ: 8.43 (d, 2H, *J* = 5.2 Hz, CH=N-CH), 7.36 (bs, 1H, NH), 7.24 (d, 1H, *J* = 7.2 Hz, N-C-CH), 7.04 (d, 2H, *J* = 6 Hz, CH=C-CH), 6.89 (t, 1H, *J* = 7.6 Hz, H20), 6.73 (t, 1H, *J* = 7.6 Hz, H21), 5.82 (d, 1H, J = 8 Hz, H22), 5.32 (m, 1H, N-CH), 3.50 (dd, 1H, J = 8.8, 15.6 Hz, CH_2), 2.91 (dd, 1H, J = 4.4, 15.6 Hz, CH_2), 1.50 (d, J = 6.4, 3H). ^{13}C NMR (100 Hz, $CDCl_3$) 169.32, 166.90 (2C carbonyls), 148.98, 142.51, 132.15, 126.19, 125.30, 123.75, 123.37, 115.69, (13C, sp^2), 60.80 (1C, aliphatic CH) 37.61 (1C, CH_2), 22.06 (1C, CH_3). ESI-MS: expected (M-H) = 304.1092, found = 304.1074.

Procedure for the synthesis of PMI-15

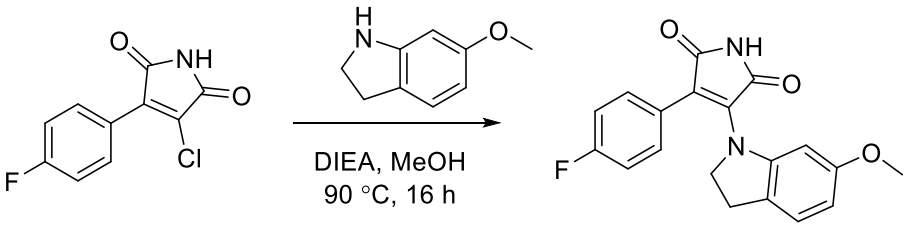


F-PMI-Cl **PMI-15**

F-PMI-Cl (20 mg, 0.087 mmol) was mixed with 2-methylindoline (14 µL, 0.11 mmol). The reaction was stirred at 90 °C for 16 h. The reaction mixture was diluted with EtOAc. The organic layer was successively washed with 3 x 1M HCl, water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel using hexanes: EtOAc (80: 20) to afford **PMI-15** (yellow solid, 15.7 mg, 55%).

PMI-15. ^{1}H NMR (400 Hz, $CDCl_3$) δ: 7.29 (bs, 1H, NH), 7.16-7.22 (nfom, 3H, N-C-CH, H1, H3, H18), 6.89-6.95 (nfom, 2H, H4, H6), 6.82 (t, 1H, *J* = 7.6 Hz, H19), 6.71 (t, 1H, *J* = 7.6 Hz, H20), 5.47 (d, 1H, *J* = 8 Hz, H21), 5.22 (m, 1H), 3.43 (dd, 1H, *J* = 9.2, 15.6 Hz), 2.87 (dd, 1H, *J* = 5.2, 15.6 Hz), 1.44 (d, 3H, *J* = 6.4 Hz). ^{13}C NMR (125 Hz, $CDCl_3$) 170.16, 167.79 (2C carbonyls), 161.06, 141.61, 136.90, 131.36, 131.16 (d, J_{CF} = 8.3 Hz), 131.12, 126.24, 125.82, 124.87, 122.48, 114.76 (app t, J_{CF} = 21.5 Hz)., 113.07 (14C, sp^2), 59.67 (1C, CH), 37.56 (1C, CH_2), 22.11 (1C, CH_3). ESI-MS: expected (M-H) = 321.1045, found = 321.1080.

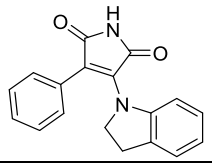
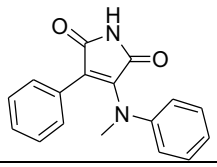
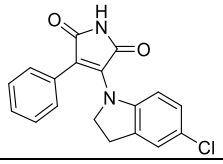
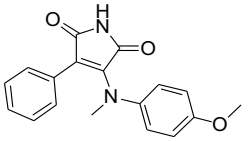
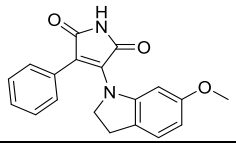
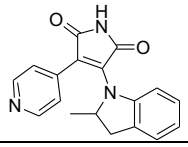
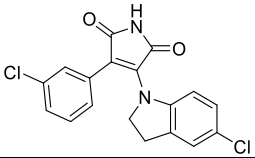
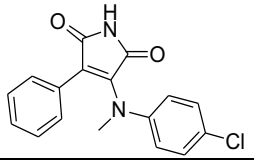
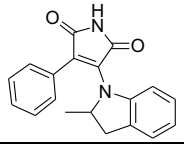
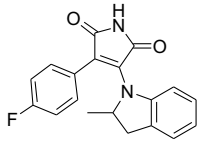
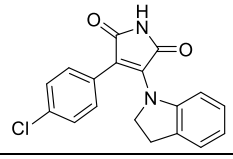
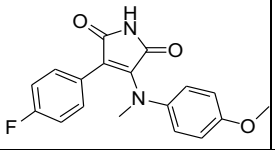
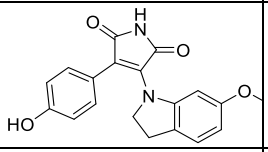
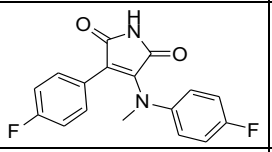
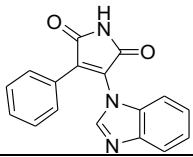
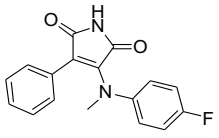
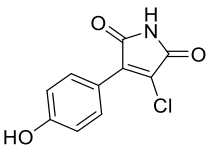
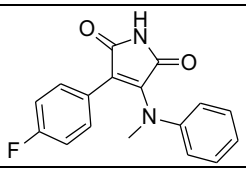
Procedure for the synthesis of PMI-20

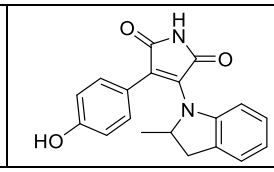
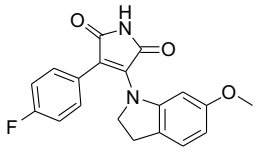


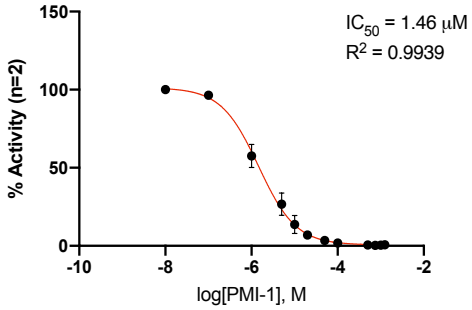
F-PMI-Cl (30 mg, 0.13 mmol) and 6-methoxyindoline (60 µL, 0.40 mmol) were dissolved in methanol (1 mL). N, N-Diisopropylethylamine (23 µL, 0.13 mmol) was added and the reaction mixture was stirred at 90 °C for 16 h. The methanol was evaporated *in vacuo*, and EtOAc was added. The organic layer was successively washed with 3 x 1M HCl, water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel using hexanes: EtOAc (90: 10) to afford **PMI-20** (orange solid, 25.4 mg, 56%).

PMI-20. ^{1}H NMR (400 Hz, $CDCl_3$) δ: 7.29 (bs, 1H, NH), 7.17-7.22 (nfom, 2H, H1, H3), 7.08 (d, 1H, J = 8.0 Hz, H20), 6.92-6.98 (nfom, 2H, H4, H6), 6.43 (dd, 1H, J = 2.4, 8.0 Hz, H19), 5.49 (d, 1H, J = 2.4 Hz, H17), 4.45 (t, 2H, J = 8 Hz, N-CH_2), 3.24 (s, 3H, OCH_3), 3.14 (t, 2H, J = 8 Hz, N-CH_2-C**H₂**). ^{13}C NMR 170.26, 167.74 (2C carbonyls), 163.39, 160.91, 158.49, 142.58, 136.88, 131.54 (d, J_{CF} = 8.1 Hz), 126.10, 124.91, 124.62, 114.77 (d, J_{CF} = 20.9 Hz), 110.01, 109.09, 101.34 (14C, sp^2), 55.05, 54.72 (1C, CH_2), 28.63 (1C, CH_3). ESI-MS: expected (M-H) = 337.0994, found = 337.1019.

Table S1. IC_{50} values of PMI compounds for HK853 and AirS inhibition. *Indicates that complete inhibition was not achieved at highest tested concentration. These values are presented as a lower bound, with the IC_{50} value being > the number indicated in GraphPad Prism.

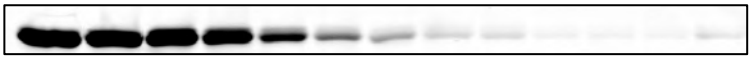
Compound		IC_{50} values (μM) (95% confidence interval) for HK853 and AirS	Compound		IC_{50} values (μM) (95% confidence interval) for HK853 and AirS
PMI-1		1.4 (1.1-1.7) > 7.6 (1.3-ambiguous)*	PMI-11		> 87 (29-334)* > 477 (90-ambiguous)*
PMI-2		1.7 (0.88-3.1) > 1000*	PMI-12		44 (3.2-60) > 41 (3.7-8699)*
PMI-3		23 (14-38) > 1000*	PMI-13		> 291 (117-1073)* > 1250
PMI-4		33 (28-39) > 1000*	PMI-14		> 419 (213-949)* > 605 (34-ambiguous)*
PMI-5		> 98 (34-280)* 153 (27-ambiguous)*	PMI-15		> 138 (110-1242)* > 130 (58-3927)*
PMI-6		4.1 (13-44) > 929 (ambiguous)*	PMI-16		> 208 (107-454)* > 1250*
PMI-7		5.4 (3.2-9.1) >1250*	PMI-17		> 236 (168-345)* > 1250*
PMI-8		7.9 (3.1-20) > 781 (77-ambiguous)*	PMI-18		> 1250* > 1250*
PMI-9		> 477 (198-1587)* 39 (17-96)	PMI-19		145 (85-270) > 1250*

PMI-10		> 198 (129-323)* 221 (668-1554)	PMI-20		28 (23-34) > 151 (21-ambiguous)*

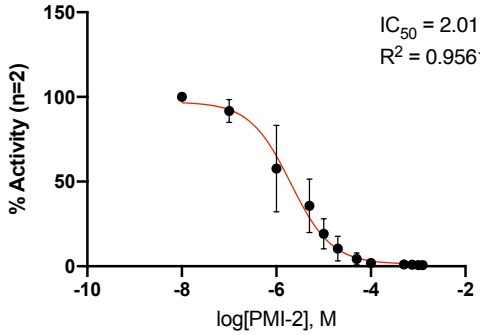


Concentration (μM) 0 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250

BODIPY-FL1

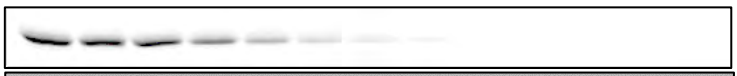


Coomassie

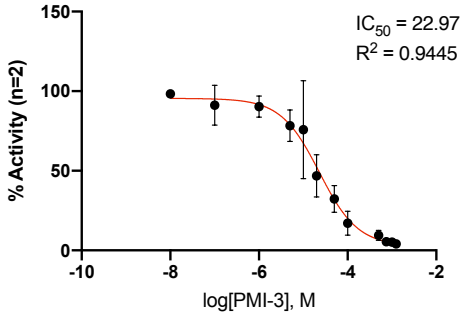


Concentration (μM) 0 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250

BODIPY-Fl

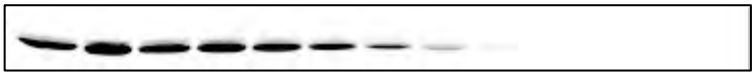


Coomassie



Concentration (μM) 0 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250

BODIPY-Fl



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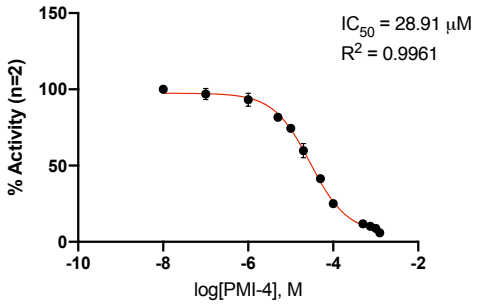
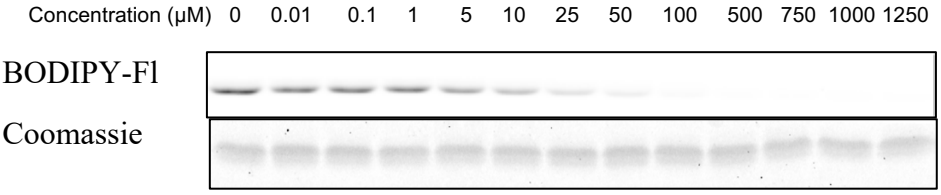
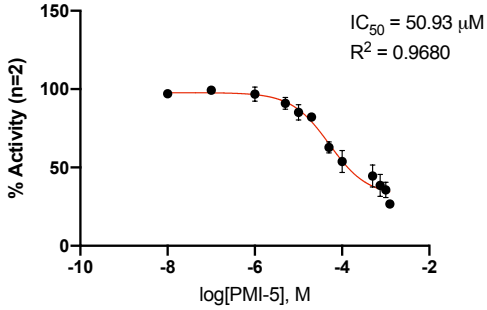
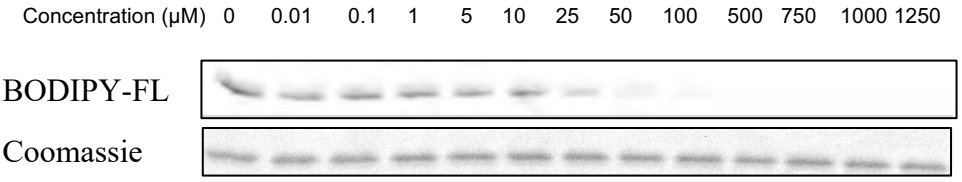
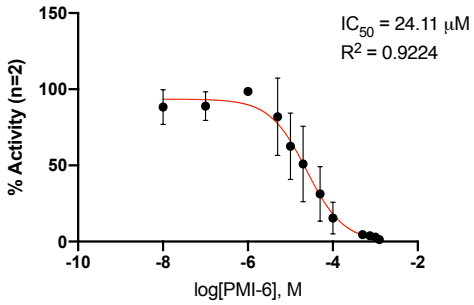
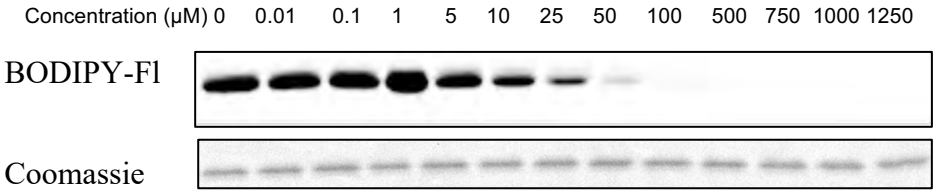

IC50 = 28.91 μM
R2 = 0.9961
% Activity (n=2)
150
100
50
0
-10
-8
-6
-4
-2
log[PMI-4], M

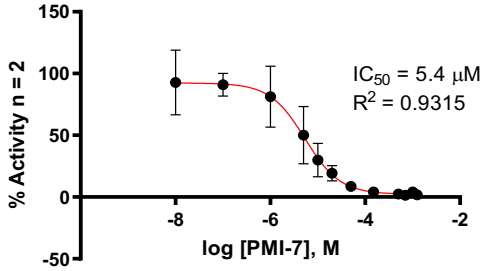

Concentration (μM) 0 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250
BODIPY-Fl
Coomassie

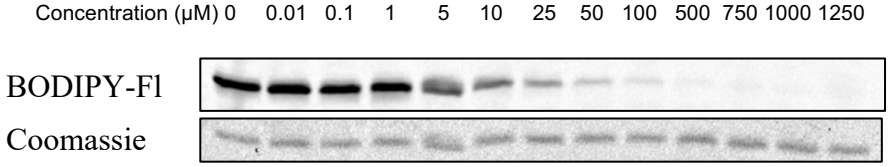

IC50 = 50.93 μM
R2 = 0.9680
% Activity (n=2)
150
100
50
0
-10
-8
-6
-4
-2
log[PMI-5], M

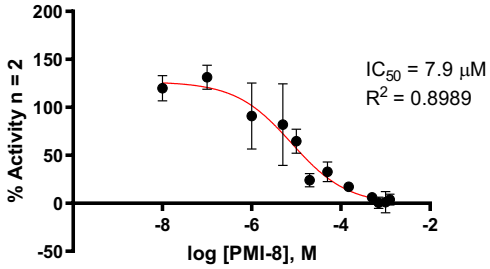

Concentration (μM) 0 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250
BODIPY-FL
Coomassie

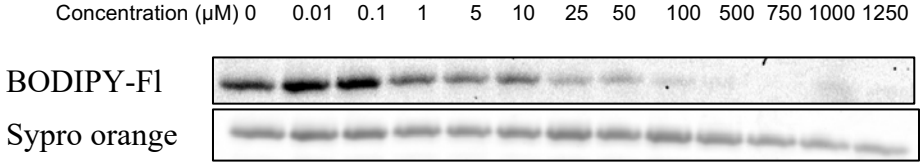

IC50 = 24.11 μM
R2 = 0.9224
% Activity (n=2)
150
100
50
0
-10
-8
-6
-4
-2
log[PMI-6], M

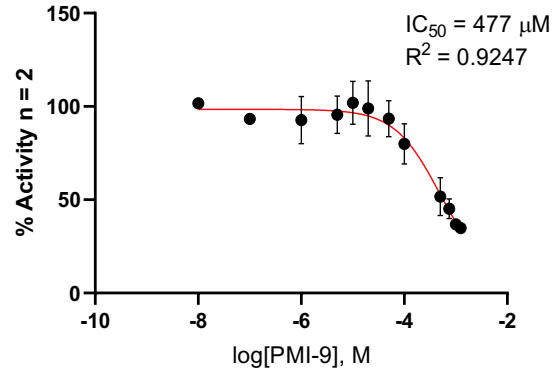

Concentration (μM) 0 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250
BODIPY-Fl
Coomassie

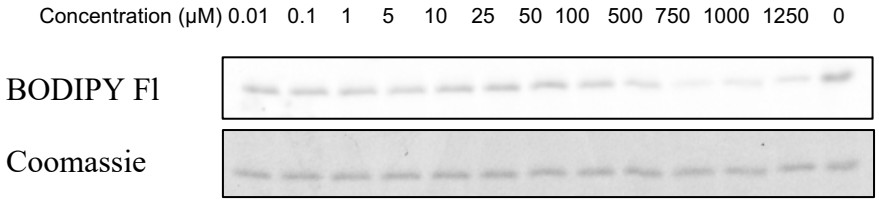

IC50 = 5.4 μM
R2 = 0.9315
% Activity n = 2
150
100
50
0
-50
-8
-6
-4
-2
log [PMI-7], M

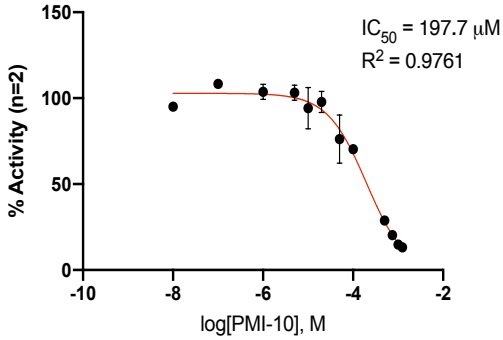

Concentration (μM) 0 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250
BODIPY-Fl
Coomassie

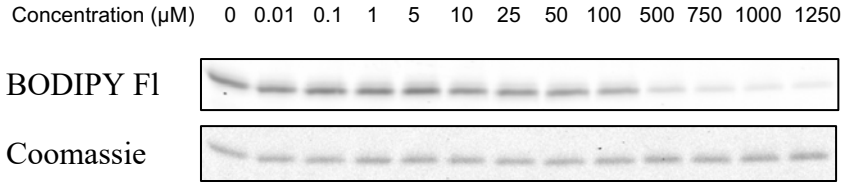

IC50 = 7.9 μM
R2 = 0.8989
% Activity n = 2
200
150
100
50
0
-50
-8
-6
-4
-2
log [PMI-8], M

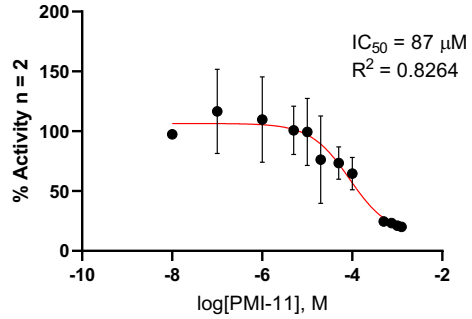

Concentration (μM) 0 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250
BODIPY-Fl
Sypro orange

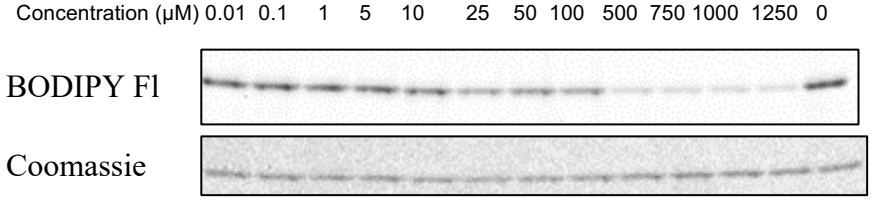

IC50 = 477 µM
R2 = 0.9247
% Activity n = 2
150
100
50
0
-10
-8
-6
-4
-2
log[PMI-9], M

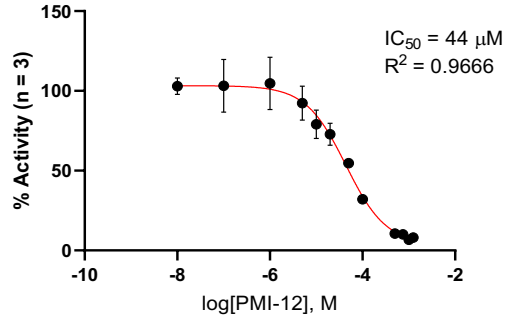

Concentration (µM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250 0
BODIPY Fl
Coomassie

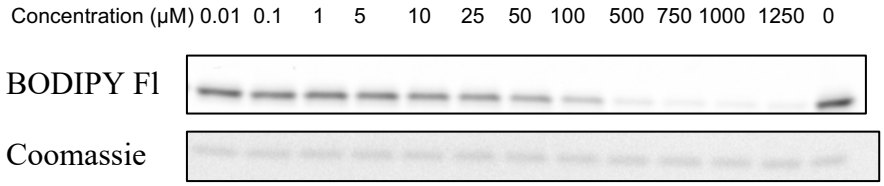

IC50 = 197.7 µM
R2 = 0.9761
% Activity (n=2)
150
100
50
0
-10
-8
-6
-4
-2
log[PMI-10], M

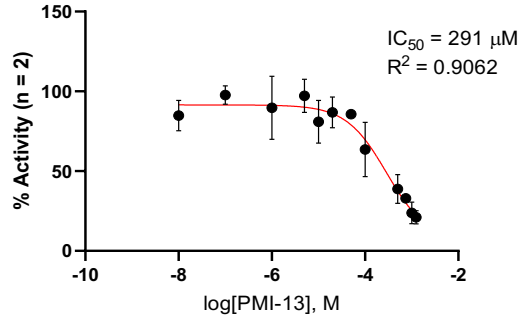

Concentration (µM) 0 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250
BODIPY Fl
Coomassie

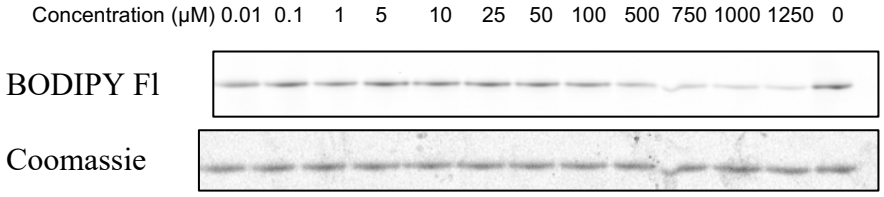

200
150
100
50
0
% Activity n = 2
-10
-8
-6
-4
-2
log[PMI-11], M
IC50 = 87 µM
R2 = 0.8264

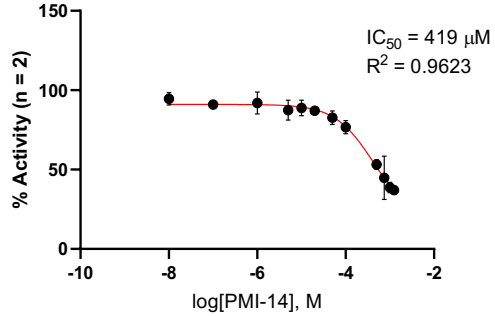

Concentration (µM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250 0
BODIPY Fl
Coomassie

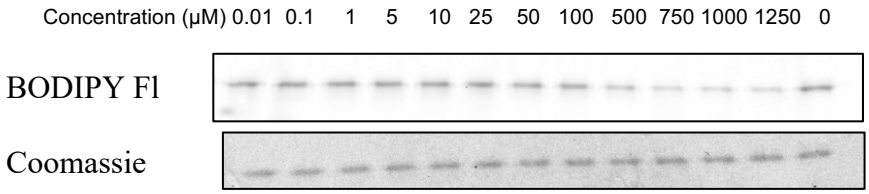

150
100
50
0
% Activity (n = 3)
-10
-8
-6
-4
-2
log[PMI-12], M
IC50 = 44 µM
R2 = 0.9666

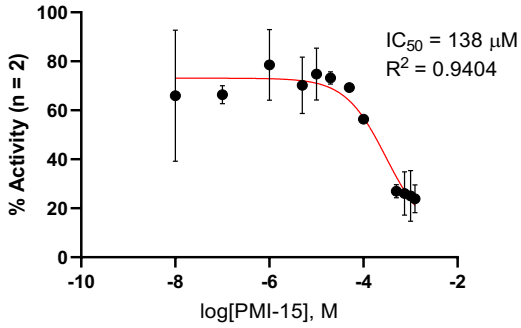

Concentration (µM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250 0
BODIPY Fl
Coomassie


150
100
50
0
% Activity (n = 2)
-10
-8
-6
-4
-2
log[PMI-13], M
IC50 = 291 µM
R2 = 0.9062


Concentration (µM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250 0
BODIPY Fl
Coomassie


150
100
50
0
% Activity (n = 2)
-10
-8
-6
-4
-2
log[PMI-14], M
IC50 = 419 µM
R2 = 0.9623

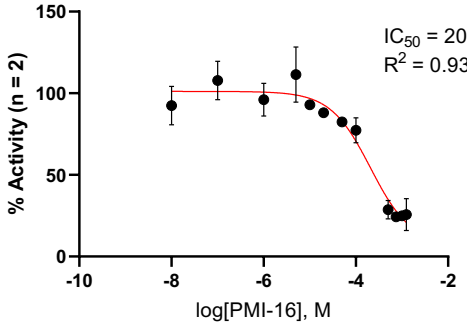

Concentration (µM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250 0
BODIPY Fl
Coomassie



Concentration (µM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250 0

BODIPY Fl

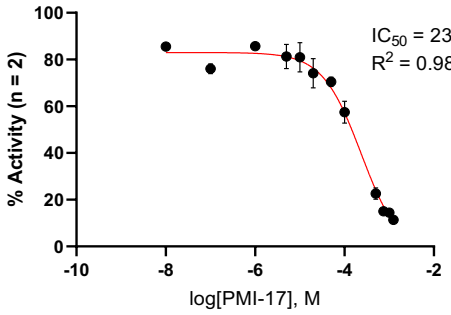
Coomassie



Concentration (µM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250 0

BODIPY Fl

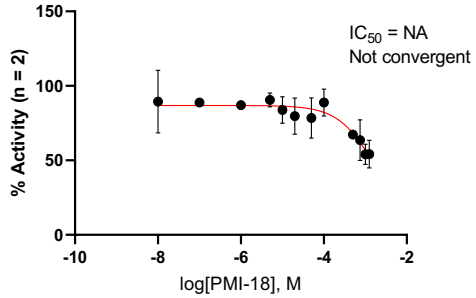
Coomassie



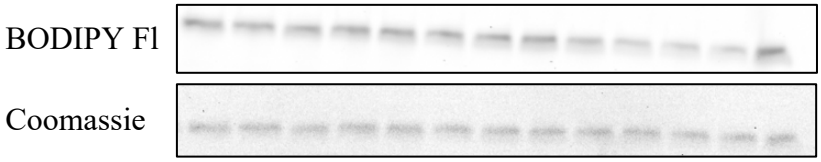
Concentration (µM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250 0

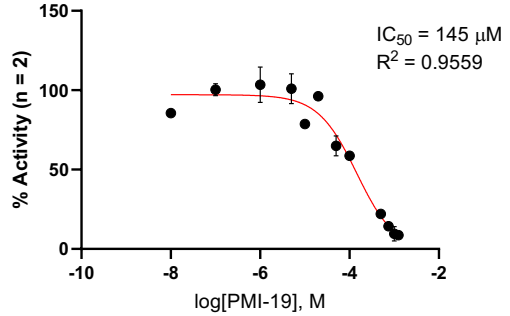
BODIPY Fl

Coomassie

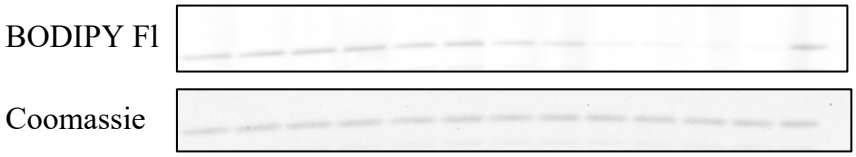


Concentration (μM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250 0





Concentration (μM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250 0



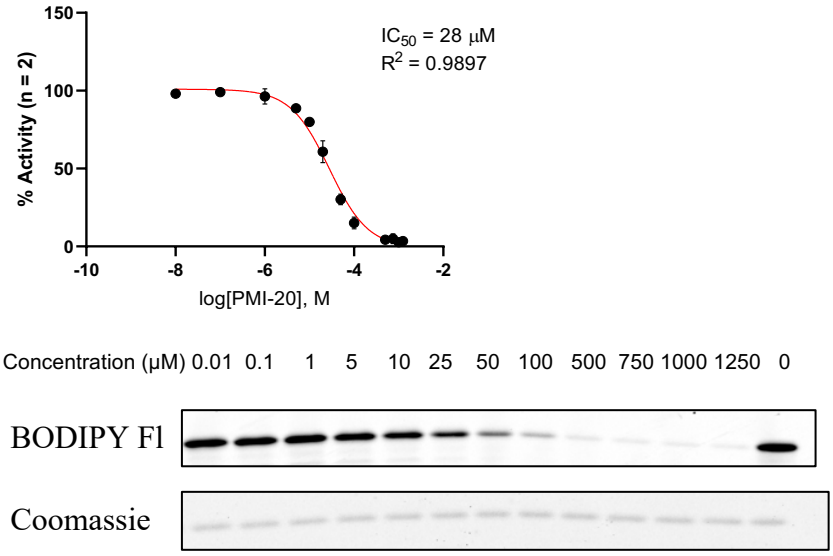
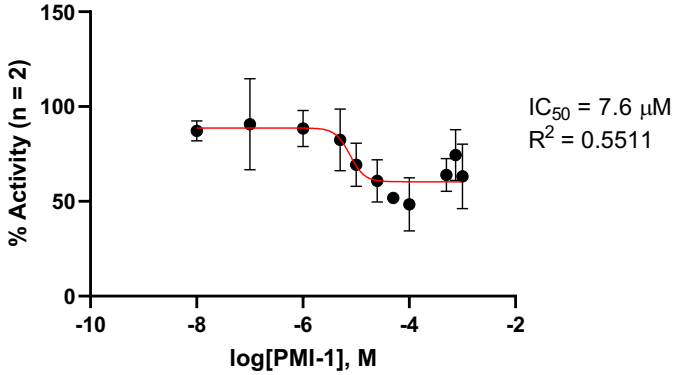
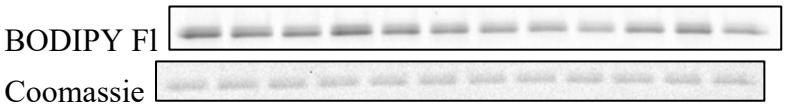
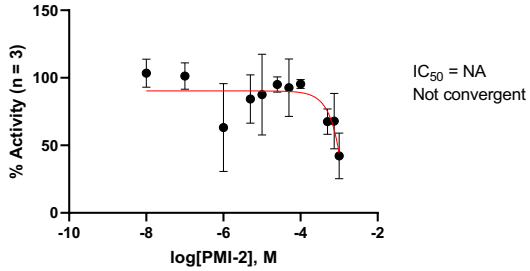


Figure S2: Dose-response curves of the inhibition of HK853 activity with **PMI-1** to **PMI-20** using B-FL-ATPγS inhibition assay.

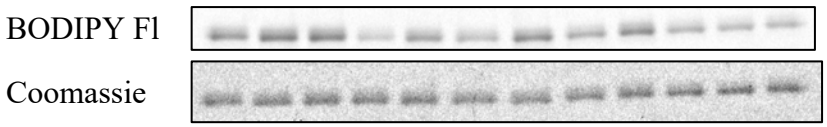


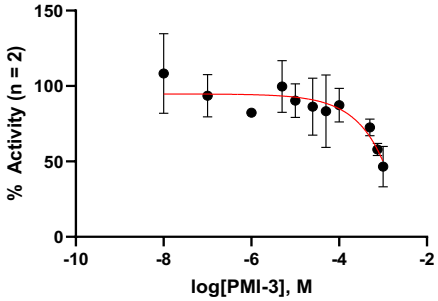
Concentration (µM) 0 0.01 0.1 1 5 10 25 50 100 500 750 1000



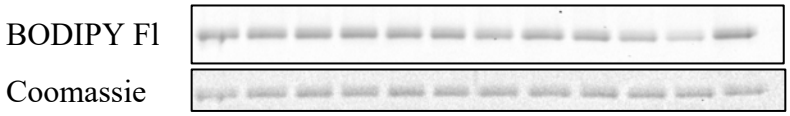


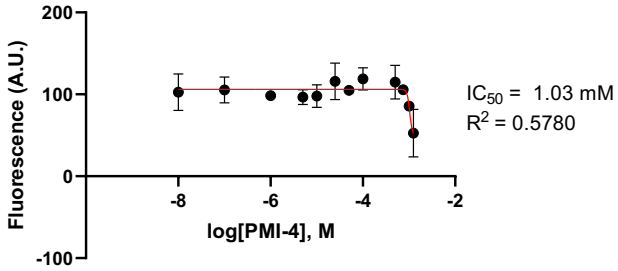
Concentration (µM) 0 0.01 0.1 1 5 10 25 50 100 500 750 1000

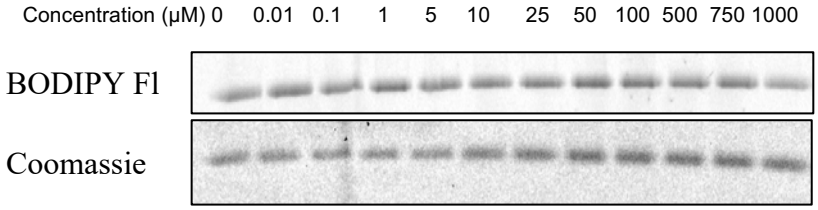


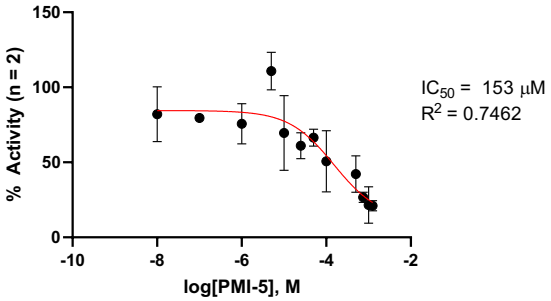


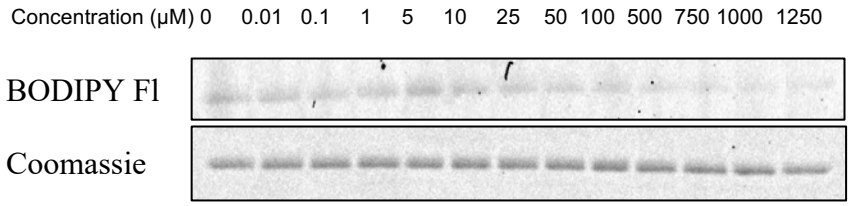
Concentration (µM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 0

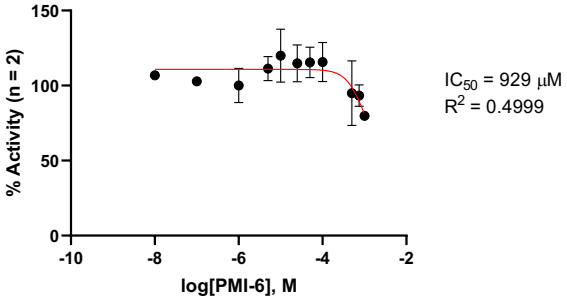


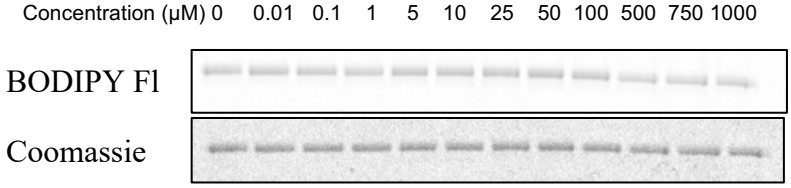

200
100
0
-100
Fluorescence (A.U.)
-8
-6
-4
-2
log[PMI-4], M
IC50 = 1.03 mM
R2 = 0.5780

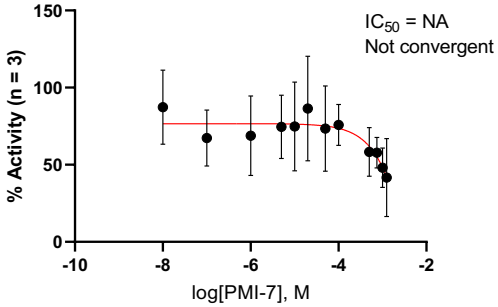

Concentration (μM) 0 0.01 0.1 1 5 10 25 50 100 500 750 1000
BODIPY Fl
Coomassie


150
100
50
0
% Activity (n = 2)
-10
-8
-6
-4
-2
log[PMI-5], M
IC50 = 153 μM
R2 = 0.7462


Concentration (μM) 0 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250
BODIPY Fl
Coomassie


150
100
50
0
% Activity (n = 2)
-10
-8
-6
-4
-2
log[PMI-6], M
IC50 = 929 μM
R2 = 0.4999

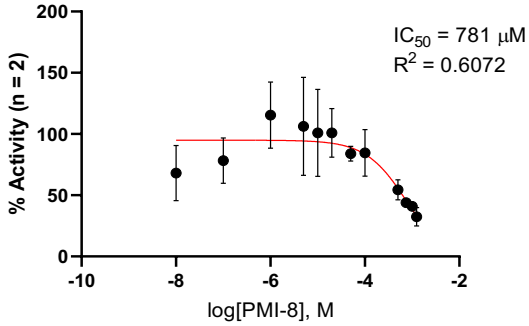

Concentration (μM) 0 0.01 0.1 1 5 10 25 50 100 500 750 1000
BODIPY Fl
Coomassie



Concentration (μM) 0 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250

BODIPY Fl

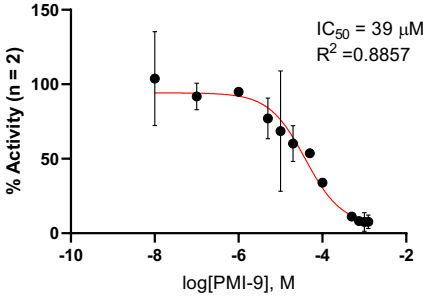
Coomassie



Concentration (μM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250 0

BODIPY Fl

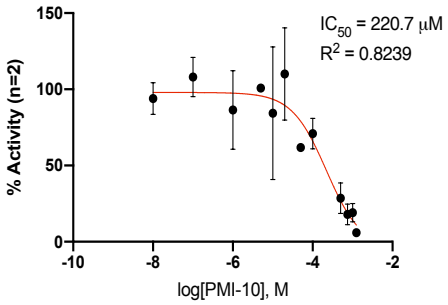
Coomassie



Concentration (μM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250 0

BODIPY Fl

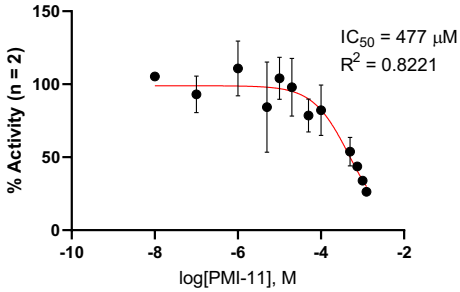
Coomassie



Concentration (µM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250 0

BODIPY Fl

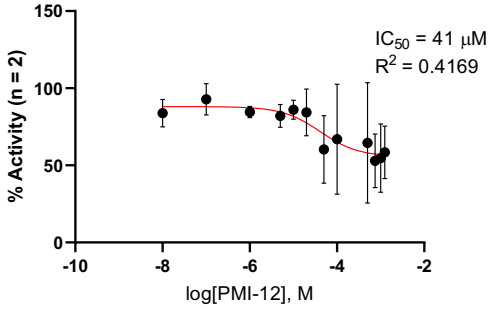
Coomassie



Concentration (µM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250 0

BODIPY Fl

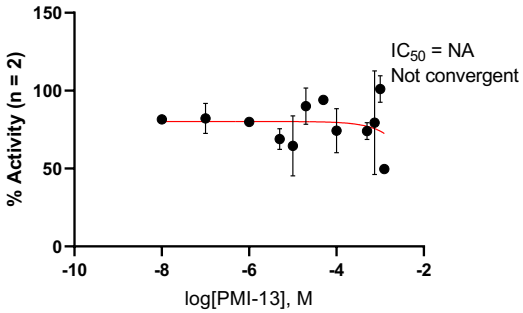
Coomassie



Concentration (µM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250 0

BODIPY Fl

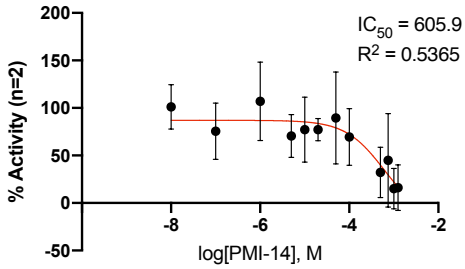
Coomassie



Concentration (μM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250 0

BODIPY Fl

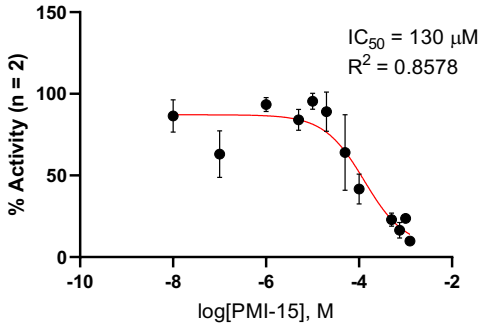
Coomassie



Concentration (μM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250 0

BODIPY Fl

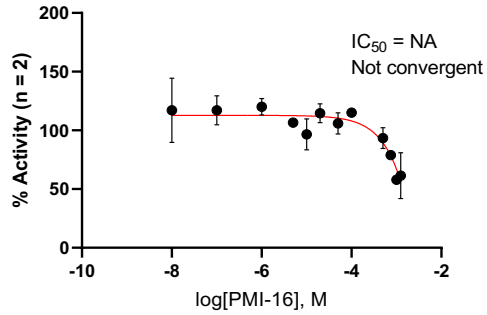
Coomassie



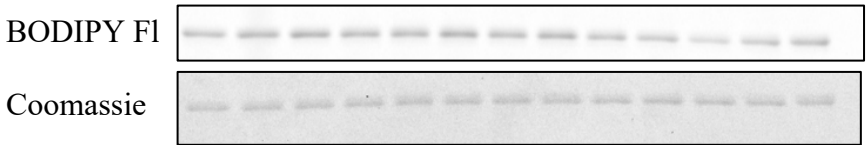
Concentration (μM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250 0

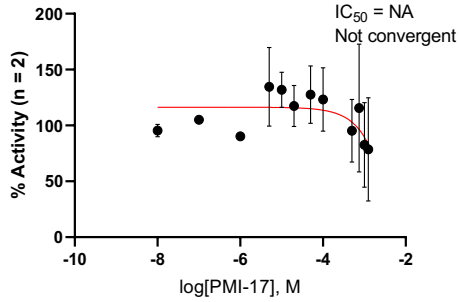
BODIPY Fl

Coomassie

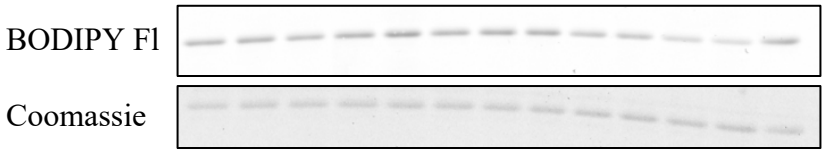


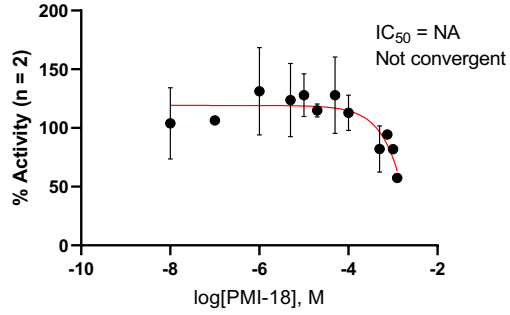
Concentration (μM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250 0



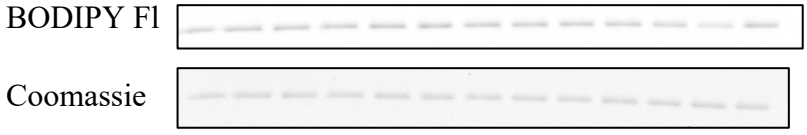


Concentration (μM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250 0





Concentration (μM) 0.01 0.1 1 5 10 25 50 100 500 750 1000 1250 0



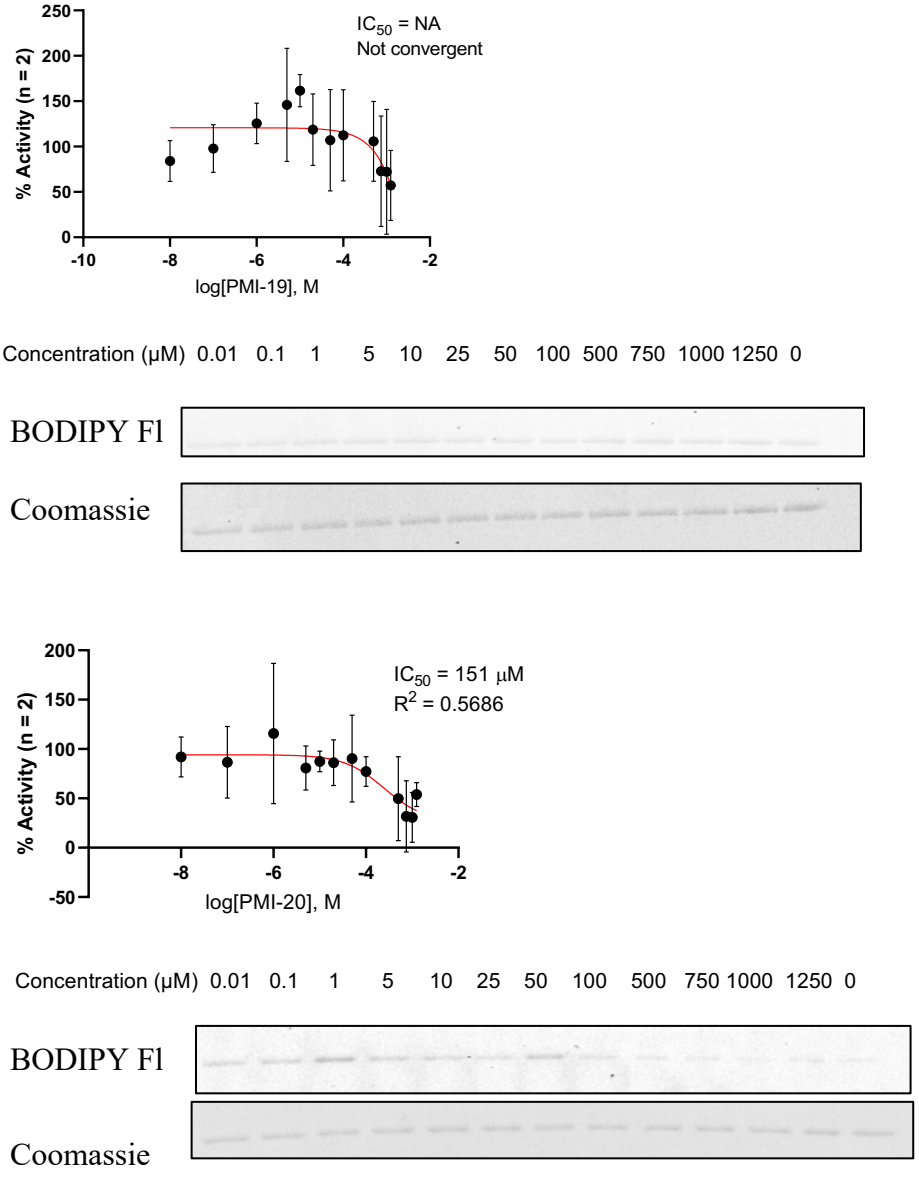


Figure S3: Dose-response curves of the inhibition of AirS activity with **PMI-1** to **PMI-20** using B-FL-ATPγS inhibition assay.

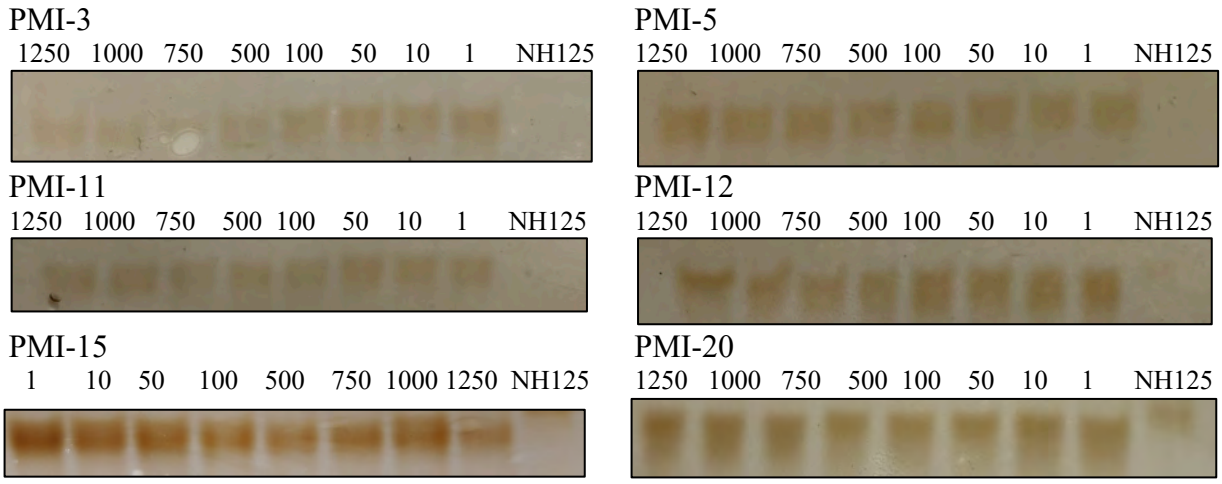


Figure S4. HK853 aggregation analysis by Native PAGE and silver staining at various concentrations of the lead PMI compounds (1 to 1250 μM). NH125 (1250 μM) was used as a positive control. Only analogs that were carried forward into advanced testing were evaluated in this assay.

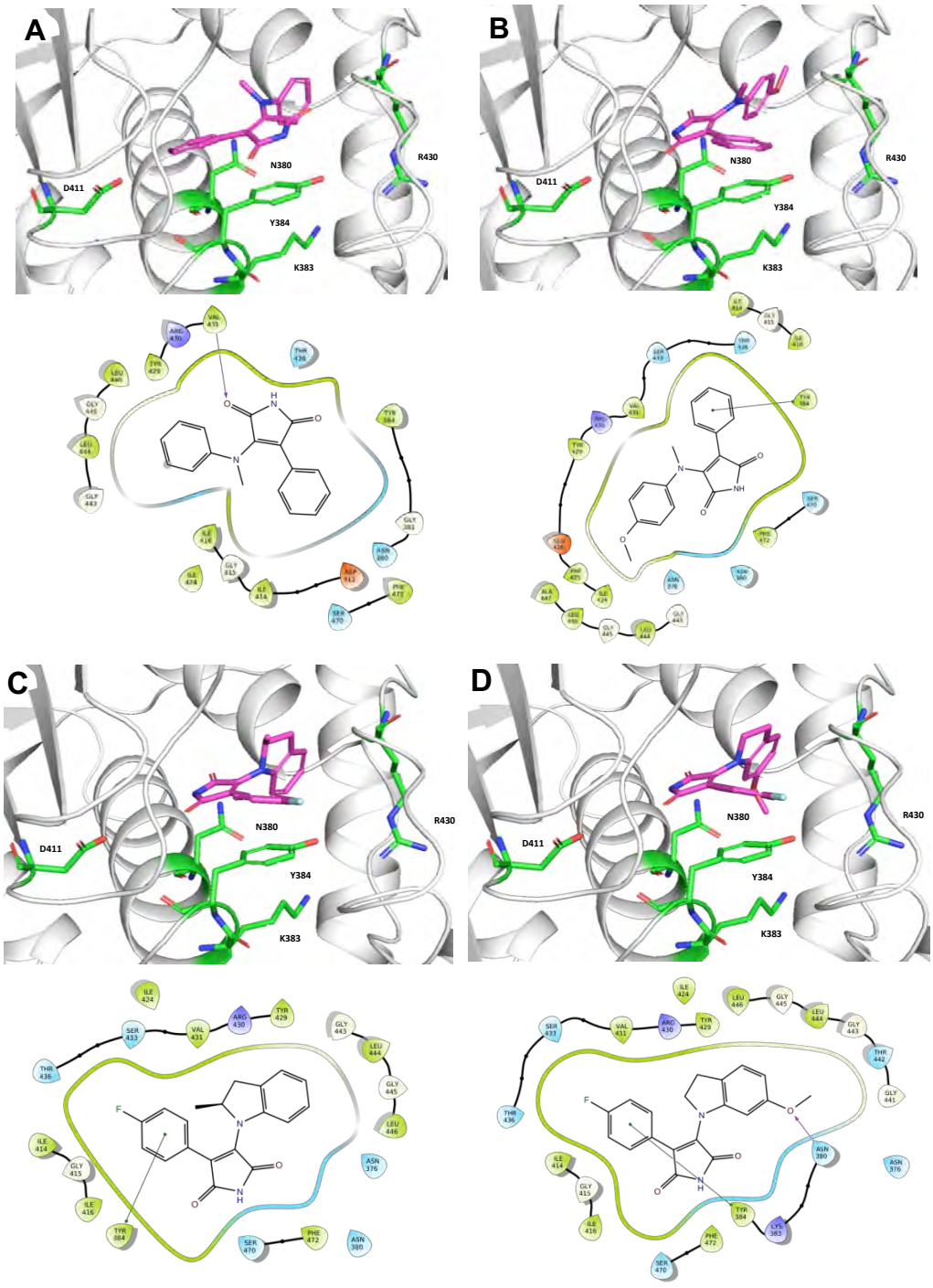


Figure S5: Docking and 2D-diagram interactions of the lead PMI compounds in the ATP-binding pocket of the catalytic domain of HK853. A. PMI-11, B. PMI-12, C. PMI-15, and D. PMI-20. In the docking figures, the critical residues known to interact with ATP are shown as sticks in green/red/blue (D411, N380, Y384, K383, and R430) and the maleimide structures are in magenta/blue/red. Magenta corresponds to carbon, blue to nitrogen, and red to oxygen. Figures generated with Schrödinger Maestro.

Table S2: Docking scores, glide gscores, and glide emodels of the lead compounds and a non-binder PMI-18 (docking interactions in the ATP-binding pocket of HK853 and 2D diagram).

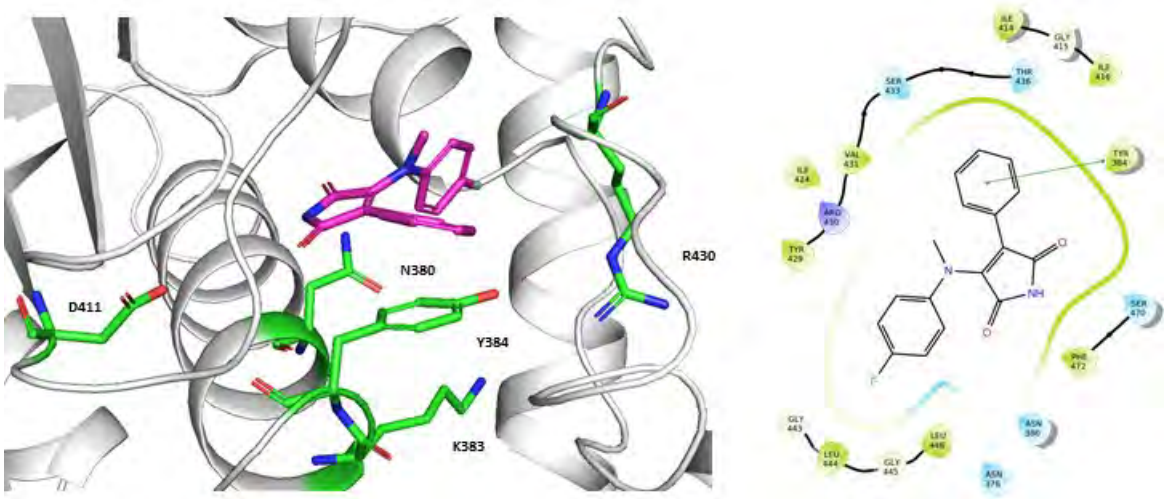
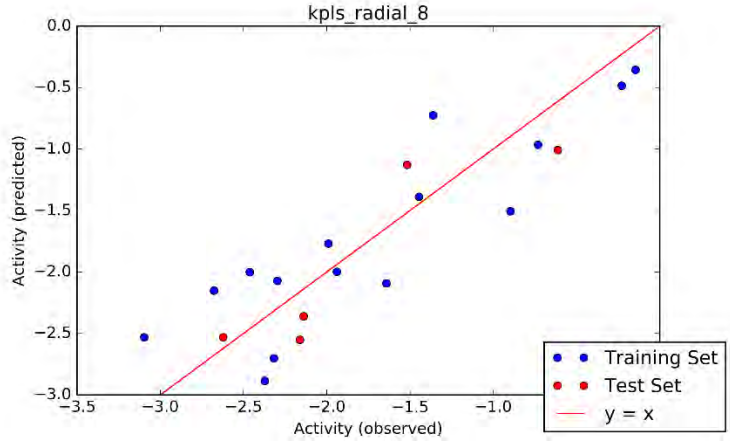
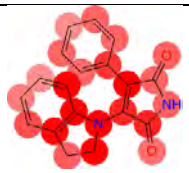
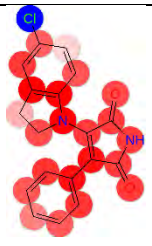
Compound	Docking score	Glide gscore	Glide emodel
PMI-3	-9.030	-9.034	-69.945
PMI-5	-7.924	-7.928	-50.600
PMI-11	-8.129	-8.134	-57.673
PMI-12	-8.322	-8.325	-66.450
PMI-15	-8.100	-8.104	-44.792
PMI-20	-8.902	-8.906	-68.093
PMI-18	-7.905	-7.909	-64.990
PMI-18 			

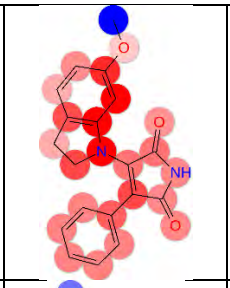
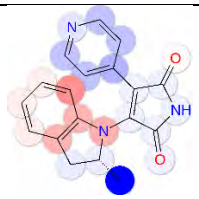
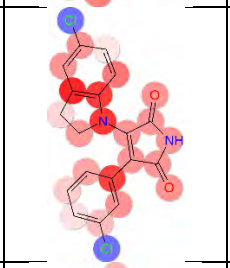
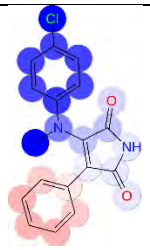
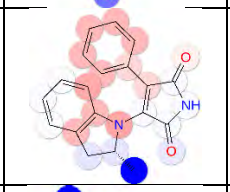
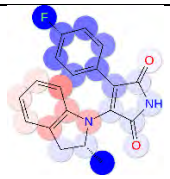
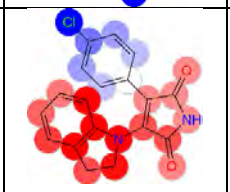
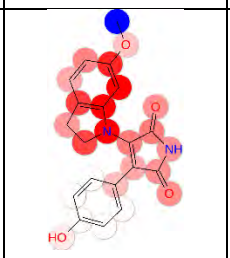
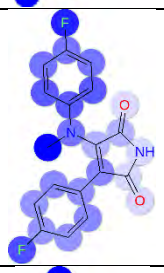
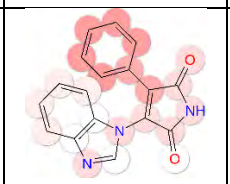
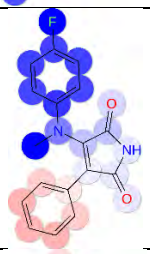
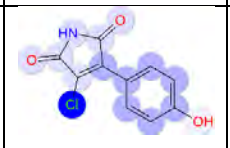
Table S3. AutoQSAR model built with all 20 PMI compounds. Compounds PMI-4, PMI-6, PMI-14, PMI-15, and PMI-19 were used as test compounds and the other molecules as the training set. Red indicates that the atom positively participates in the activity of the molecule whereas blue means that the atom negatively affects its activity. The color saturation represents the magnitude of the contribution.

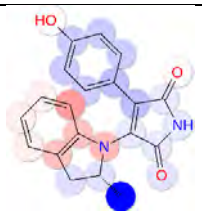
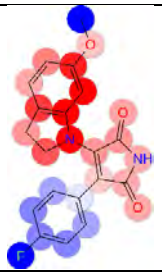
Report for Numeric Model kpls_radial_8
Ranking score = 0.788991

Training Set		Test Set	
S.D.	R^2	RMSE	Q^2
0.4361	0.7784	0.3217	0.7854



PMI	Structure	Predicted	Observed	PMI	Structure	Predicted	Observed
1		-0.353	-0.146	11		-1.996	-1.940
2		-0.486	-0.230	12		-2.093	-1.643

3		-0.725	-1.362	13		-2.000	-2.464
4		-1.129	-1.519	14		-2.530	-2.622
5		-1.767	-1.991	15		-2.360	-2.140
6		-1.008	-0.613	16		-2.702	-2.318
7		-0.964	-0.732	17		-2.885	-2.375
8		-1.506	-0.898	18		-2.530	-3.097
9		-2.150	-2.679	19		-2.351	-2.161

10		-2.071	-2.297	20		-1.388	-1.447

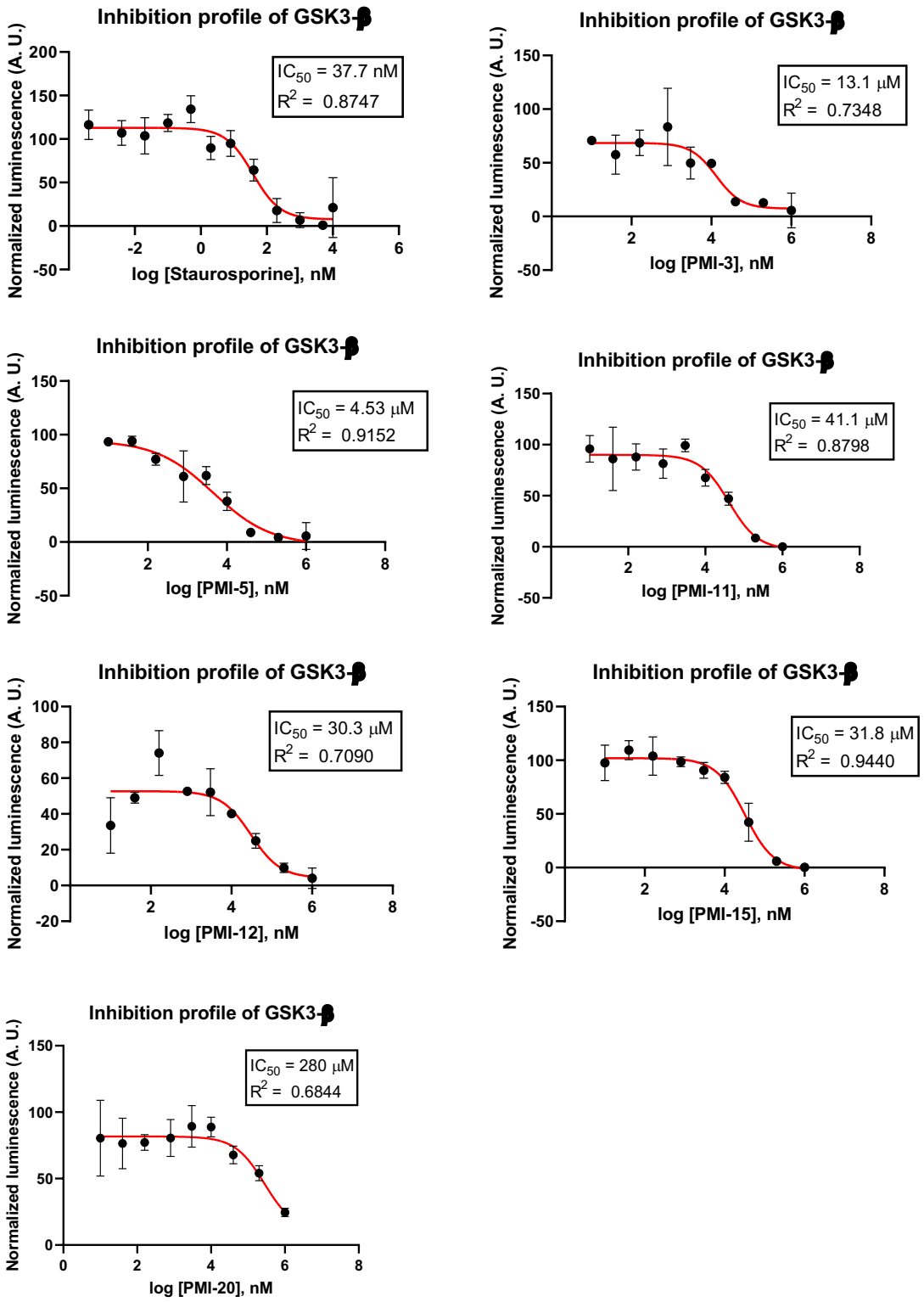


Figure S6: Inhibition profile of GSK-3β by PMI-3, PMI-5, PMI-11, PMI-12, PMI-15, PMI-20, and staurosporine, a known kinase inhibitor, using the ADP-Glo™ assay. Each reaction contains GSK3β (1 ng), ATP (25 μM), the kinase substrate (0.2 μg/μL), and the test compounds (10 nM to

1 mM) or the inhibitor dilution solution (DMSO 25%/kinase buffer 75%). A positive control without any inhibitor and a sample containing only the pre-mixed ATP/substrate was used to determine respectively the maximum luminescence signal and the background luminescence signal.[3]

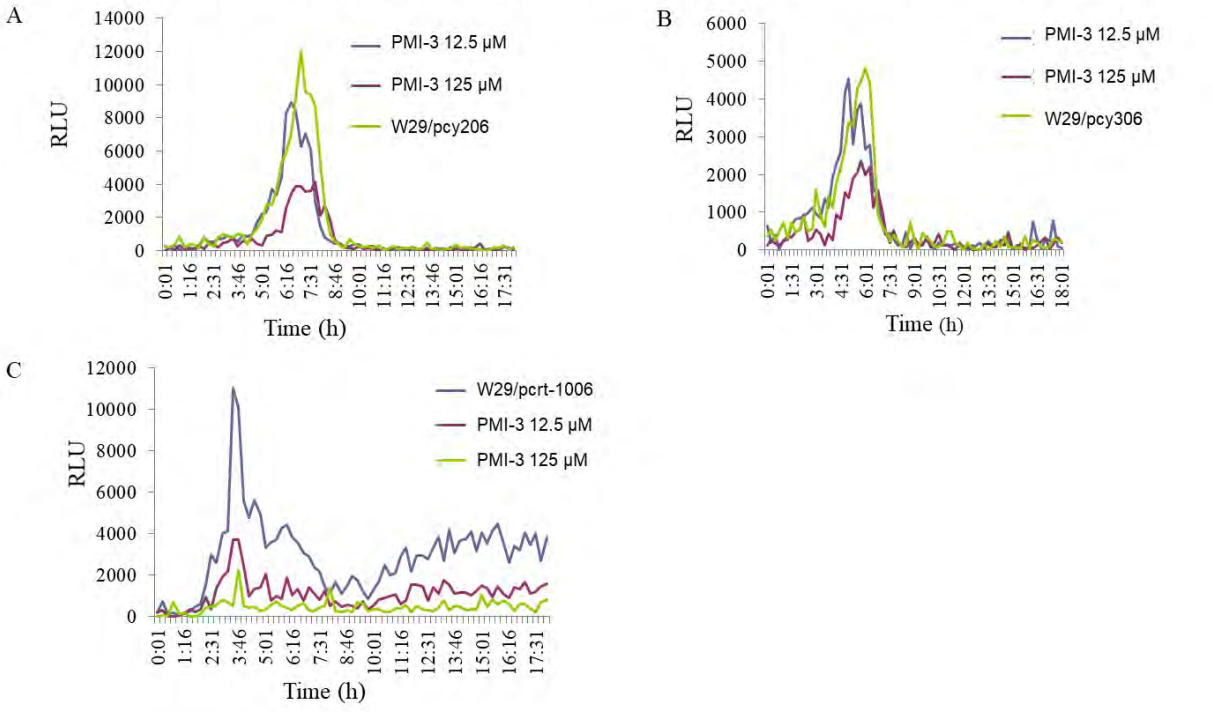
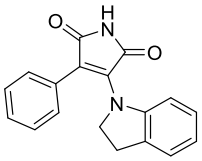
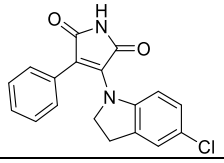
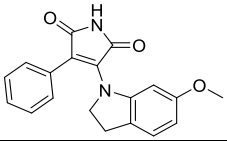
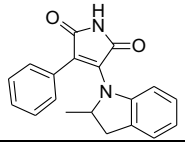


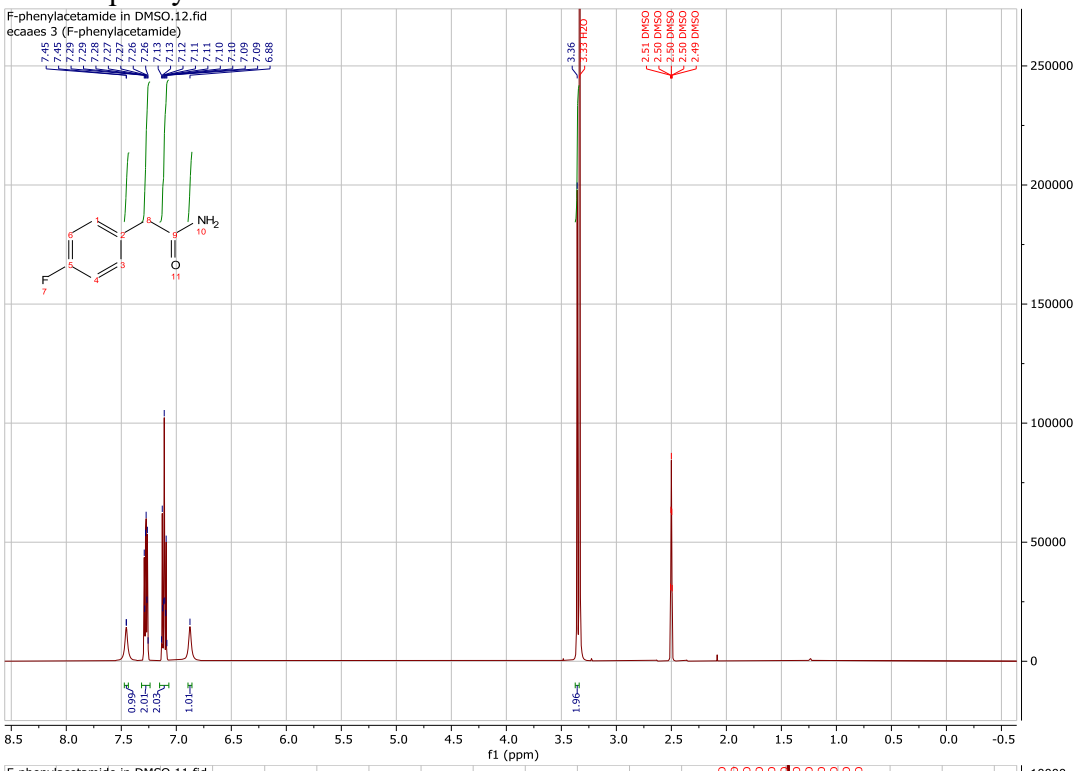
Figure S7: Effect of **PMI-3** on the transcription of *walk/yycG* (A), *saeS* (B), and *agr* (C).

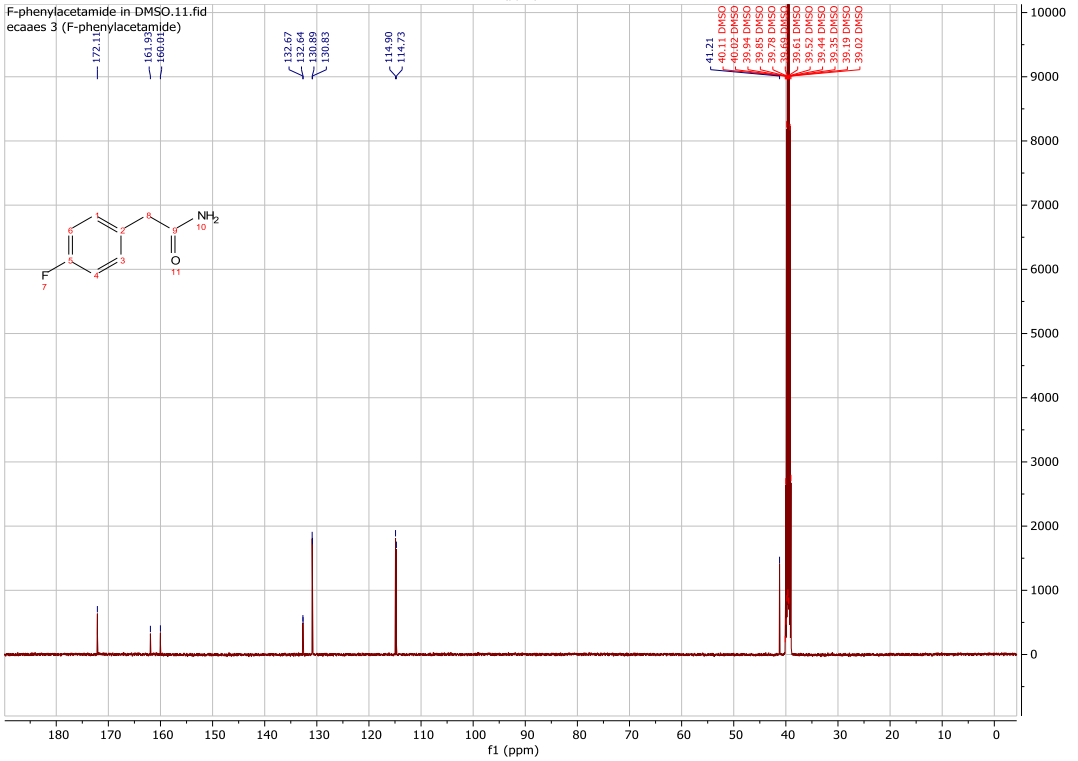
Table S4. Summary of data for lead inhibitors. *Indicates that complete inhibition was not achieved at highest tested concentration. These values are presented as a lower bound, with the IC_{50} value being > than the number indicated in GraphPad Prism. Hemolytic activity of the PMI compounds evaluated at 500 μM in sheep red blood cells. Cell viability evaluated with A549 cells exposed to compounds (250 μM). Hemolysis activity of *S. aureus* evaluated in WCUH29 and CA-MRSA 923 pre-exposed to inhibitors (50 μM) and the supernatants used for cytotoxicity assays in sheep red blood cells. DMSO-treated WCUH29 was positive control, PBS-only was negative control. % Hemolysis = [(A450 test sample − A450 negative control) /(A450 positive control − A450 negative control)] × 100. Values of lysed cells obtained from three independent experiments. Bacterial burden in infected skin compares WCUH29-infected group with vehicle control treatment to WCUH29-infected group with **PMI-5** treatment. ND: Not determined.

Compound		**IC_{50} values (μM) (95% confidence interval) for HK853 and AirS**	**% Hemolytic activity (molecule alone)**	**% A549 cell viability (molecule alone)**	**% Decrease in hemolysis by *S. aureus* WCUH29**	**% Decrease in hemolysis by *S. aureus* CA-MRSA 923**	**Effect on lesion formation caused by WCUH29**
PMI-1		1.4 (1.1-1.7) > 7.6 (1.3-ambiguous)*	3.37	>99	66.1	72.3	ND
PMI-2		1.7 (0.88-3.1) > 1000*	1.53	2.6	86.9	91.6	ND
PMI-3		23 (14-38) > 1000*	0.79	>99	74.2	12.7	ND
PMI-5		> 98 (34-280)* 153 (27-ambiguous)*	2.42	>99	69.5	85.6	65% lesion size reduction

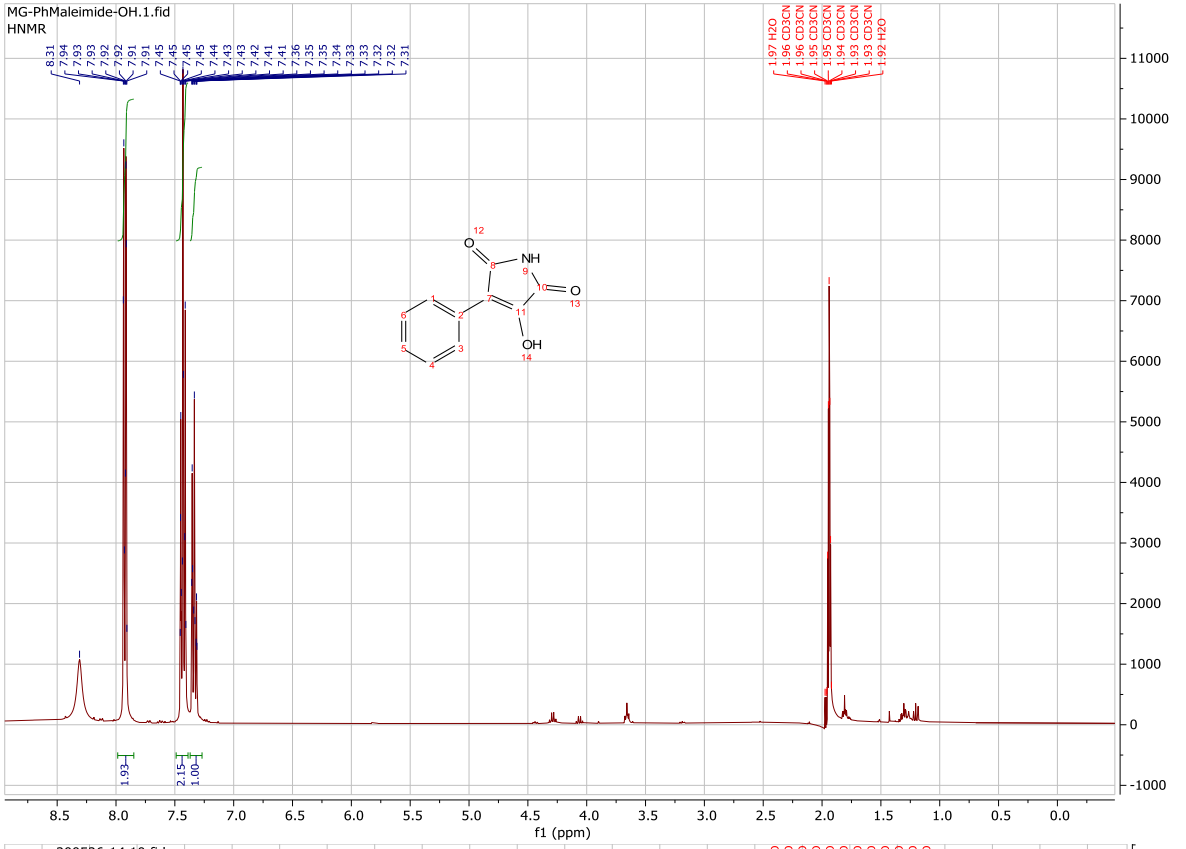
^{1}H and ^{13}C spectra of compounds PMI-1 to PMI-20 and key intermediates

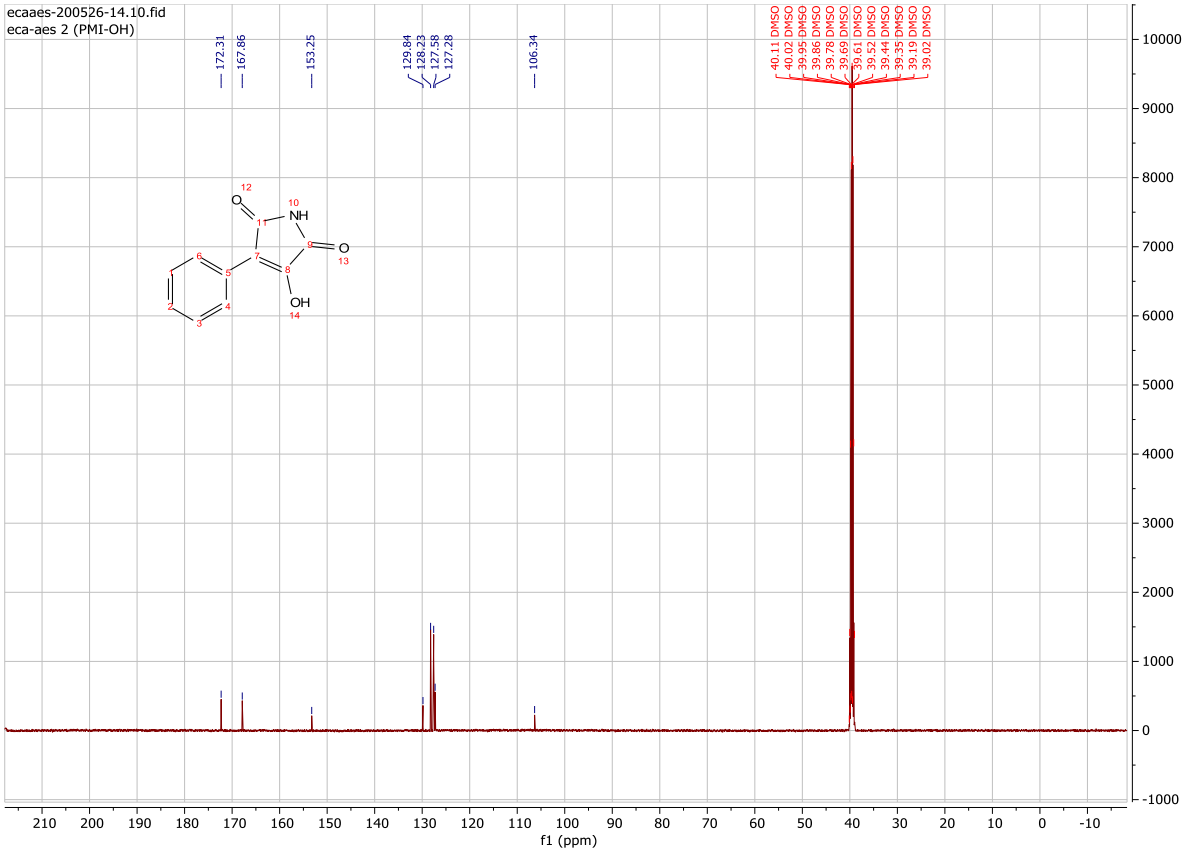
4-fluorophenylacetamide



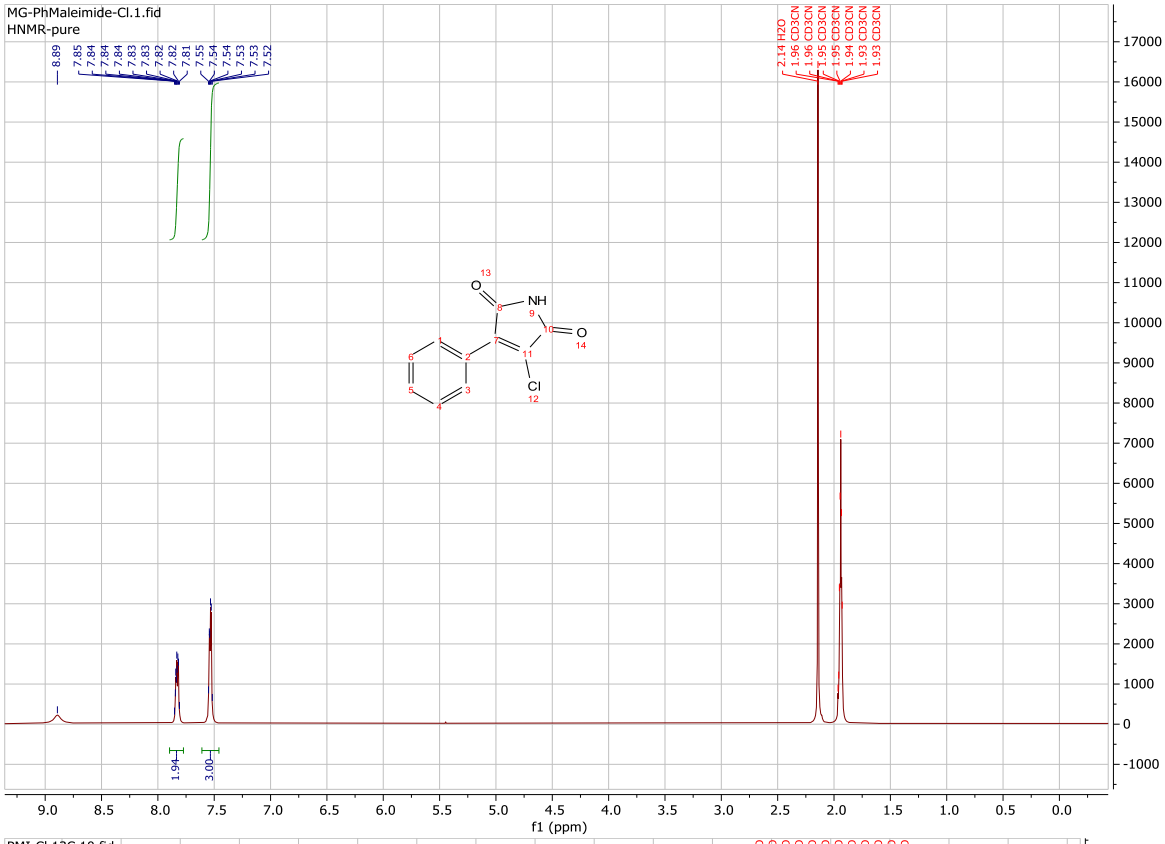


PMI-OH


MG-PhMaleimide-OH.1.fid
HNMR
f1 (ppm)


ecaaes-200526-14.10.fid
eca-aes 2 (PMI-OH)
f1 (ppm)

PMI-Cl


MG-PhMaleimide-Cl.1.fid
HNMR-pure
8.89
7.85
7.84
7.84
7.84
7.83
7.83
7.82
7.82
7.81
7.55
7.54
7.54
7.53
7.53
7.52
2.14 H2O
1.96 CD3CN
1.96 CD3CN
1.96 CD3CN
1.95 CD3CN
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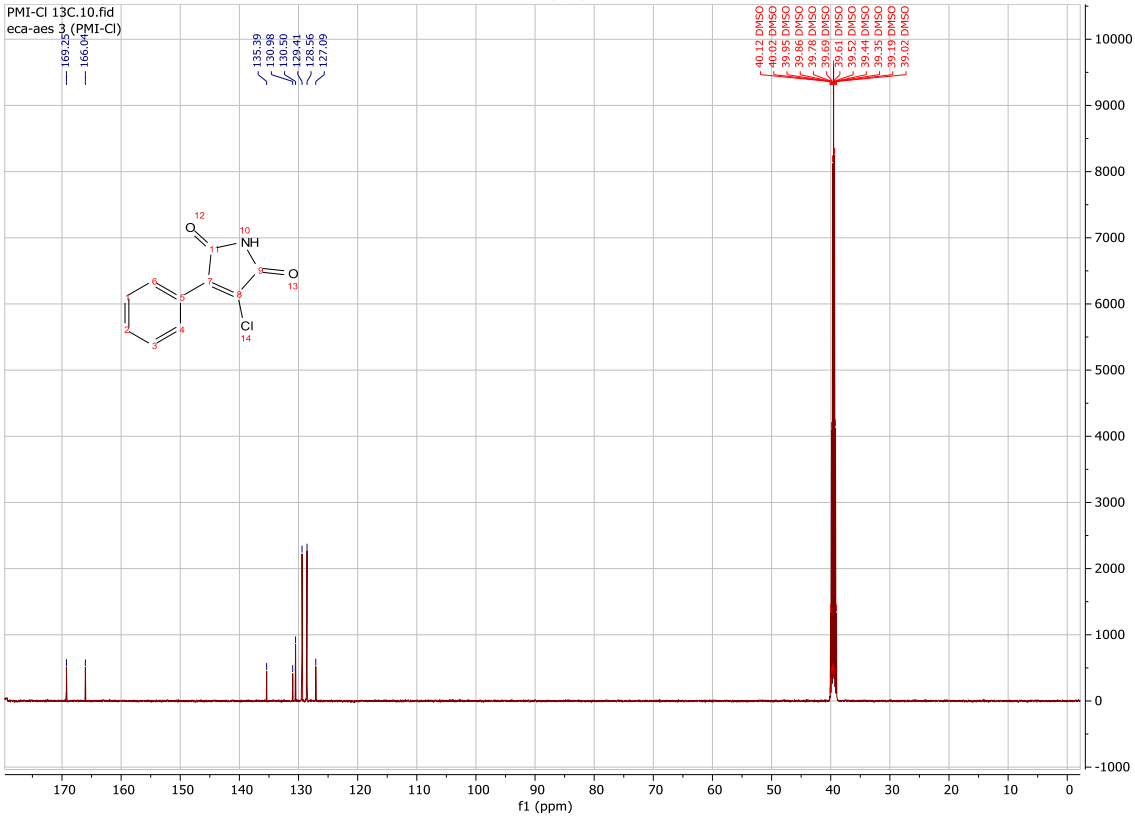
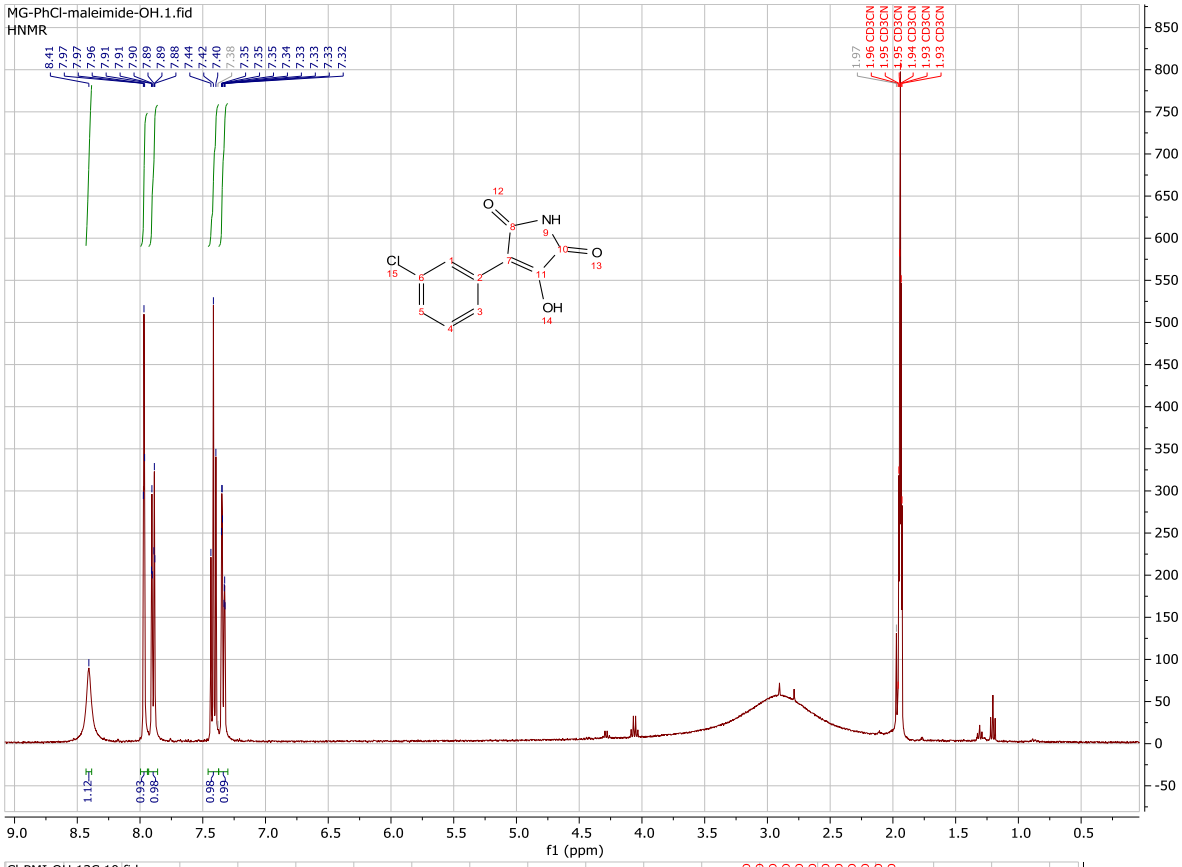
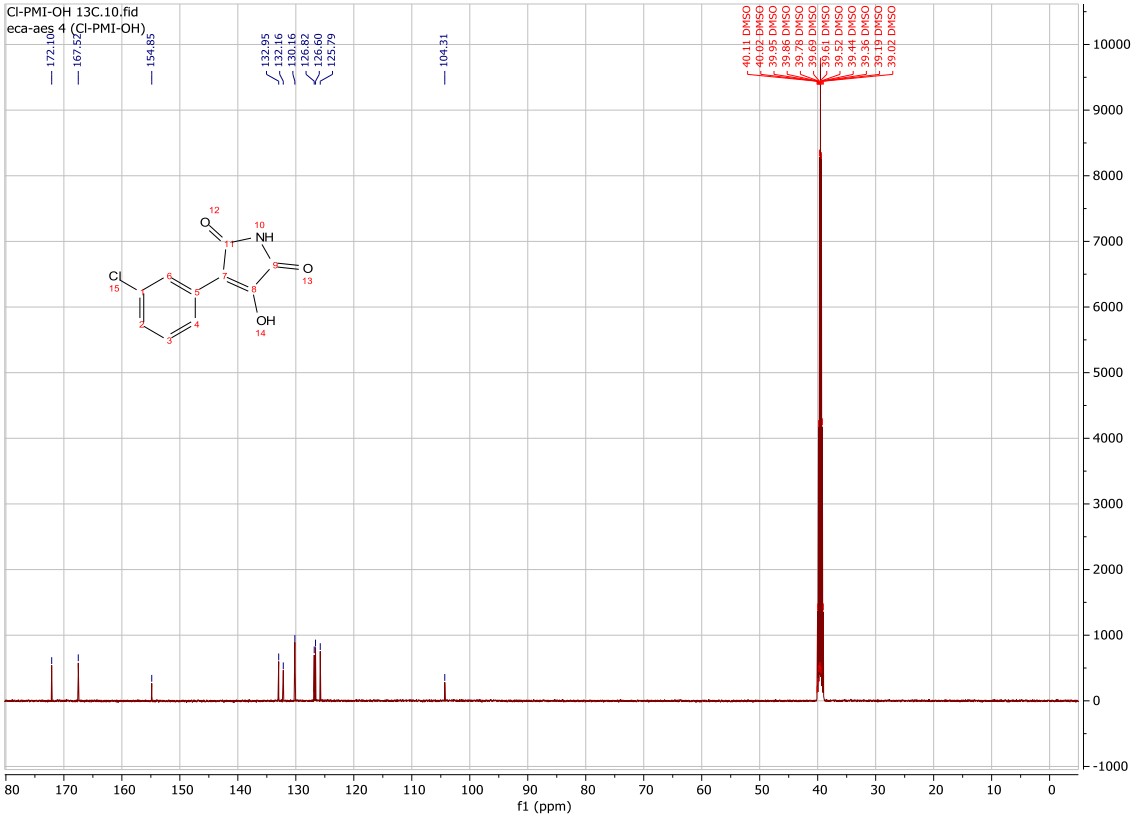
1.93 CD3CN
1.94
3.00
f1 (ppm)

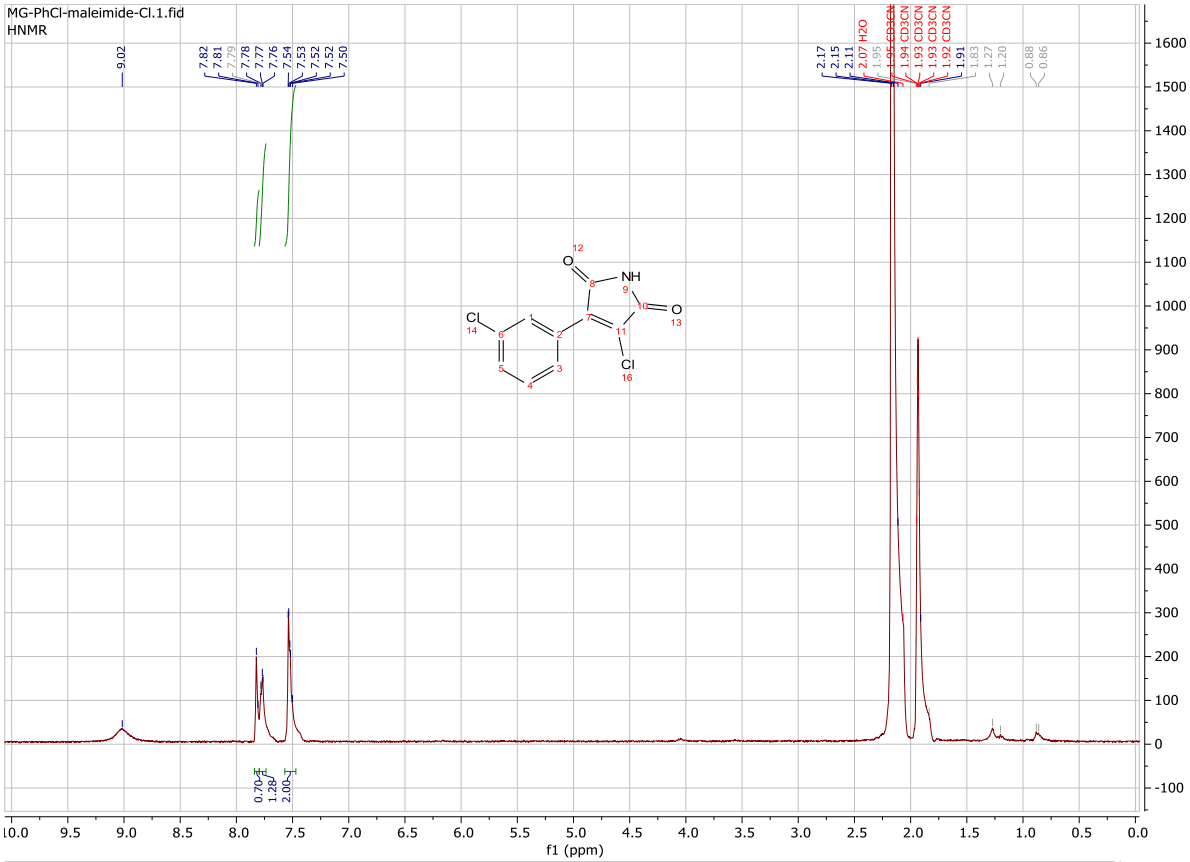

PMI-Cl 13C.10.fid
eca-aes 3 (PMI-Cl)
169.25
166.04
135.39
130.98
130.50
129.41
128.56
127.09
40.12 DMSO
40.02 DMSO
39.95 DMSO
39.86 DMSO
39.78 DMSO
39.69 DMSO
39.61 DMSO
39.52 DMSO
39.44 DMSO
39.35 DMSO
39.19 DMSO
39.02 DMSO
f1 (ppm)

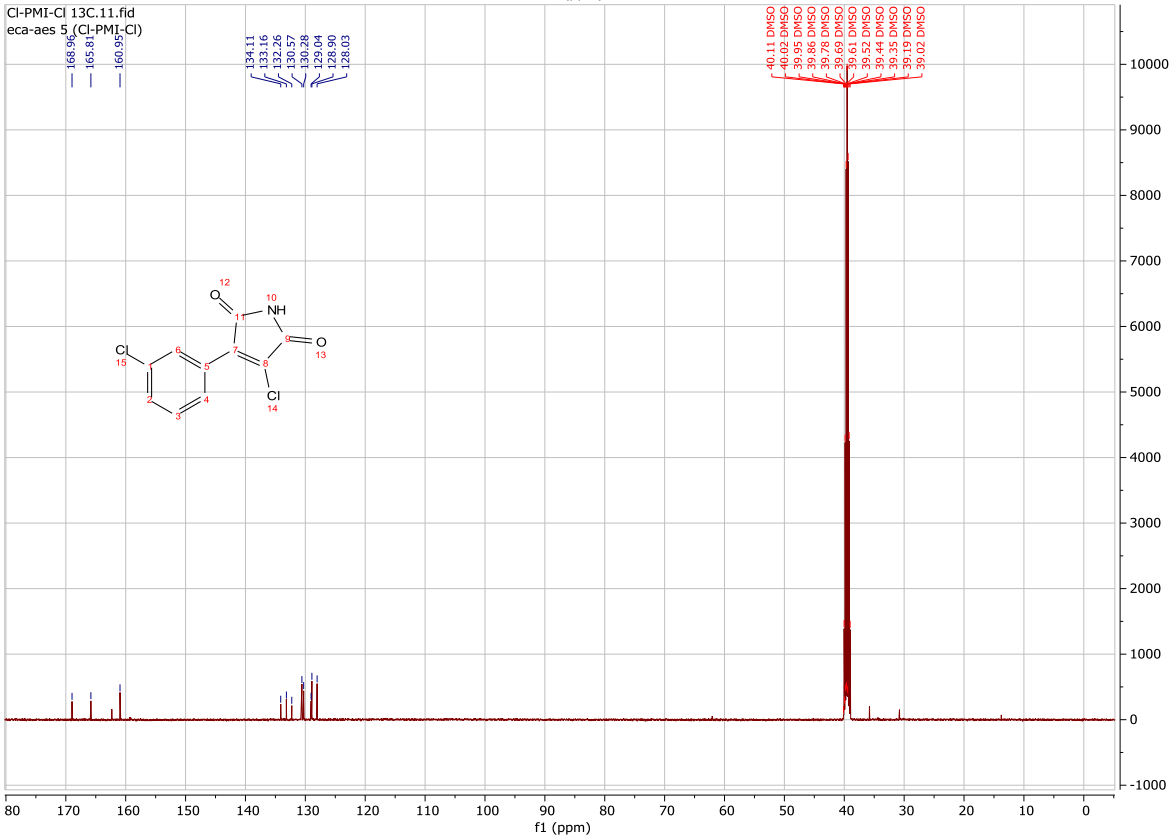
Cl-PMI-OH


MG-PhCl-maleimide-OH.1.fid
HNMR
f1 (ppm)

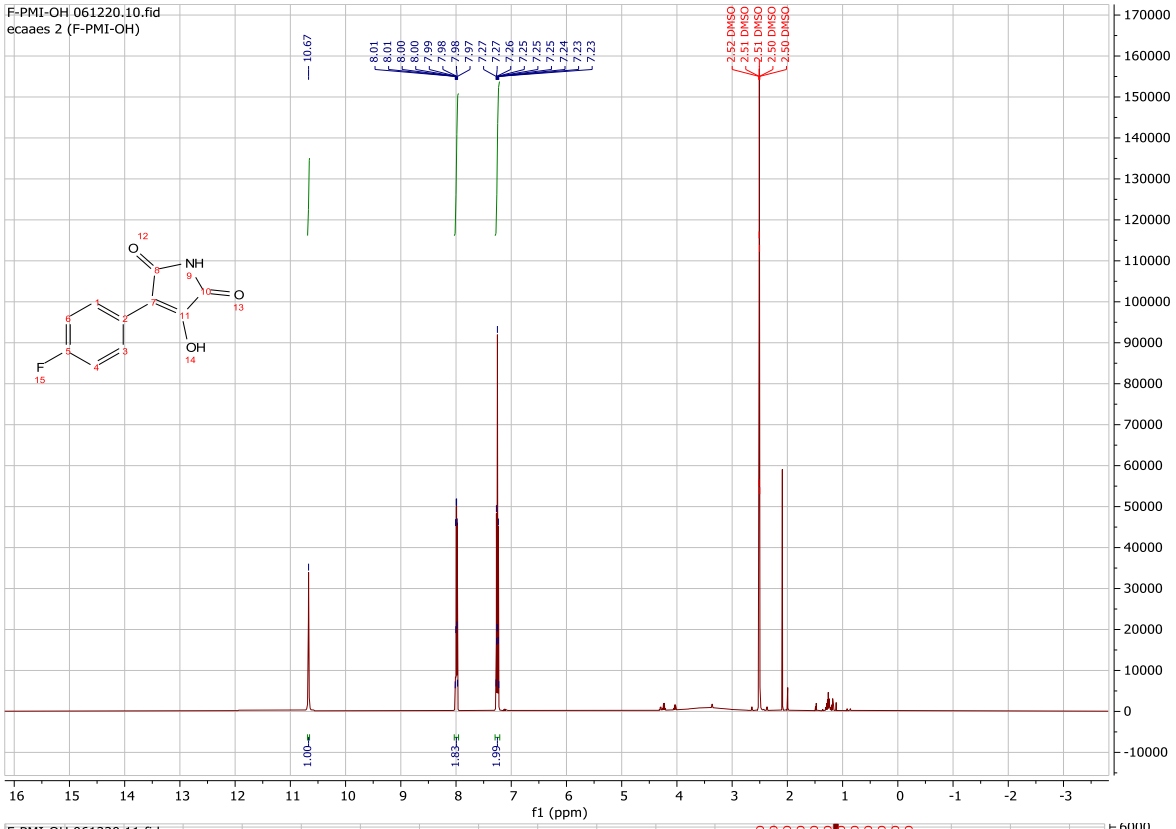

Cl-PMI-OH 13C.10.fid
eca-aes 4 (Cl-PMI-OH)
f1 (ppm)

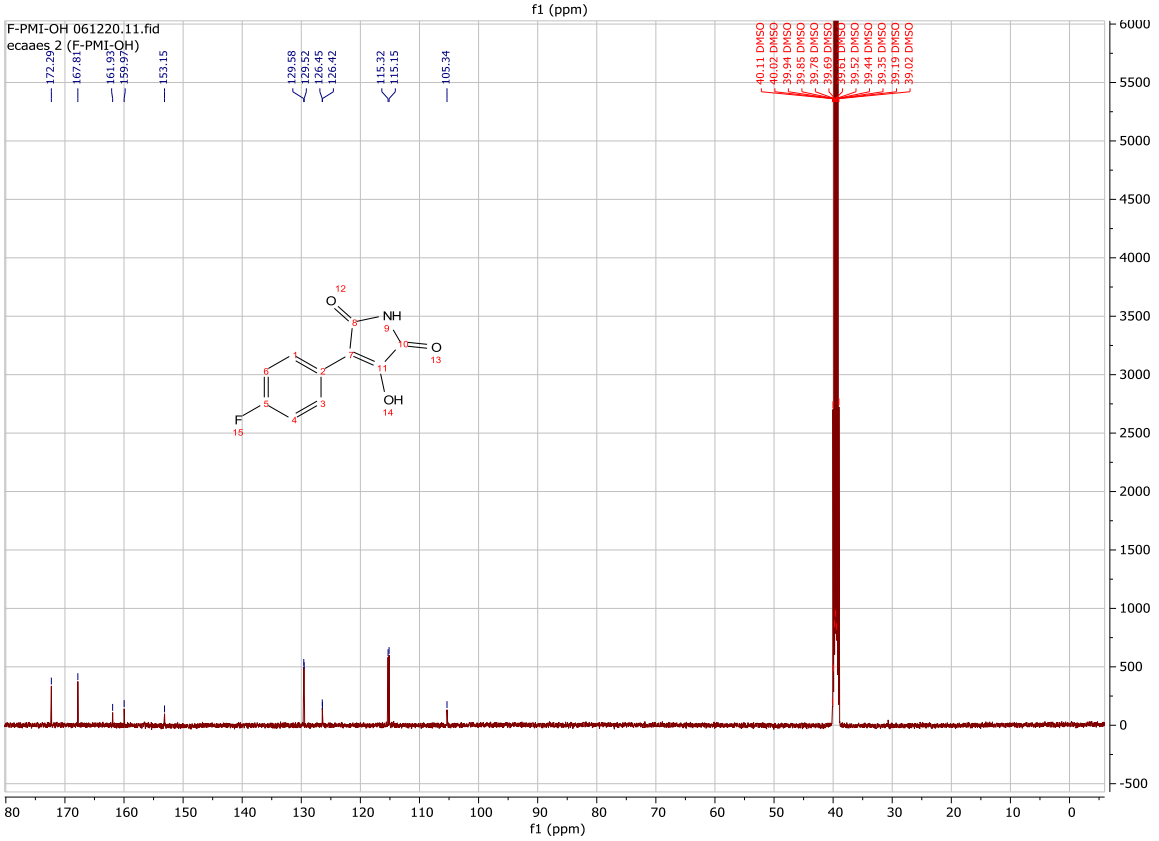
Cl-PMI-Cl


MG-PhCl-maleimide-Cl.1.fid
HNMR
f1 (ppm)

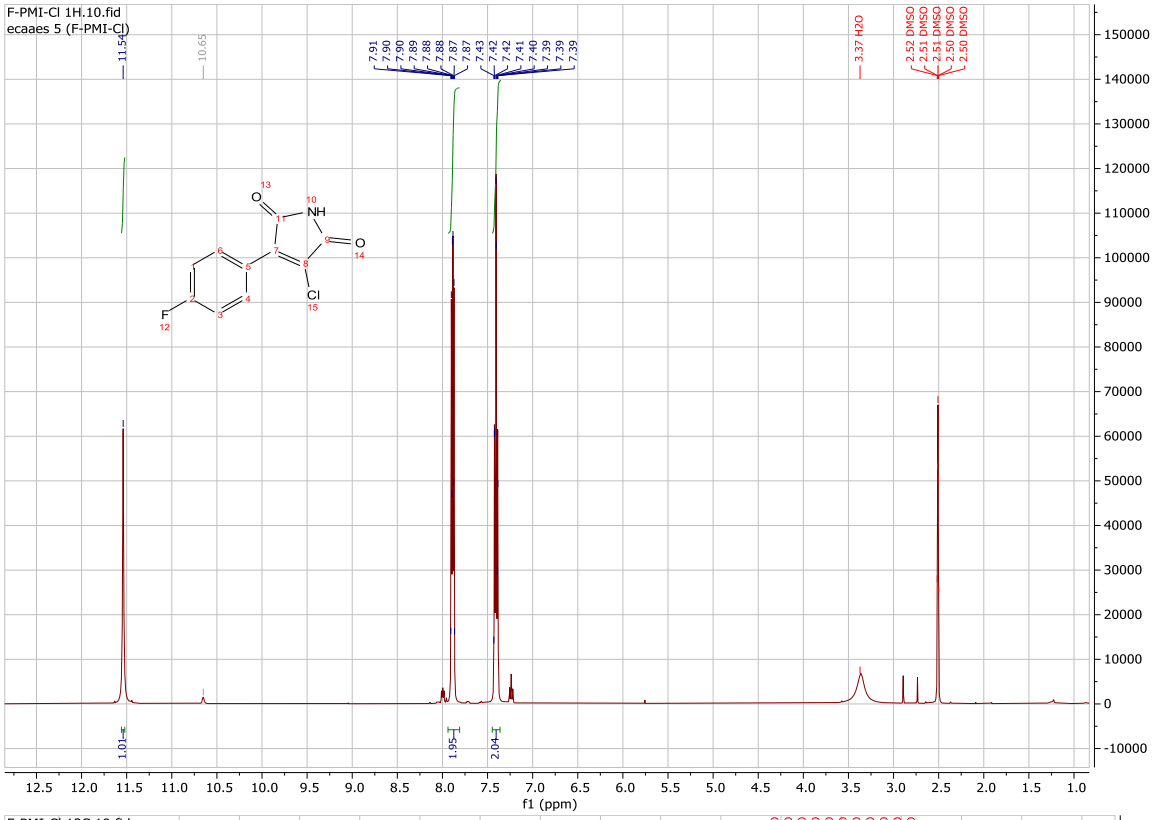

Cl-PMI-Cl 13C.11.fid
eca-aes 5 (Cl-PMI-Cl)
f1 (ppm)

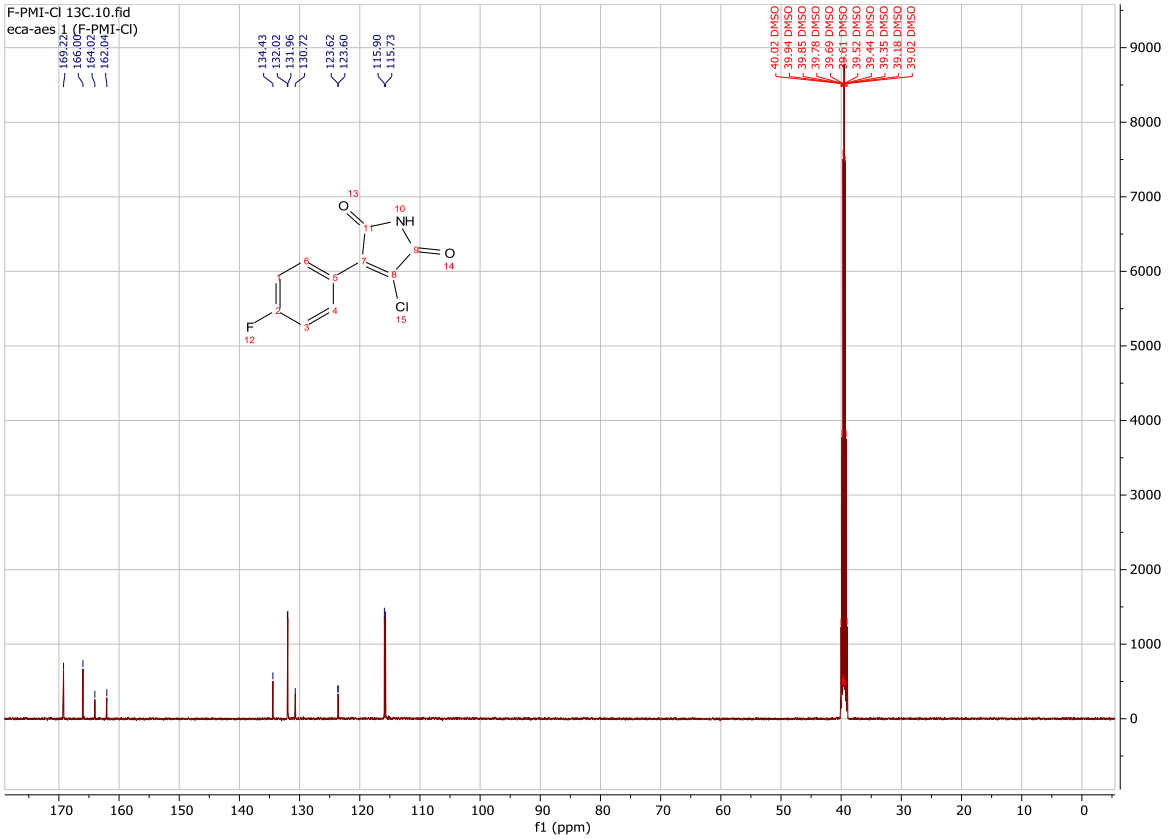
F-PMI-OH


F-PMI-OH 061220.10.fid
ecaaes 2 (F-PMI-OH)
10.67
8.01
8.01
8.00
8.00
7.99
7.98
7.98
7.97
7.27
7.27
7.26
7.25
7.25
7.25
7.24
7.23
7.23
2.52 DMSO
2.51 DMSO
2.51 DMSO
2.50 DMSO
2.50 DMSO
1.00
1.83
1.99
f1 (ppm)

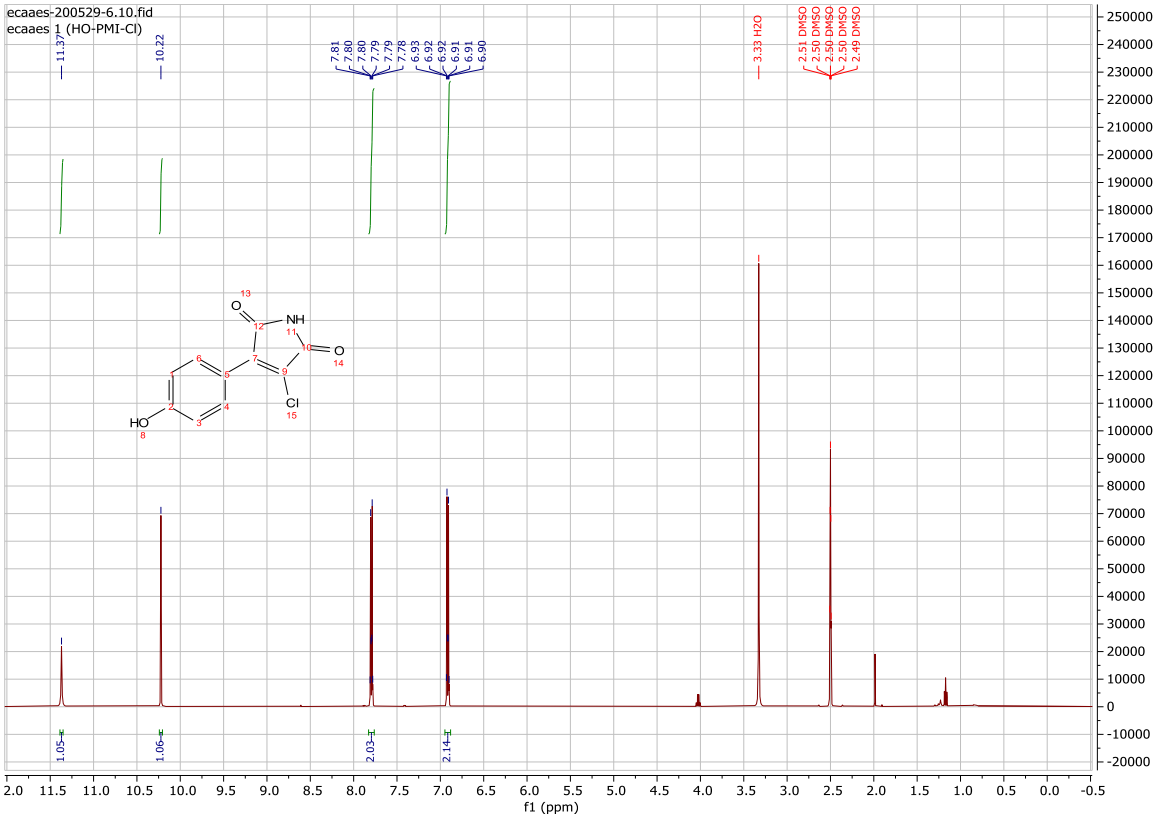

F-PMI-OH 061220.11.fid
ecaaes 2 (F-PMI-OH)
172.29
167.81
161.93
159.97
153.15
129.58
129.52
126.45
126.42
115.32
115.15
105.34
40.11 DMSO
40.02 DMSO
39.94 DMSO
39.85 DMSO
39.78 DMSO
39.69 DMSO
39.61 DMSO
39.52 DMSO
39.44 DMSO
39.35 DMSO
39.19 DMSO
39.02 DMSO
f1 (ppm)

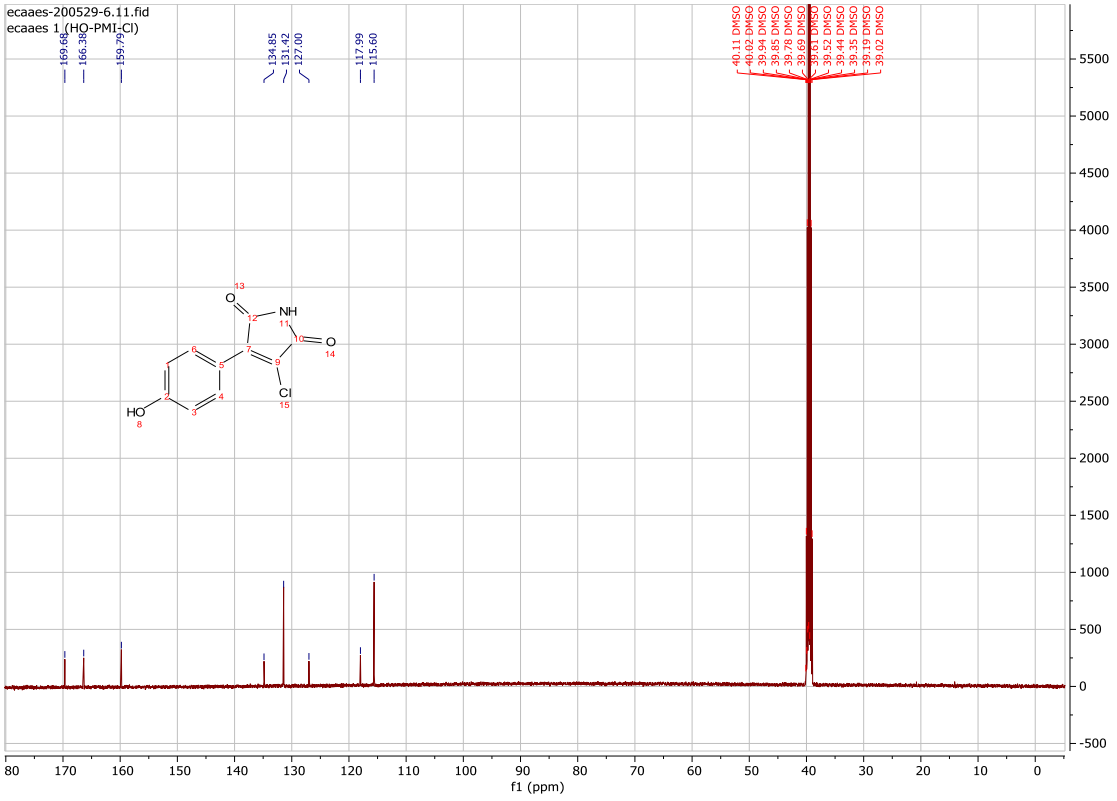
F-PMI-Cl


F-PMI-Cl 1H.10.fid
ecaaes 5 (F-PMI-Cl)
11.54
10.65
7.91
7.90
7.90
7.89
7.88
7.88
7.87
7.87
7.43
7.42
7.42
7.41
7.40
7.39
7.39
7.39
3.37 H2O
2.52 DMSO
2.51 DMSO
2.51 DMSO
2.50 DMSO
2.50 DMSO
1.01
1.95
2.04
f1 (ppm)

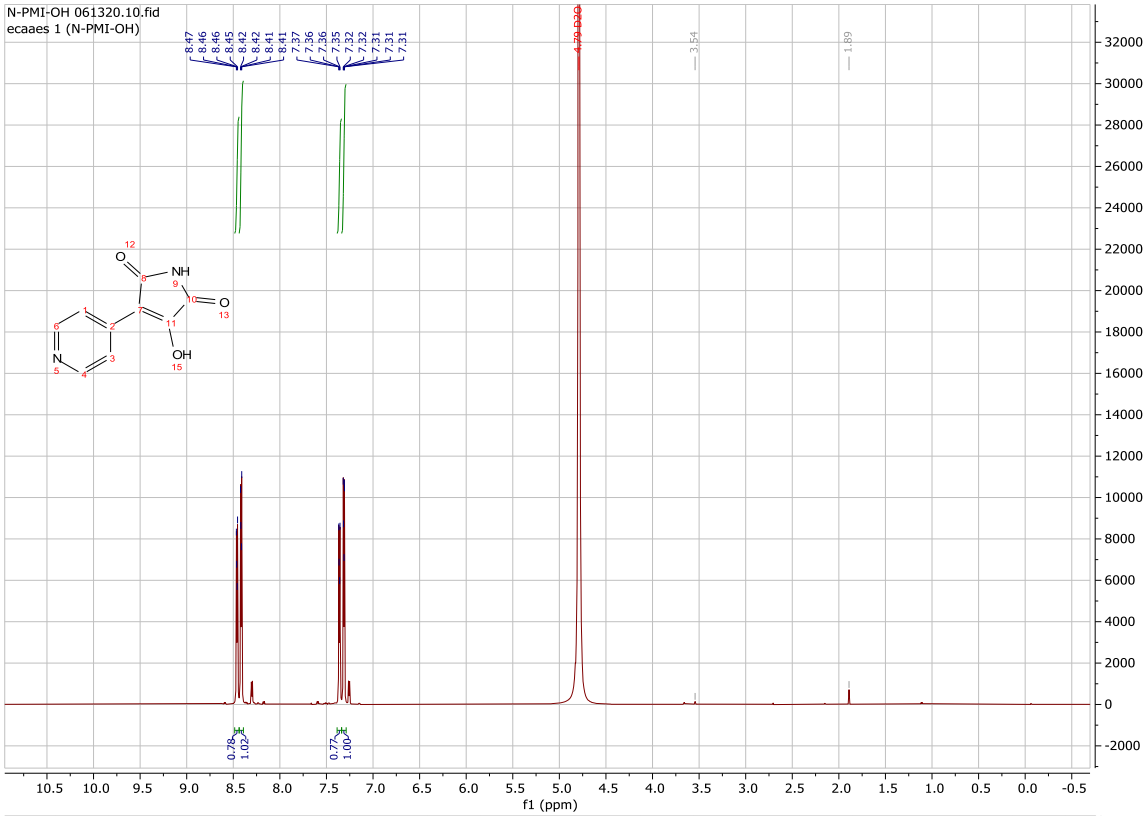

F-PMI-Cl 13C.10.fid
eca-aes 1 (F-PMI-Cl)
169.22
166.00
164.02
162.04
134.43
132.02
131.96
130.72
123.62
123.60
115.90
115.73
40.02 DMSO
39.94 DMSO
39.85 DMSO
39.78 DMSO
39.69 DMSO
39.61 DMSO
39.52 DMSO
39.44 DMSO
39.35 DMSO
39.18 DMSO
39.02 DMSO
f1 (ppm)

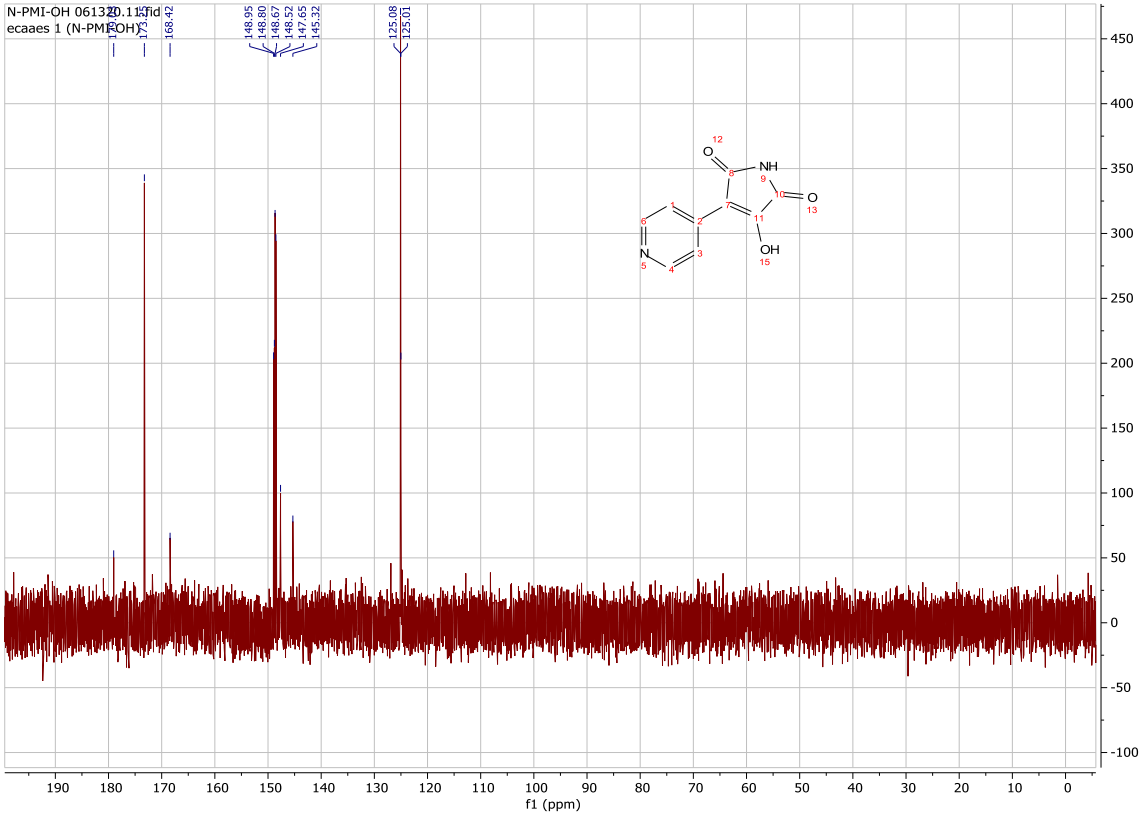
PMI-9 or HO-PMI-Cl



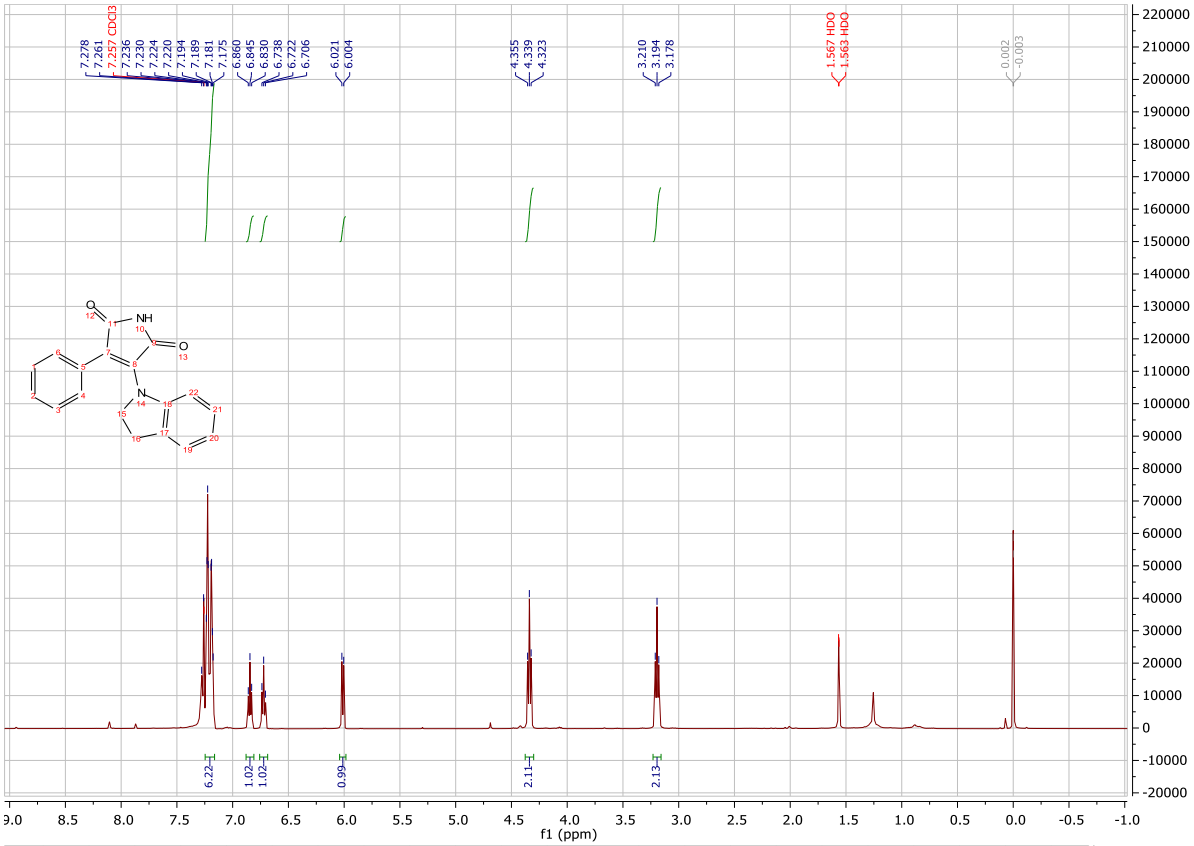


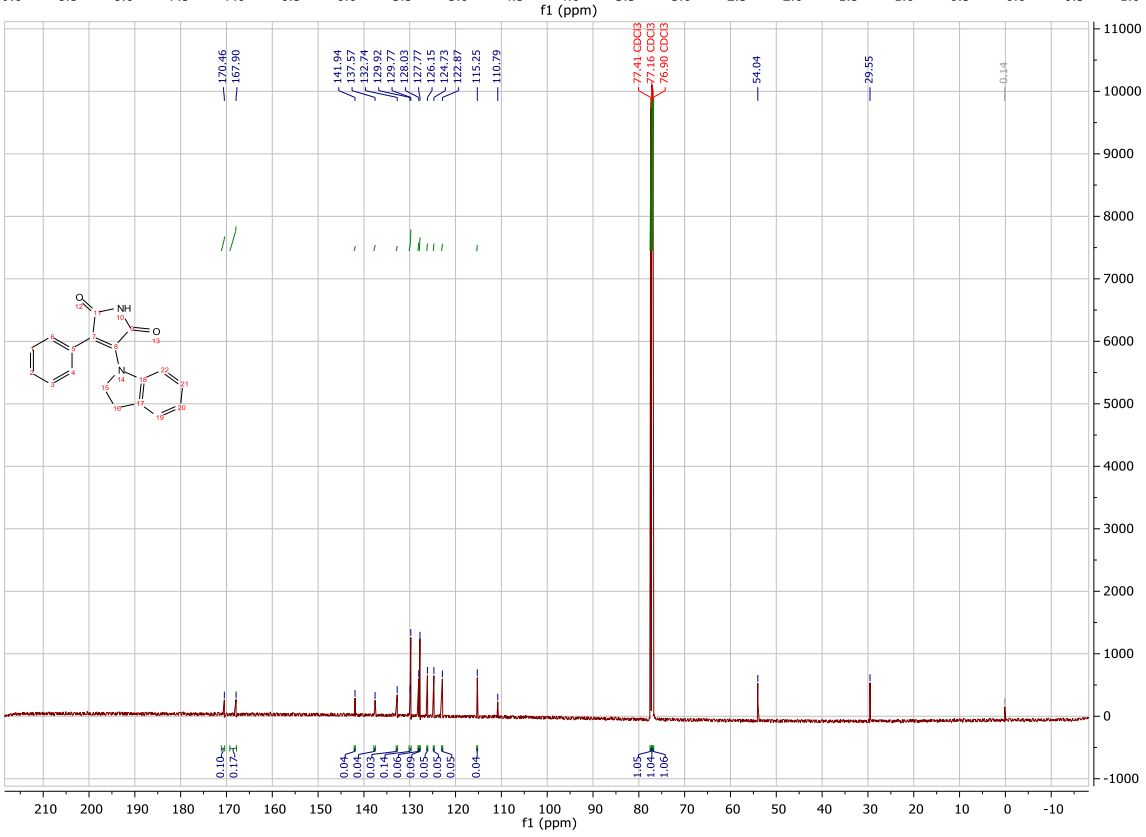
N-PMI-OH


N-PMI-OH 061320.10.fid
ecaaes 1 (N-PMI-OH)
8.47
8.46
8.46
8.45
8.42
8.42
8.41
8.41
7.37
7.36
7.36
7.35
7.32
7.32
7.31
7.31
7.31
7.31
4.29
3.54
1.89
0.78
1.02
0.77
1.00
f1 (ppm)

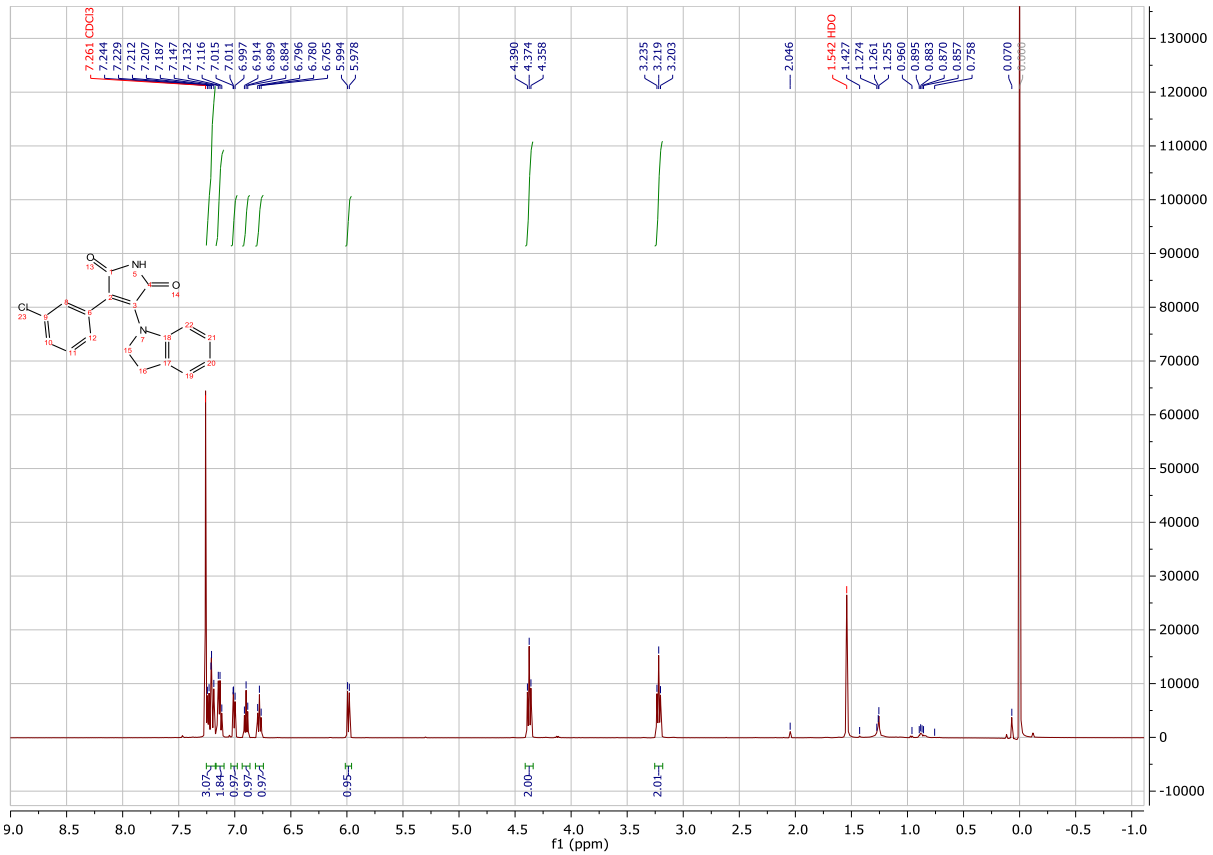

N-PMI-OH 061320.11.fid
ecaaes 1 (N-PMI-OH)
168.42
148.95
148.80
148.67
148.52
147.65
145.32
125.08
125.01
f1 (ppm)

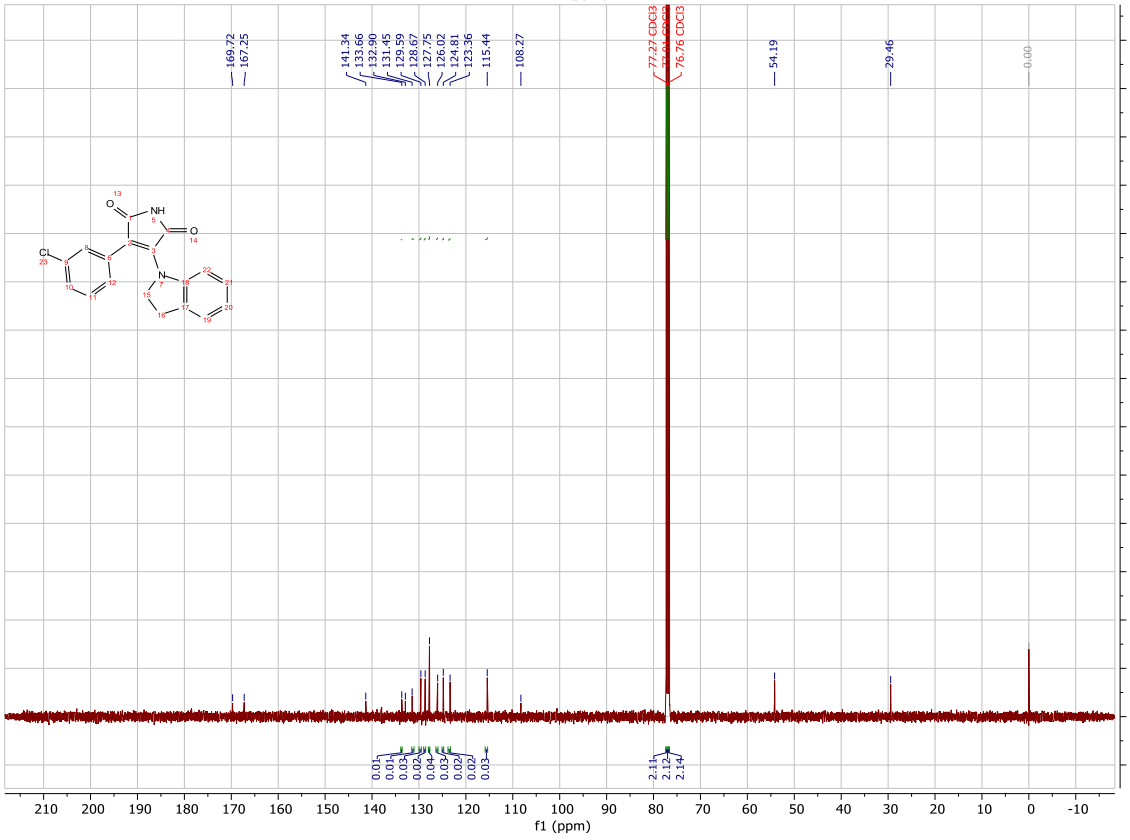
PMI-1


7.278
7.261
7.257 CDCl3
7.236
7.230
7.224
7.220
7.194
7.189
7.181
7.175
6.860
6.845
6.830
6.738
6.722
6.706
6.021
6.004
4.355
4.339
4.323
3.210
3.194
3.178
1.567 HDO
1.563 HDO
0.002
-0.003
6.22
1.02
1.02
0.99
2.11
2.13
f1 (ppm)

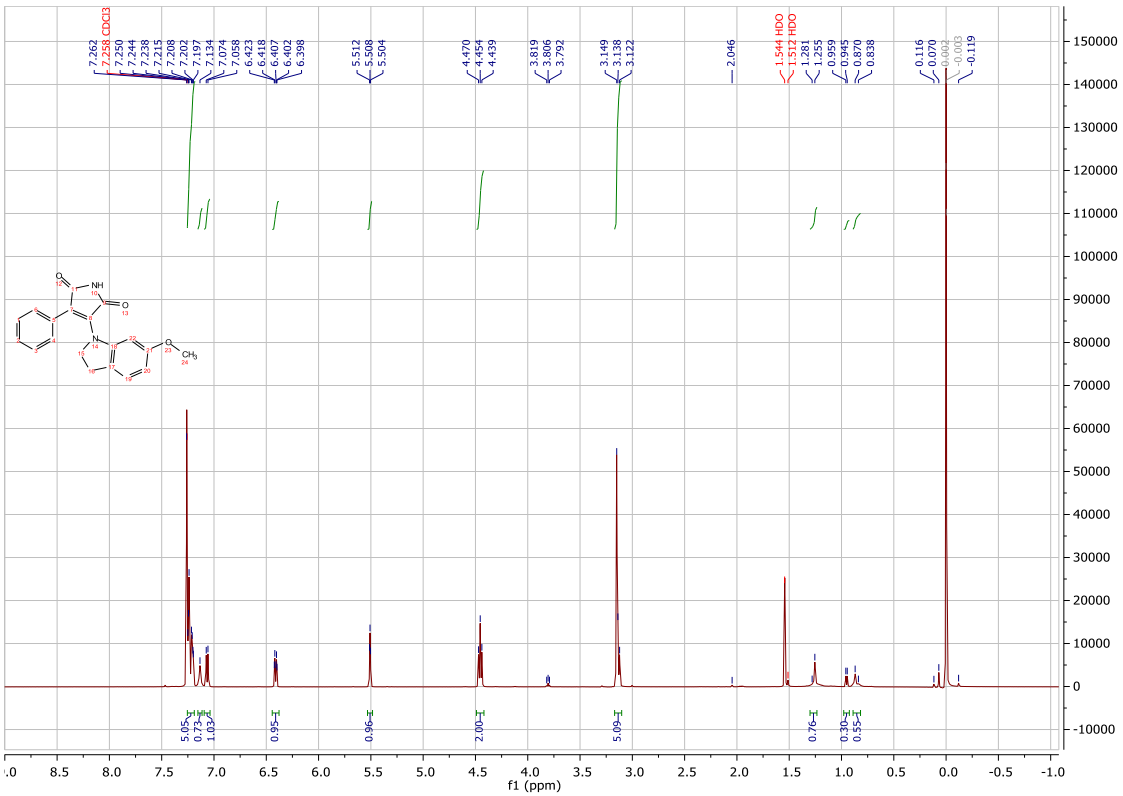

170.46
167.90
141.94
137.57
132.74
129.92
129.77
128.03
127.77
126.15
124.73
122.87
115.25
110.79
77.41 CDCl3
77.16 CDCl3
76.90 CDCl3
54.04
29.55
0.14
0.10
0.17
0.04
0.04
0.03
0.14
0.06
0.09
0.05
0.05
0.05
0.04
1.05
1.04
1.06
f1 (ppm)

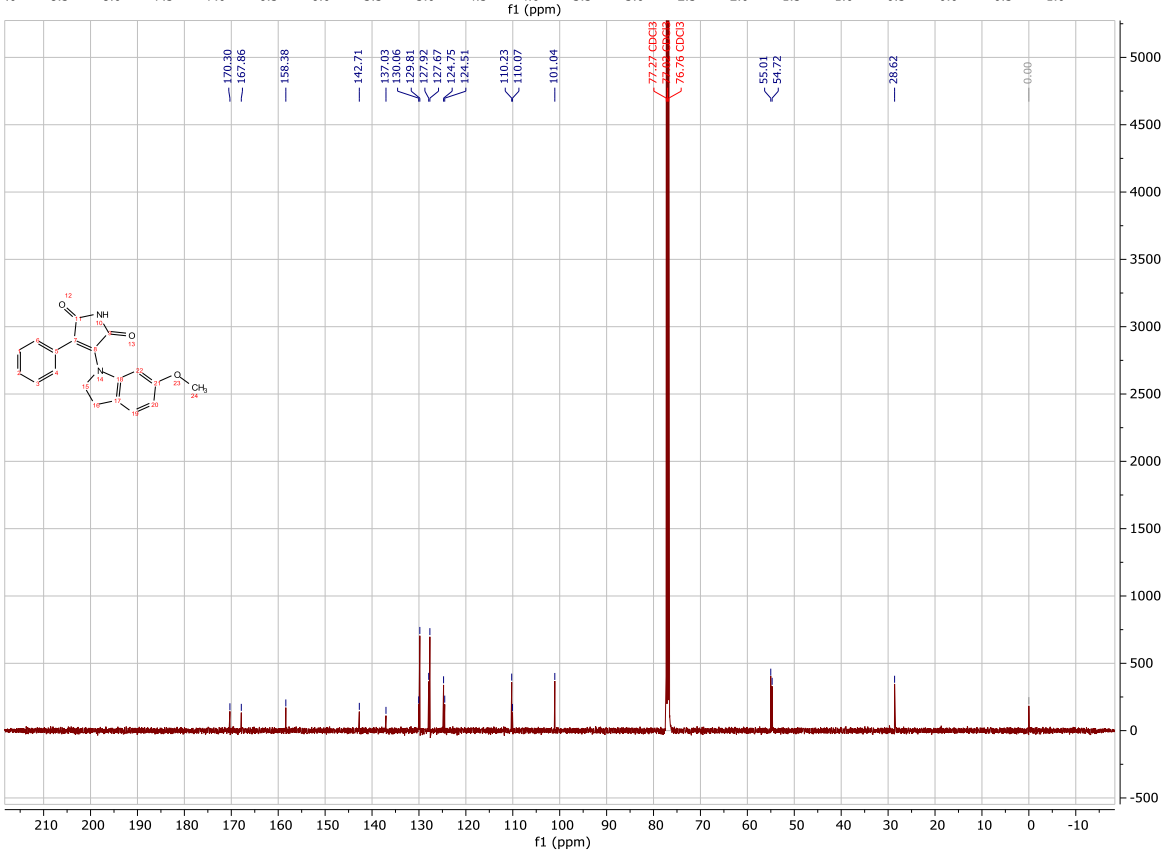
PMI-2


7.261 CDCl3
1.542 HDO
f1 (ppm)

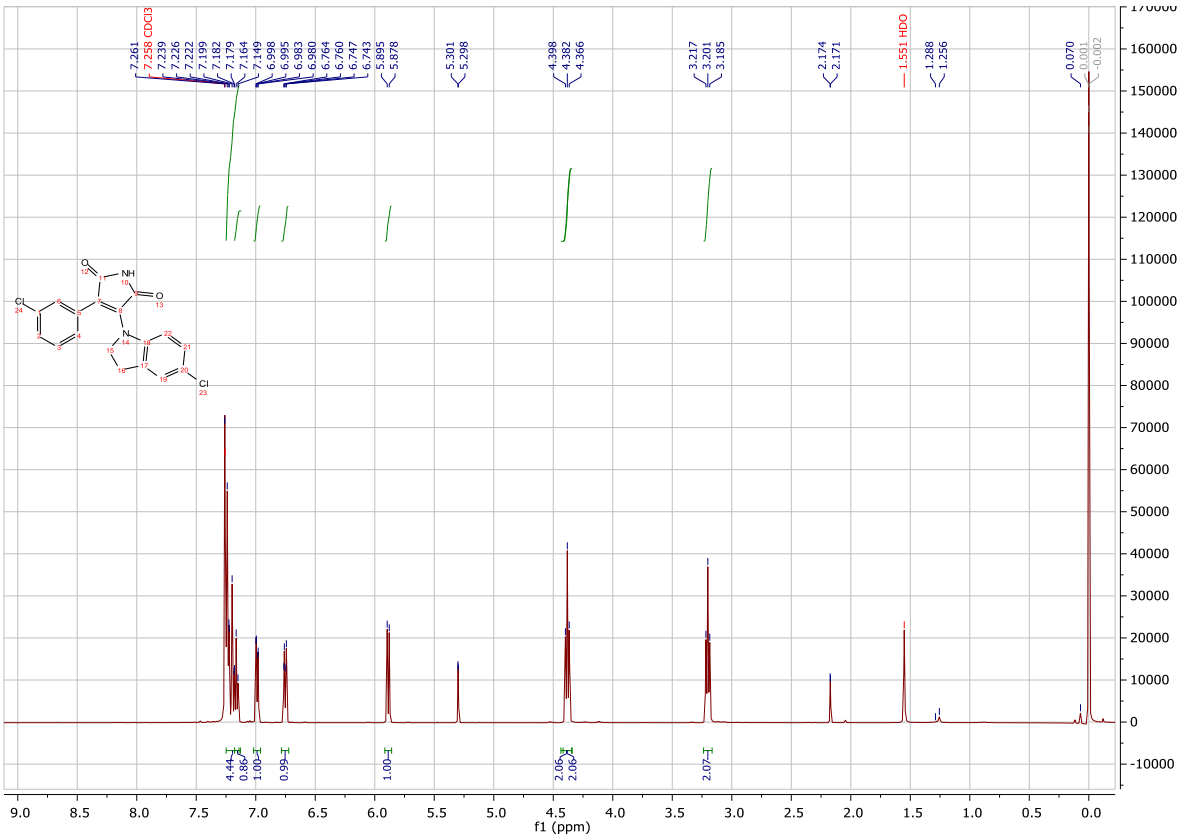

169.72
167.25
141.34
133.66
133.00
131.45
129.59
128.67
127.75
126.02
124.81
123.36
115.44
108.27
77.22 CDCl3
76.76 CDCl3
54.19
29.46
0.00
f1 (ppm)

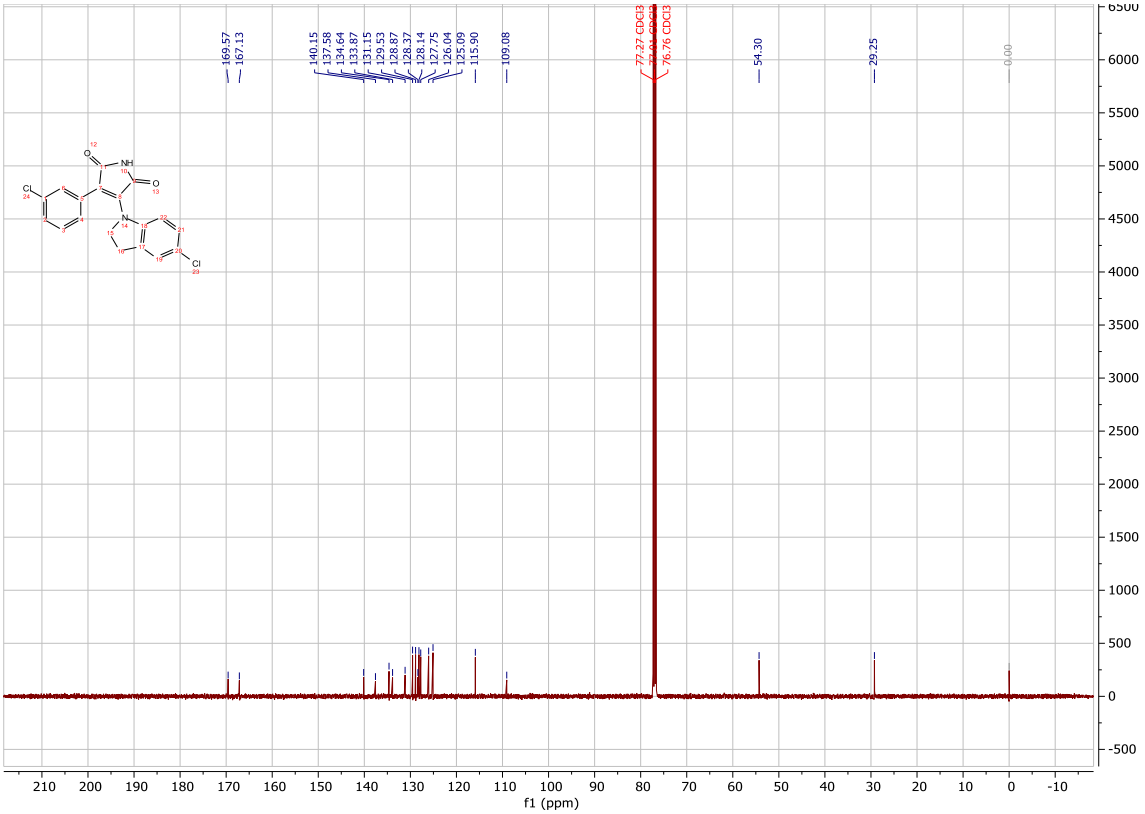
PMI-3


7.262 CDCl3
7.250
7.244
7.238
7.215
7.208
7.202
7.197
7.134
7.074
7.058
6.423
6.418
6.407
6.402
6.398
5.512
5.508
5.504
4.470
4.454
4.439
3.819
3.806
3.792
3.149
3.138
3.122
2.046
1.544 HDO
1.512 HDO
1.281
1.255
0.959
0.945
0.870
0.838
0.116
0.070
0.000
-0.003
-0.119
5.05
0.73
1.03
0.95
0.96
2.00
5.09
0.76
0.30
0.55
f1 (ppm)

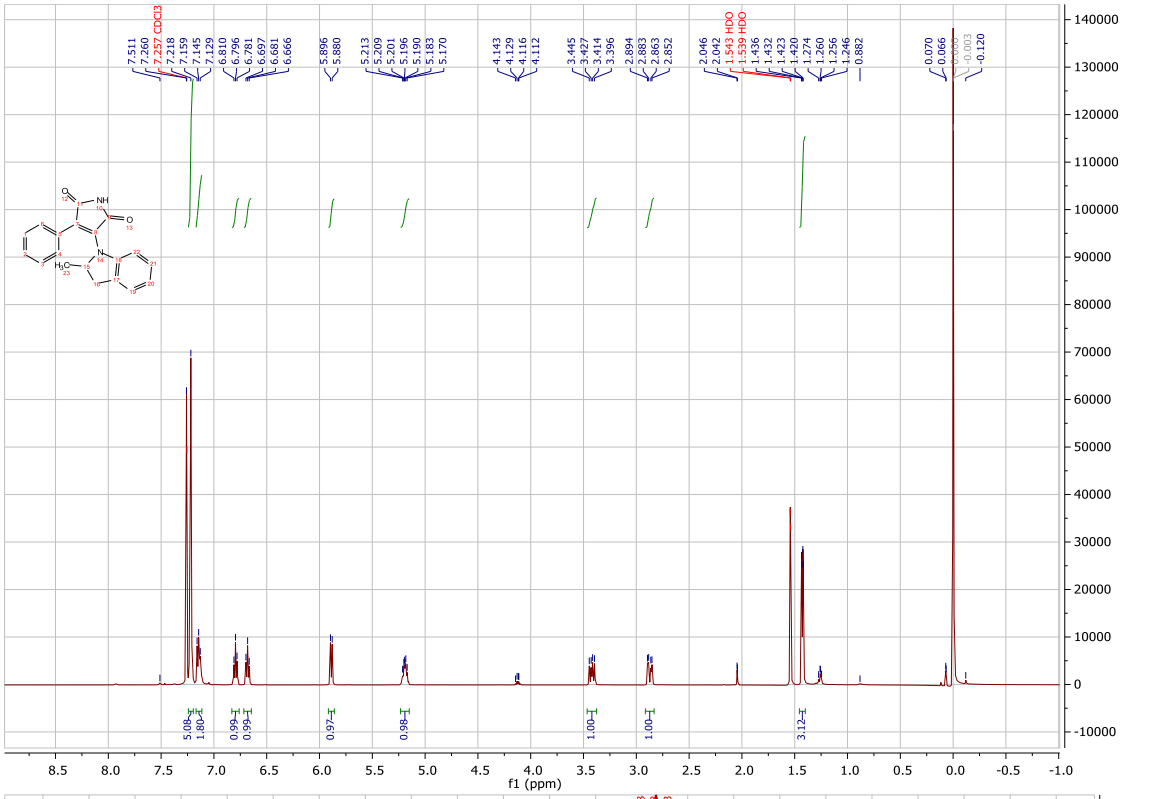

170.30
167.86
158.38
142.71
137.03
130.06
129.81
127.92
127.67
124.75
124.51
110.23
110.07
101.04
77.27 CDCl3
77.01 CDCl3
76.76 CDCl3
55.01
54.72
28.62
0.00
f1 (ppm)

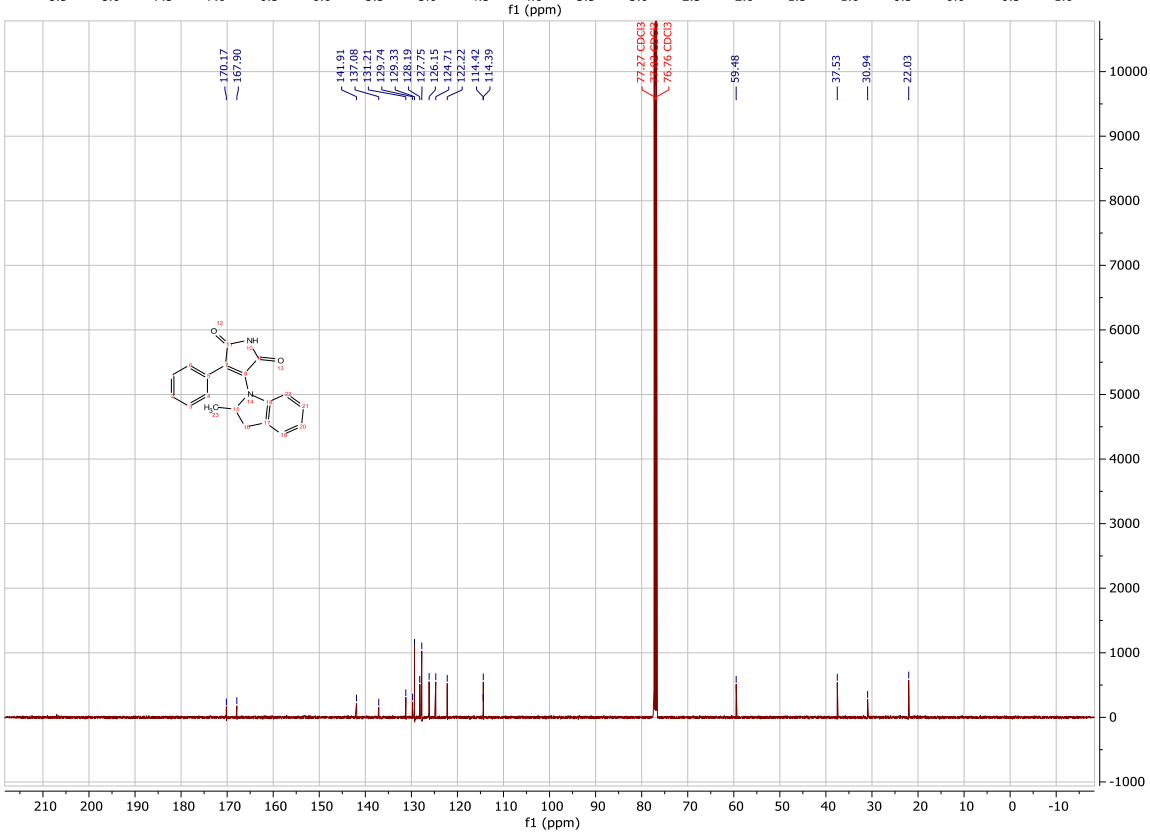
PMI-4


7.261
7.238 CDCl3
7.236
7.226
7.222
7.199
7.182
7.179
7.164
7.149
6.998
6.995
6.983
6.980
6.764
6.760
6.747
6.743
5.895
5.878
5.301
5.298
4.398
4.382
4.366
3.217
3.201
3.185
2.174
2.171
1.551 HDO
1.288
1.256
0.070
0.001
-0.002
4.44
0.86
1.00
0.99
1.00
2.06
2.06
2.07
f1 (ppm)

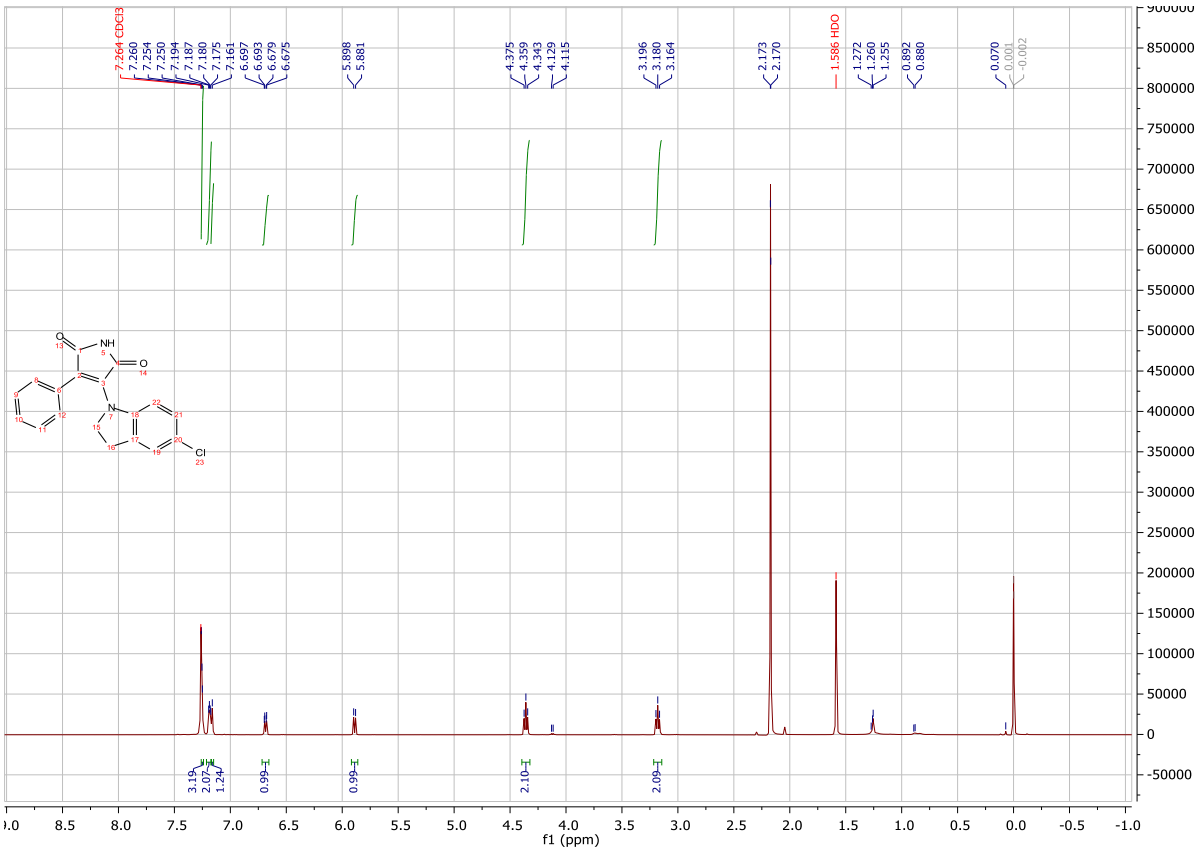

169.57
167.13
140.15
137.58
134.64
133.87
131.15
129.53
128.87
128.37
128.14
127.75
126.04
125.09
115.90
109.08
77.27 CDCl3
77.01 CDCl3
76.76 CDCl3
54.30
29.25
0.00
f1 (ppm)

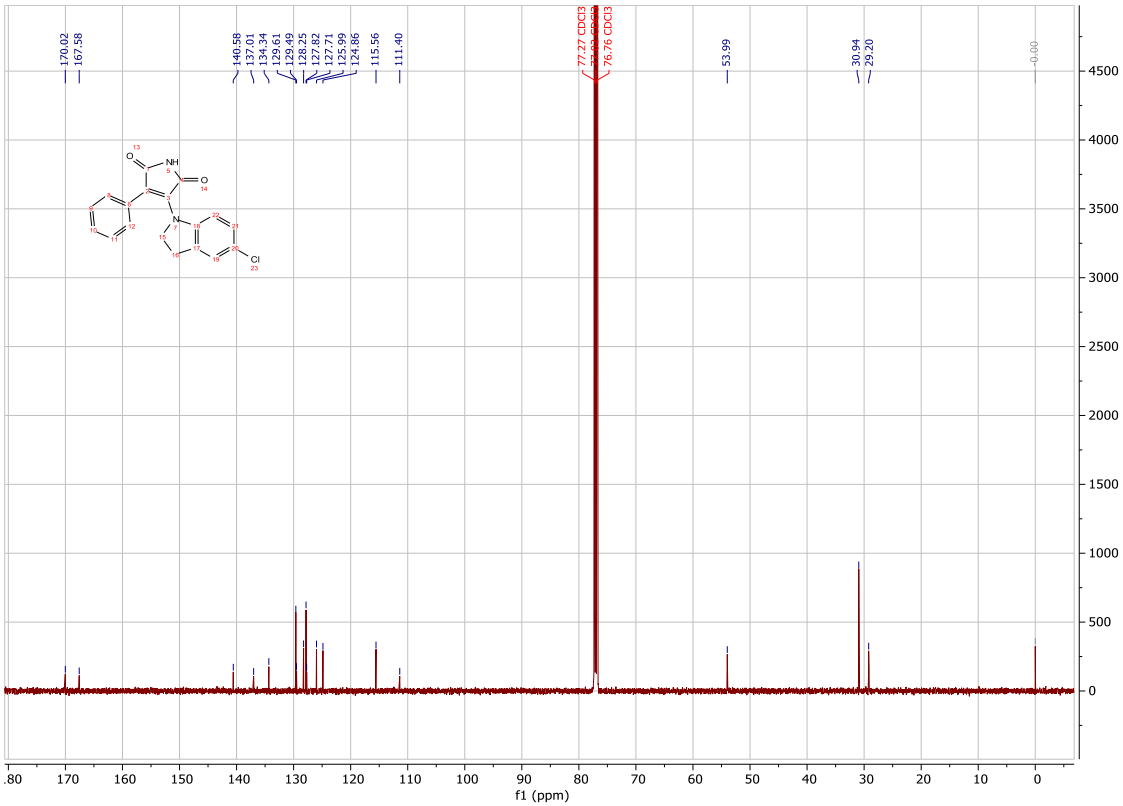
PMI-5


7.511
7.260
7.257 CDCl3
7.218
7.159
7.145
7.129
6.810
6.796
6.781
6.697
6.681
6.666
5.896
5.880
5.213
5.209
5.201
5.196
5.190
5.183
5.170
4.143
4.129
4.116
4.112
3.445
3.427
3.414
3.396
2.894
2.883
2.863
2.852
2.046
2.042
1.543 HDO
1.539 HDO
1.436
1.432
1.423
1.420
1.274
1.260
1.256
1.246
0.882
0.070
0.066
-0.003
-0.120
5.08
1.80
0.99
0.99
0.97
0.98
1.00
1.00
3.12
f1 (ppm)

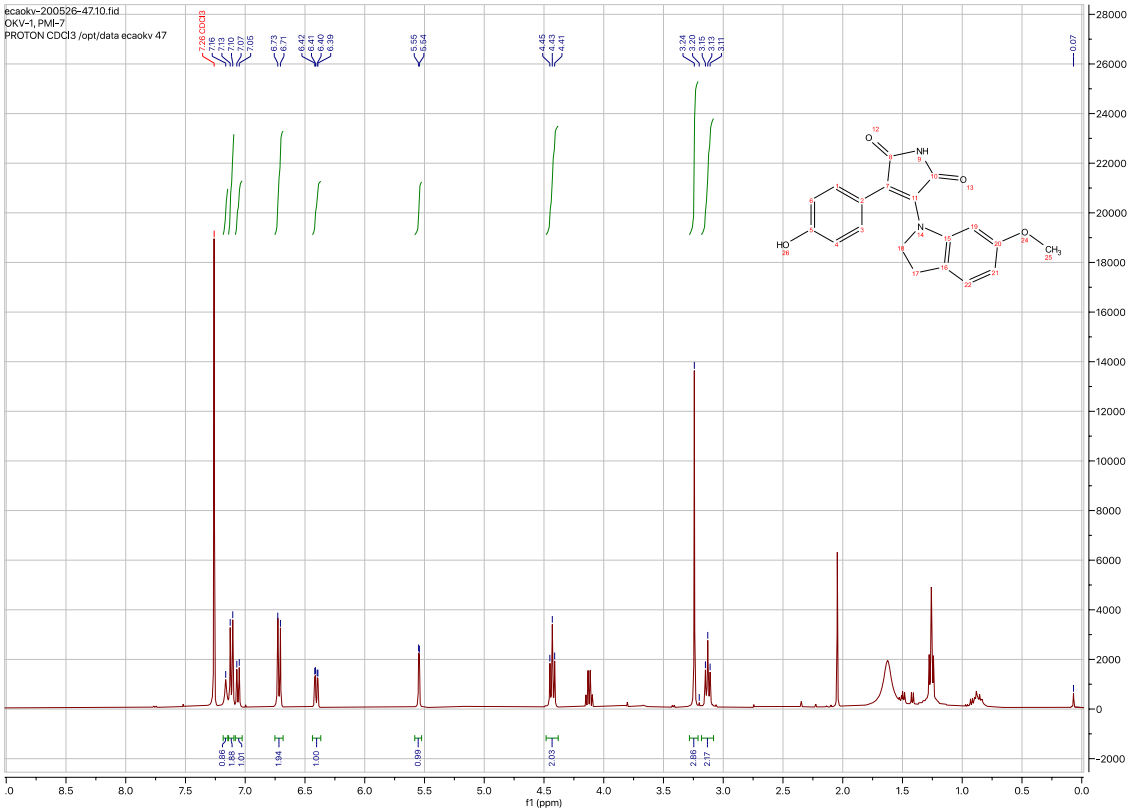

170.17
167.90
141.91
137.08
131.21
129.74
129.33
128.19
127.75
126.15
124.71
122.22
114.42
114.39
77.27 CDCl3
77.02 CDCl3
76.76 CDCl3
59.48
37.53
30.94
22.03
f1 (ppm)

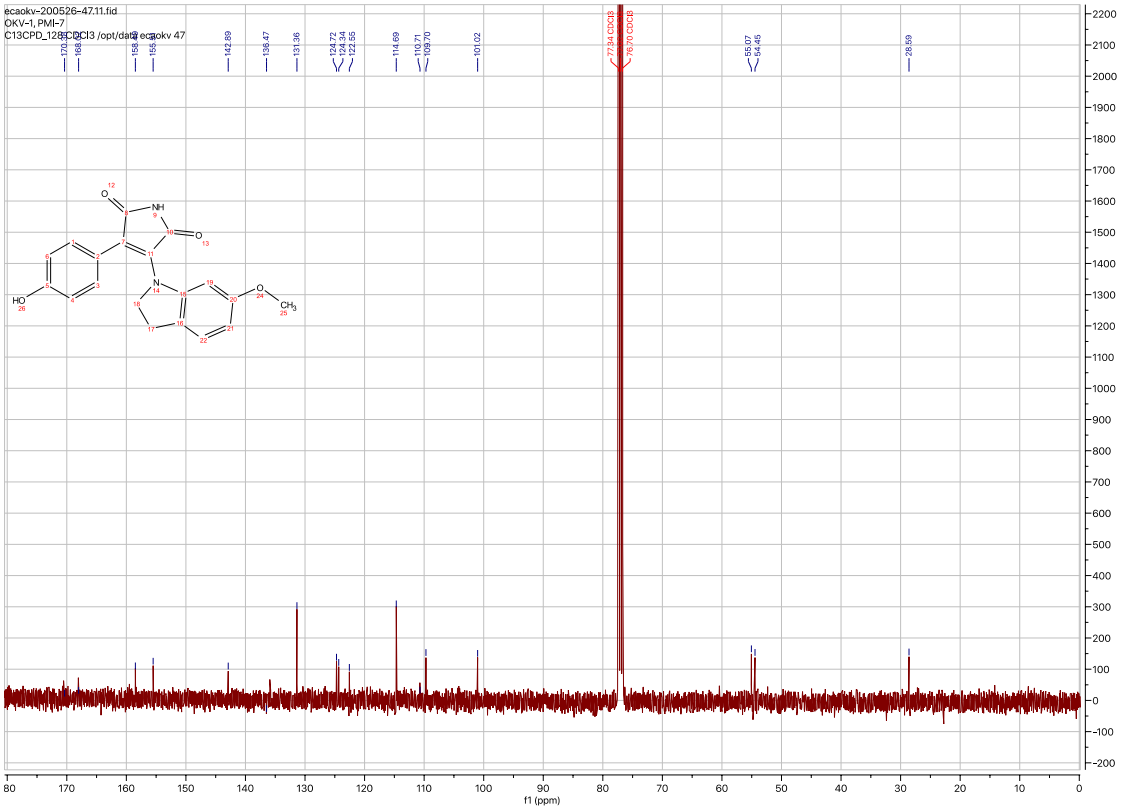
PMI-6


7.264 CDCl3
7.260
7.254
7.250
7.194
7.187
7.180
7.175
7.161
6.697
6.693
6.679
6.675
5.898
5.881
4.375
4.359
4.343
4.129
4.115
3.196
3.180
3.164
2.173
2.170
1.586 HDO
1.272
1.260
1.255
0.892
0.880
0.070
0.001
-0.002
3.19
2.07
1.24
0.99
0.99
2.10
2.09
f1 (ppm)

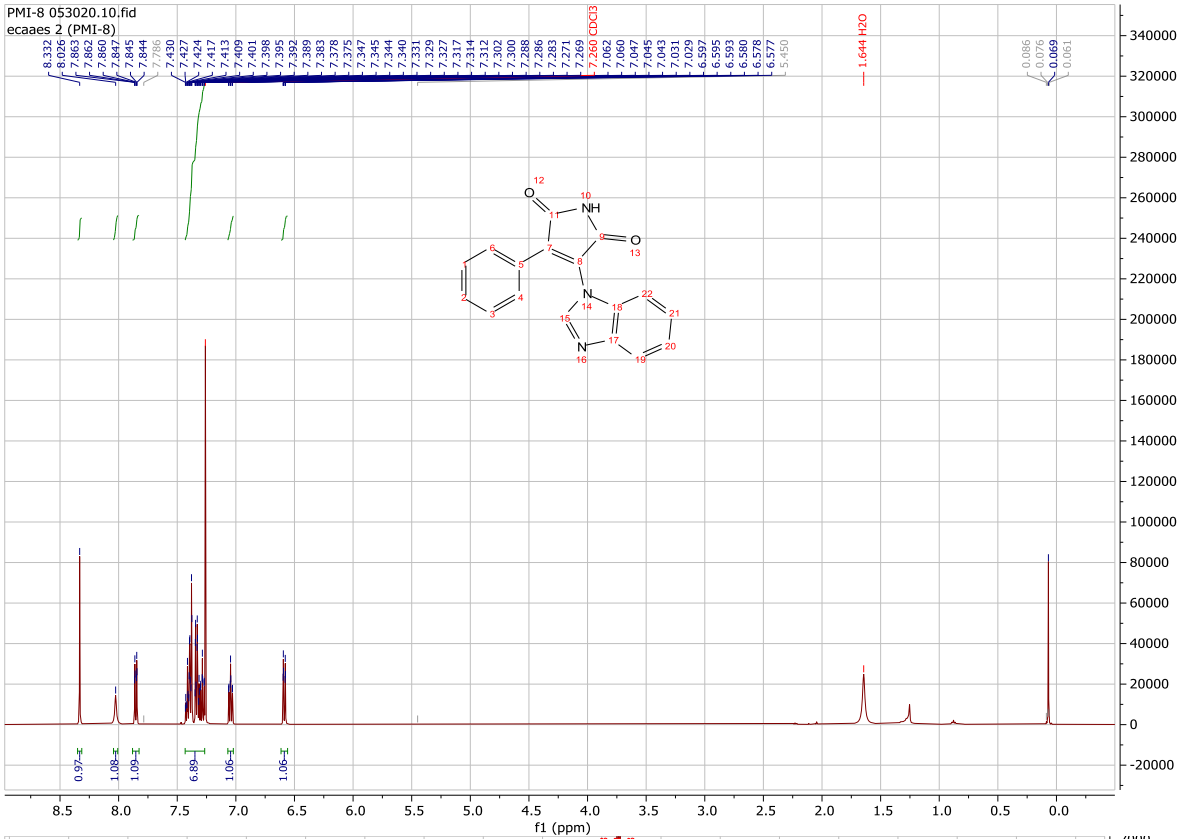

170.02
167.58
140.58
137.01
134.34
129.61
129.49
128.25
127.82
127.71
125.99
124.86
115.56
111.40
77.27 CDCl3
76.76 CDCl3
53.99
30.94
29.20
-0.00
f1 (ppm)

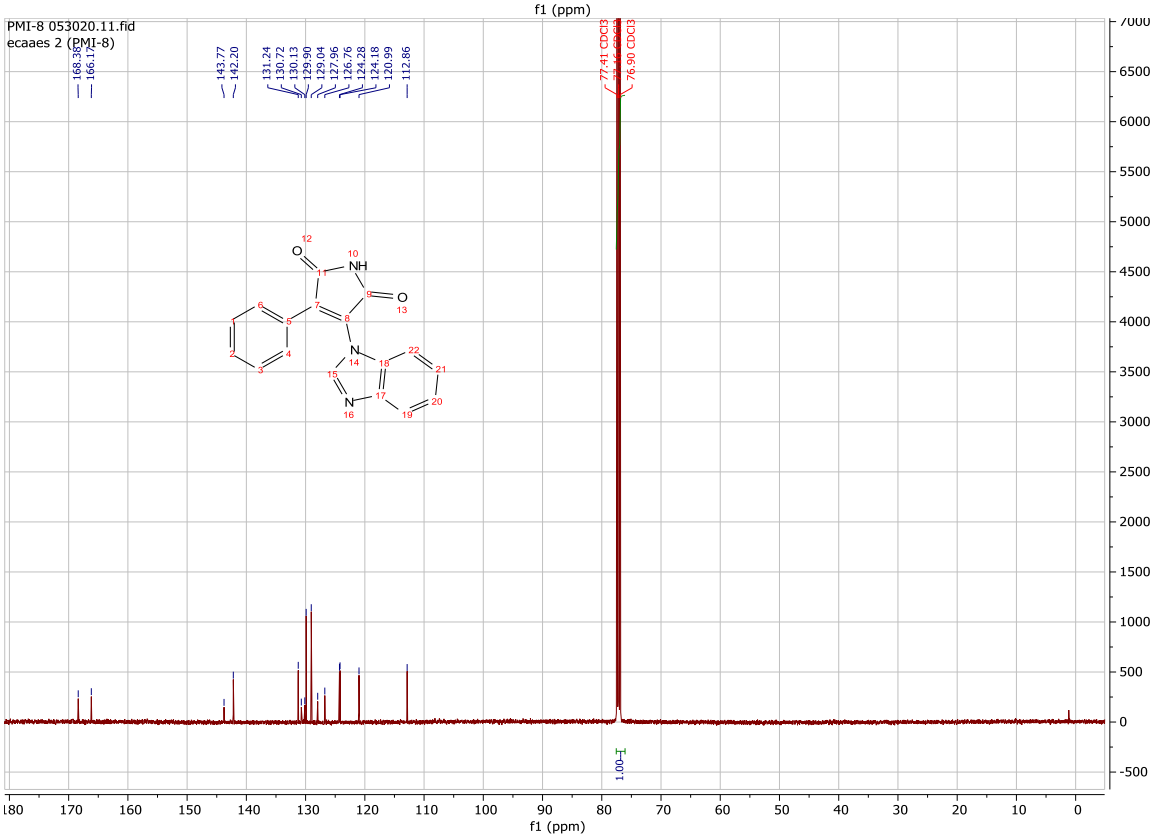
PMI-7


ecaokv-200526-47.10.fid
OKV-1, PMI-7
PROTON CDCl3 /opt/data ecaokv 47
f1 (ppm)

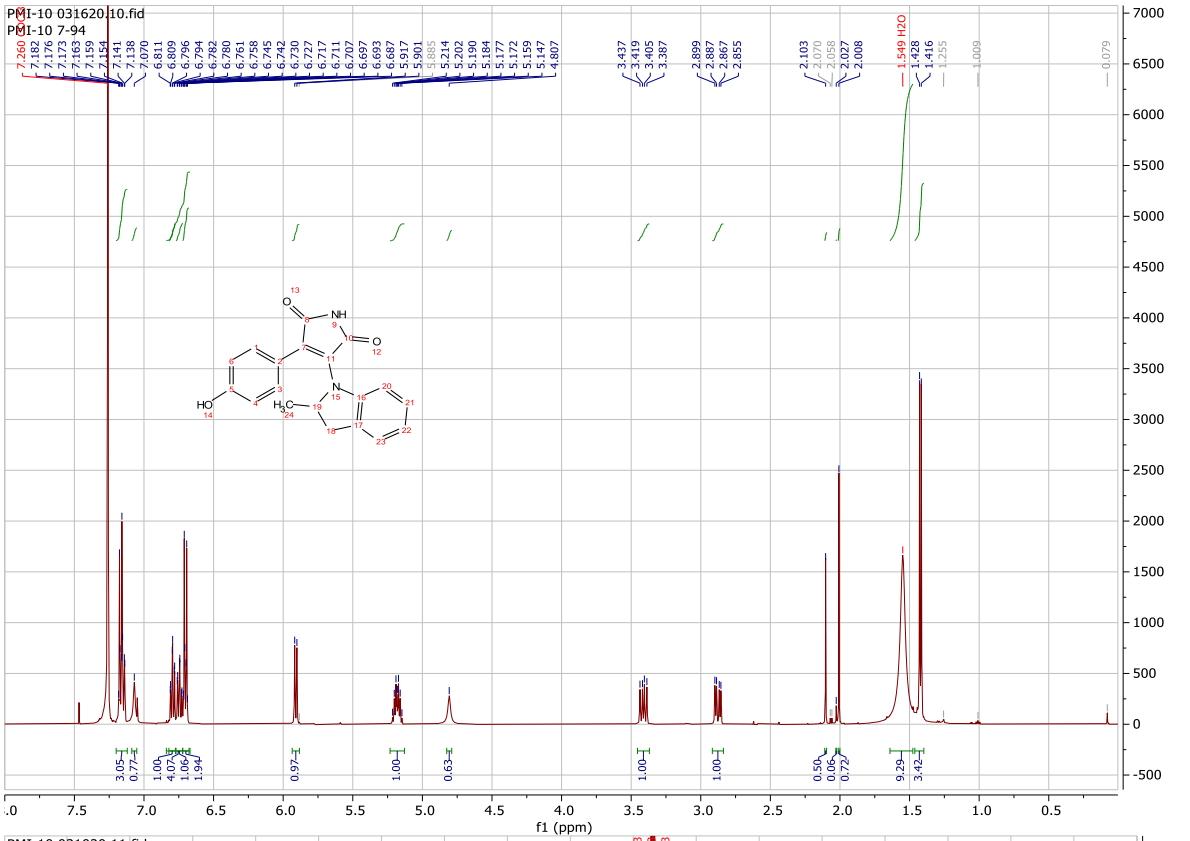

ecaokv-200526-47.11.fid
OKV-1, PMI-7
C13CPD_128_CDCl3 /opt/data ecaokv 47
f1 (ppm)

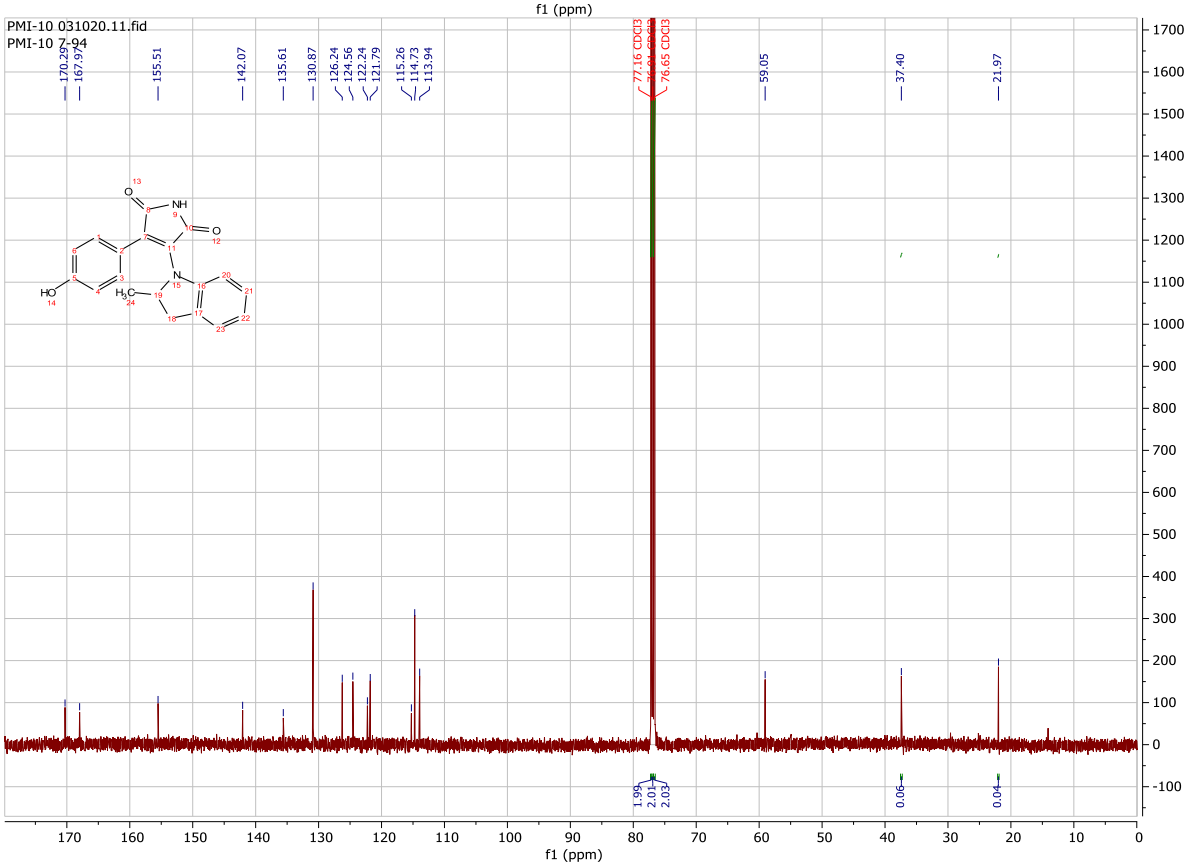
PMI-8


PMI-8 053020.10.fid
ecaaes 2 (PMI-8)
7.260 CDCl3
1.644 H2O
0.069
0.97
1.08
1.09
6.89
1.05
1.06
f1 (ppm)

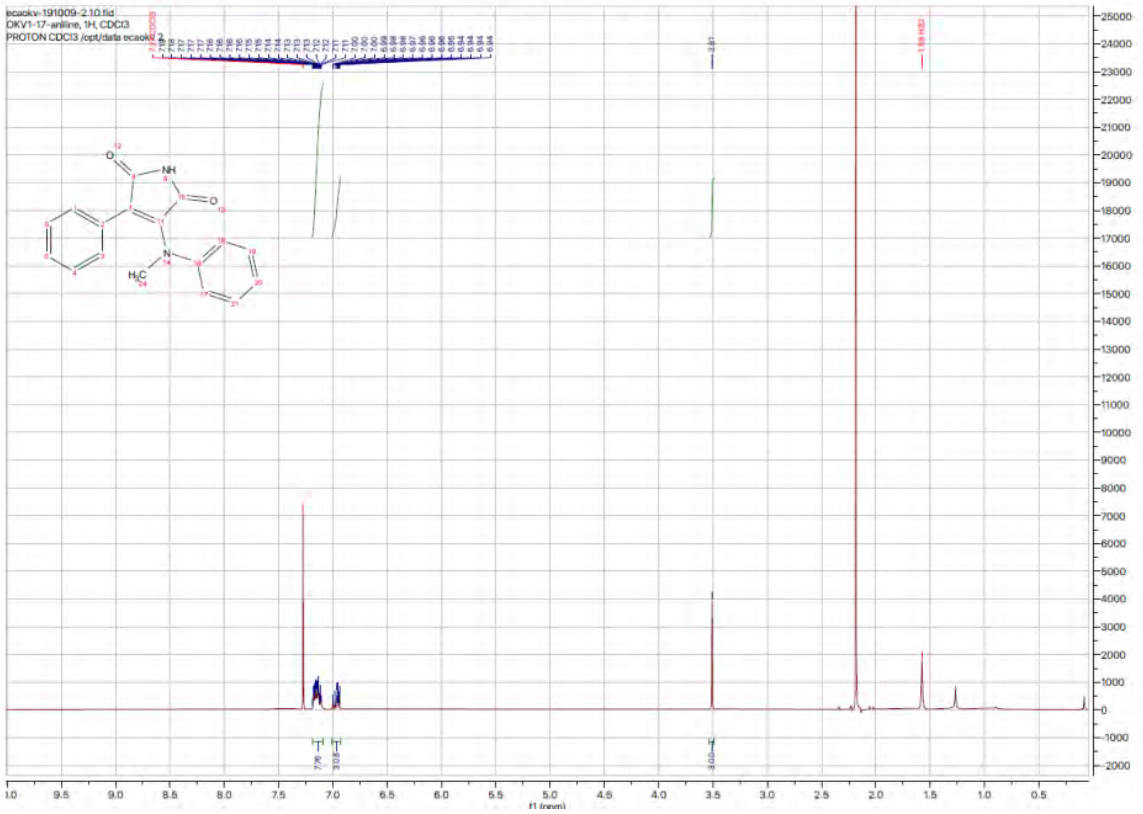

PMI-8 053020.11.fid
ecaaes 2 (PMI-8)
168.36
166.17
143.77
142.20
131.24
130.72
130.13
129.90
129.04
127.96
126.76
124.28
124.18
120.99
112.86
77.41 CDCl3
76.90 CDCl3
1.00
f1 (ppm)

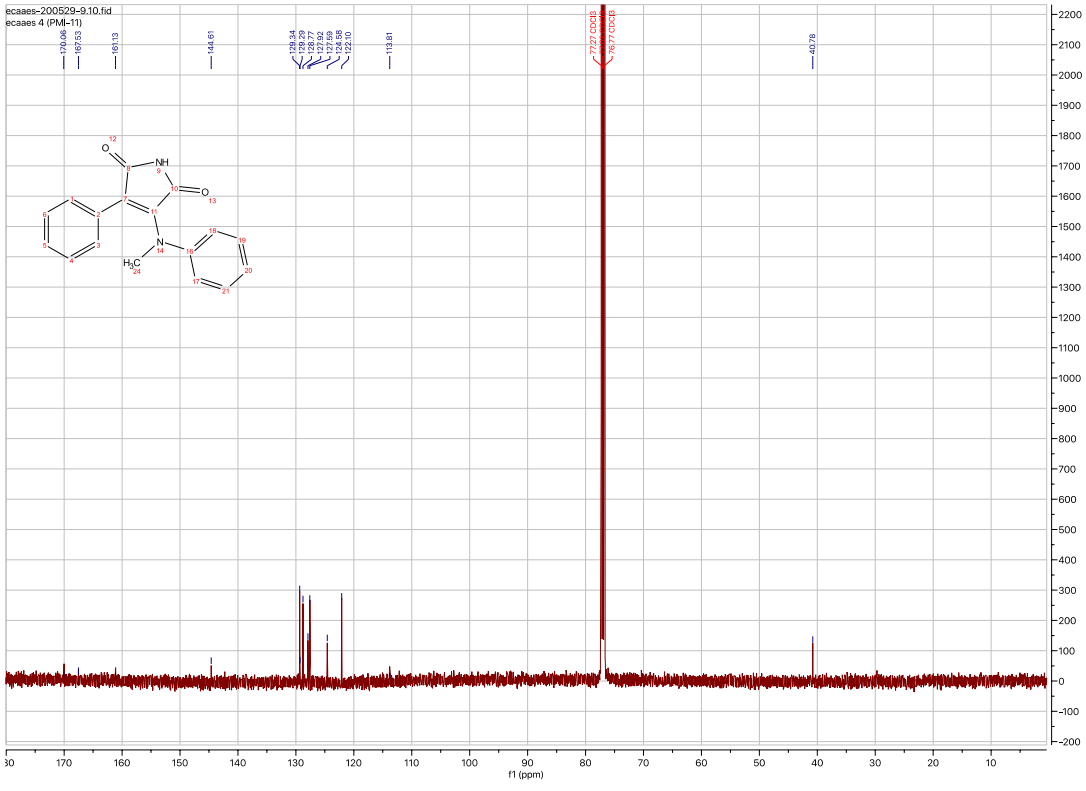
PMI-10


PMI-10 031620.10.fid
PMI-10 7-94
f1 (ppm)

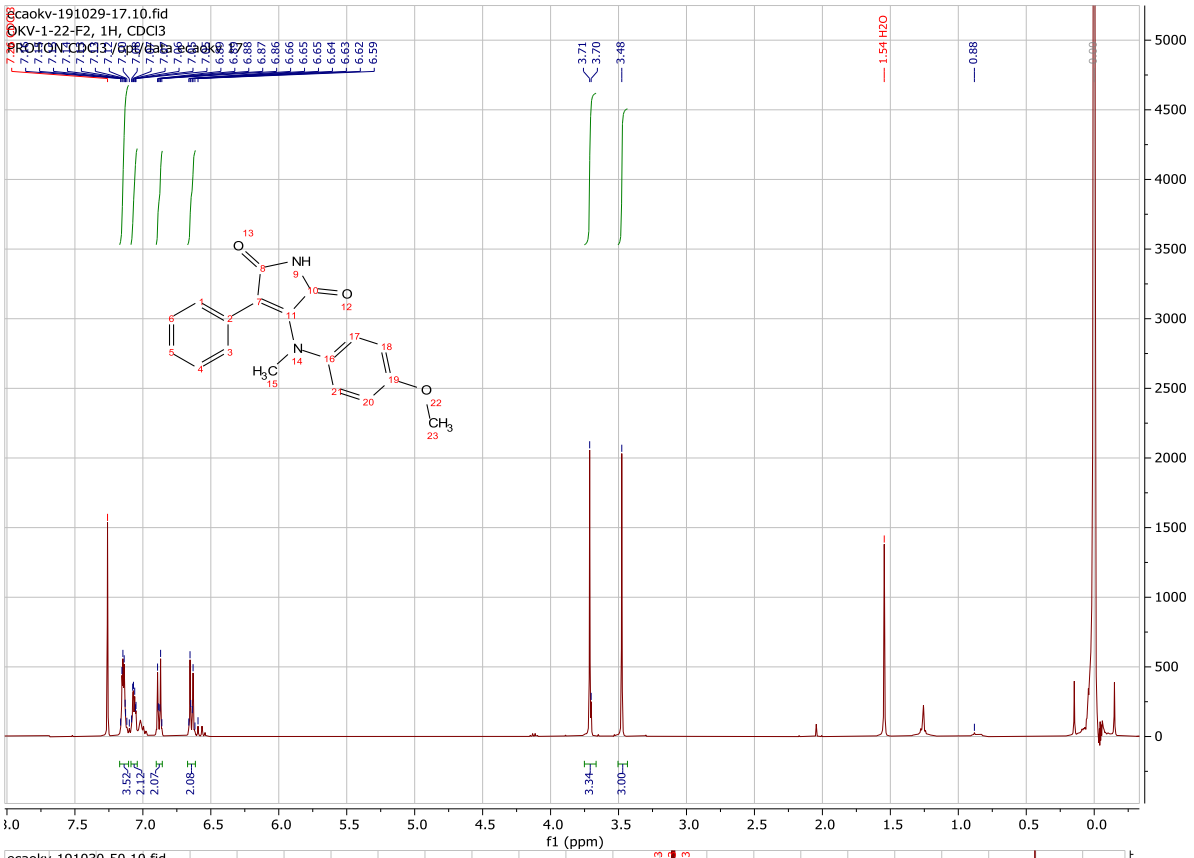

PMI-10 031020.11.fid
PMI-10 7-94
f1 (ppm)

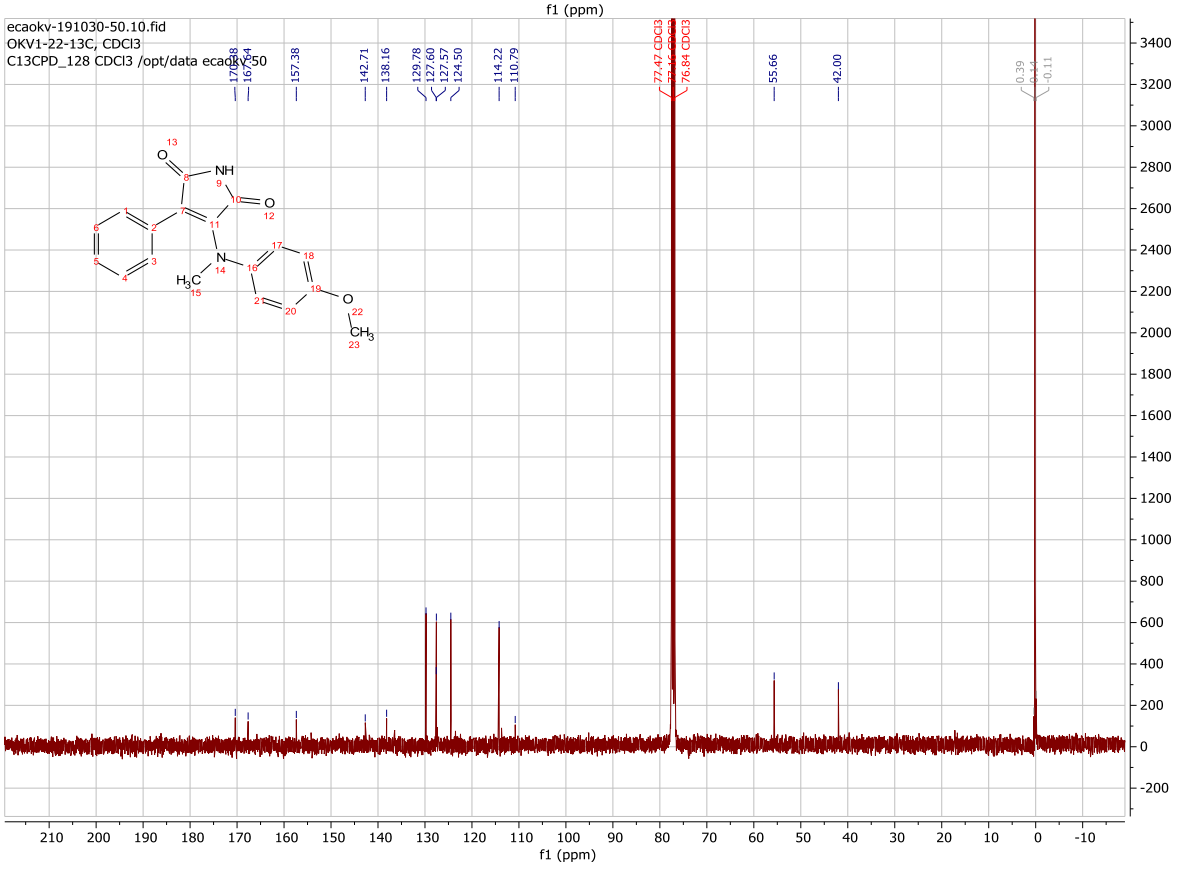
PMI-11


ecaokv-191009-2.10.fid
OKV1-17-aniline, 1H, CDCl3
PROTON CDCl3 /opt/data ecaokv 2

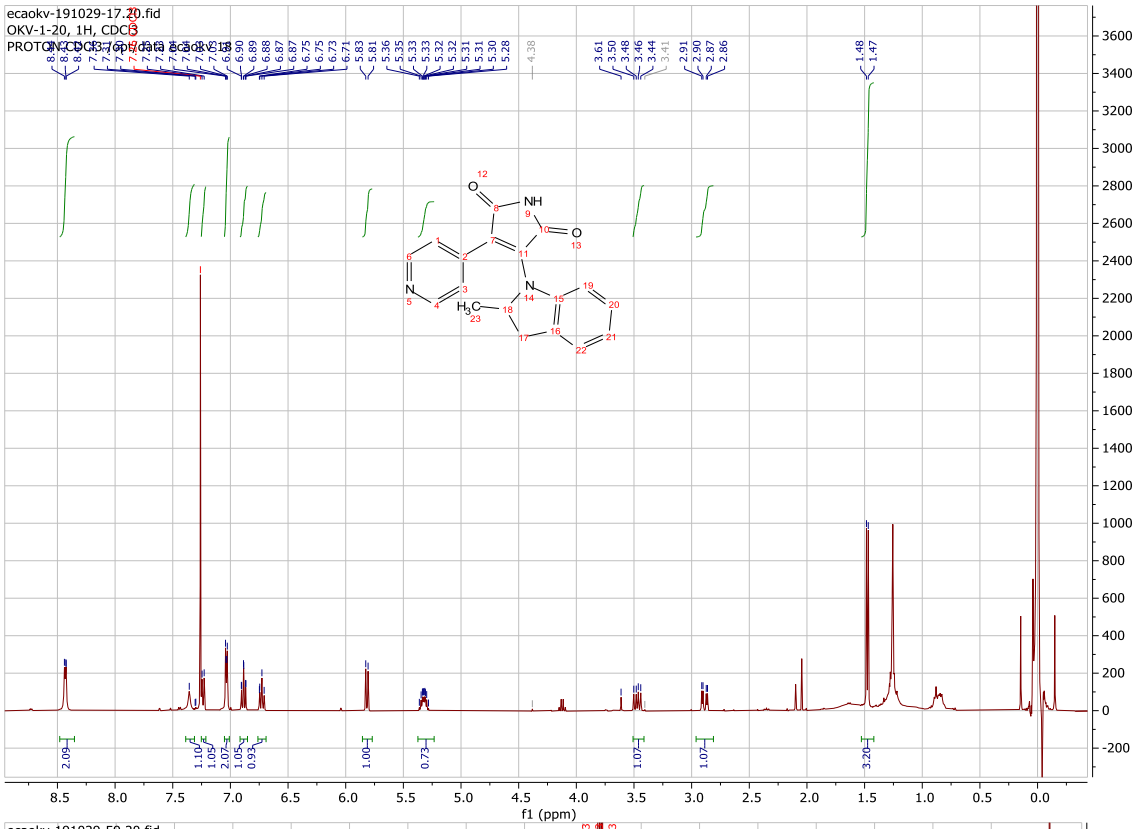

ecaaes-200529-9.10.fid
ecaaes 4 (PMI-11)
f1 (ppm)

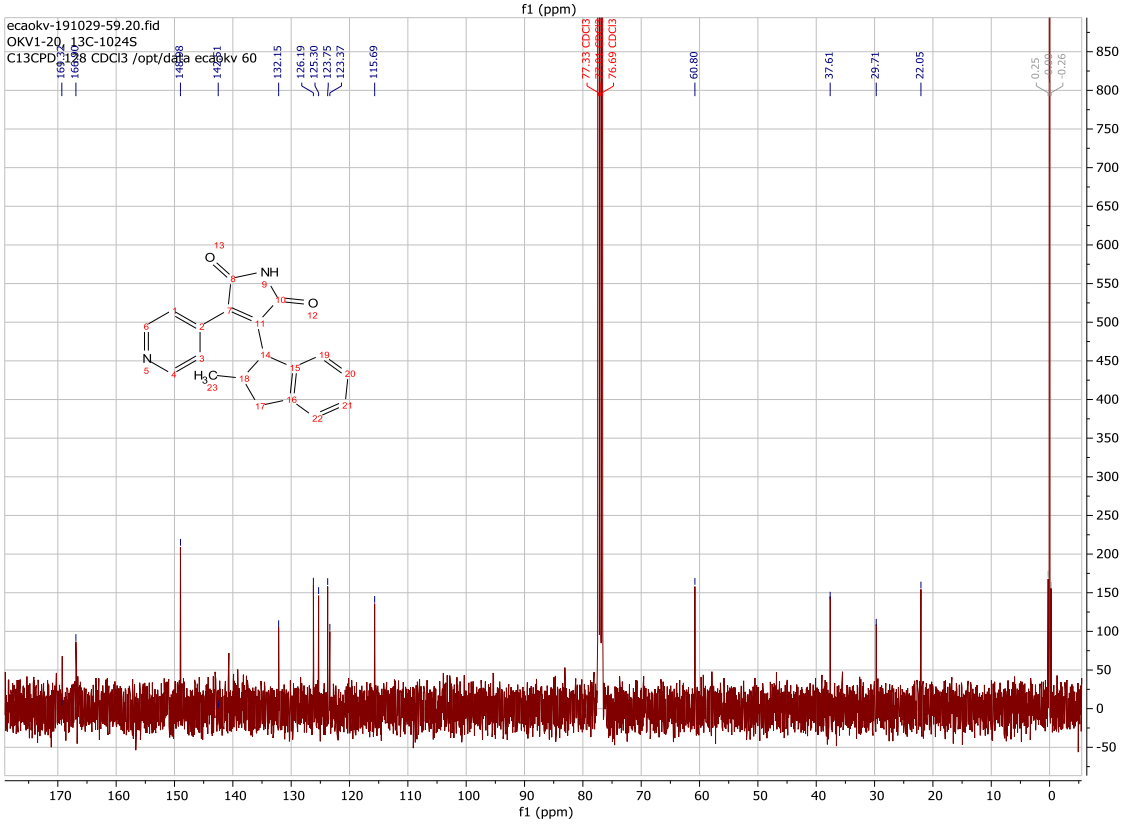
PMI-12


ecaokv-191029-17.10.fid
OKV-1-22-F2, 1H, CDCl3
PROTON_CDCl3 /opt/data ecaokv 17
3.71
3.70
3.48
1.54 H2O
0.88
3.52
2.12
2.07
2.08
3.34
3.00
f1 (ppm)

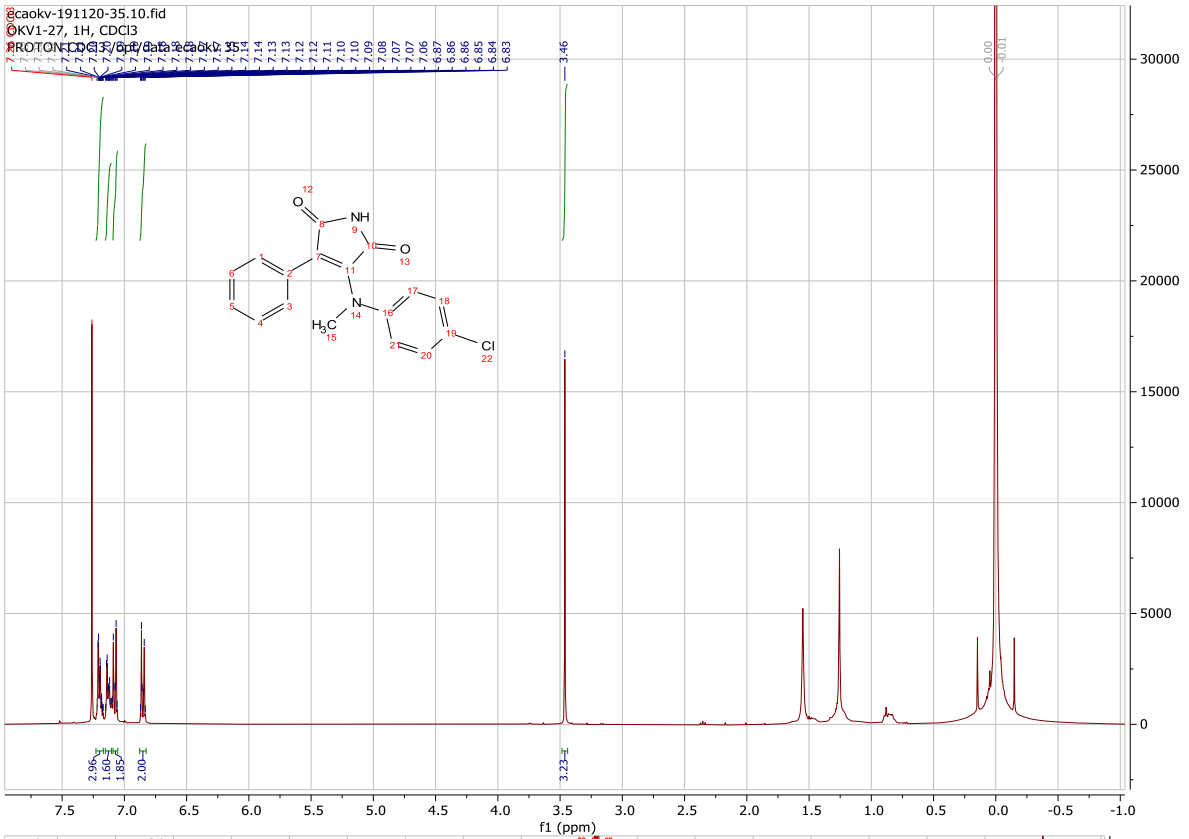

ecaokv-191030-50.10.fid
OKV1-22-13C, CDCl3
C13CPD_128 CDCl3 /opt/data ecaokv 50
170.88
167.64
157.38
142.71
138.16
129.78
127.60
127.57
124.50
114.22
110.79
77.47 CDCl3
76.84 CDCl3
55.66
42.00
0.39
0.14
-0.11
f1 (ppm)

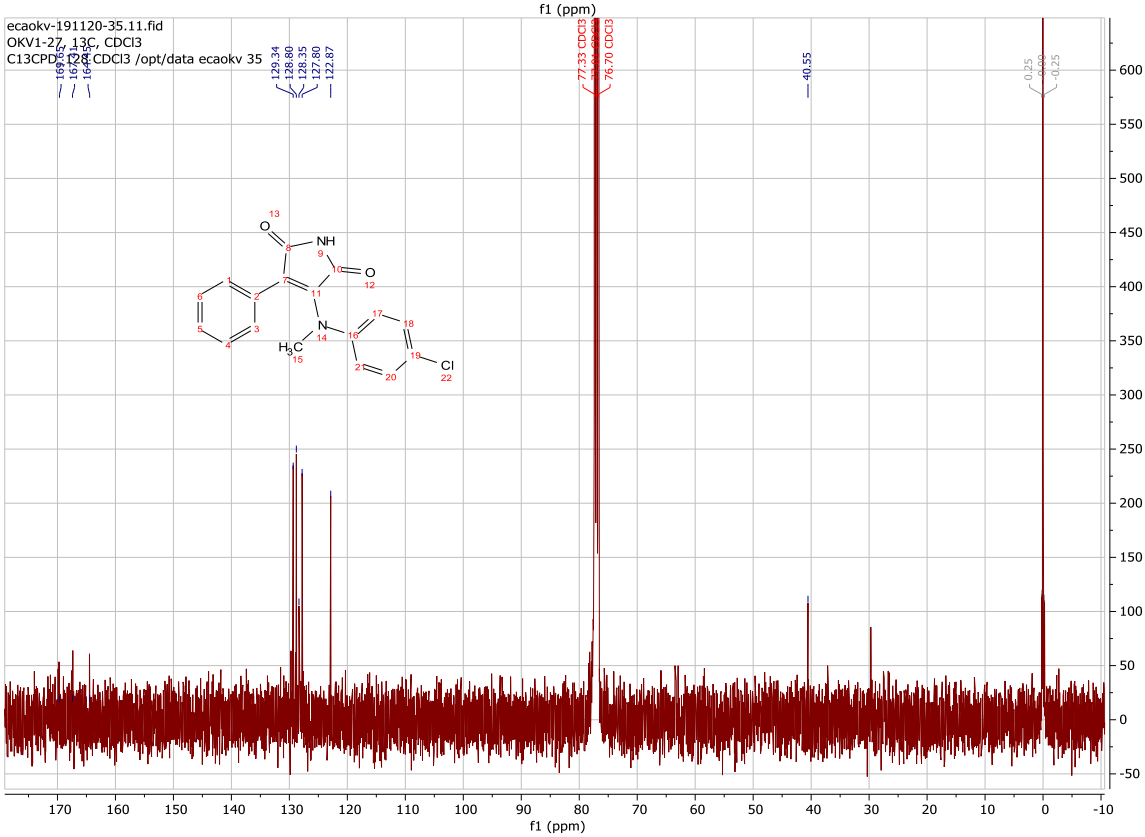
PMI-13


ecaokv-191029-17.20.fid
OKV-1-20, 1H, CDCl3
PROTON,CDCl3 /opt/data ecaokv 18
f1 (ppm)

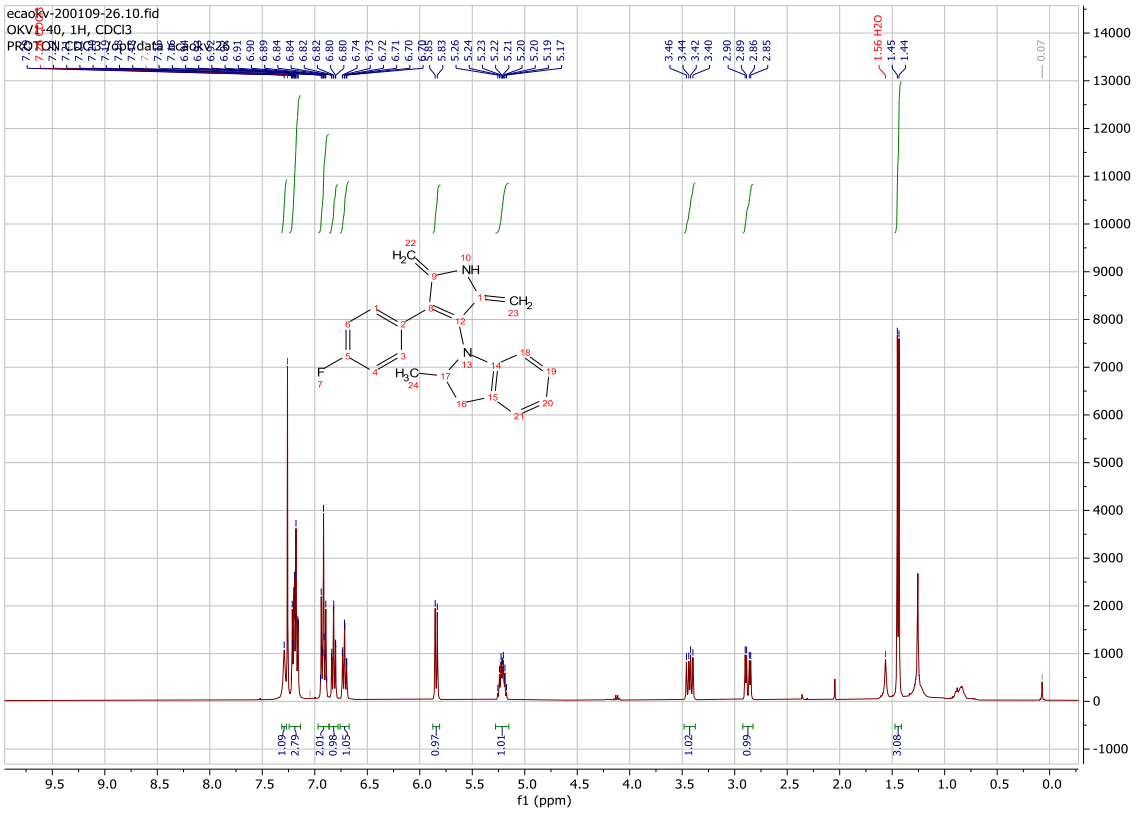

ecaokv-191029-59.20.fid
OKV1-20, 13C-1024S
C13CPD,128 CDCl3 /opt/data ecaokv 60
f1 (ppm)

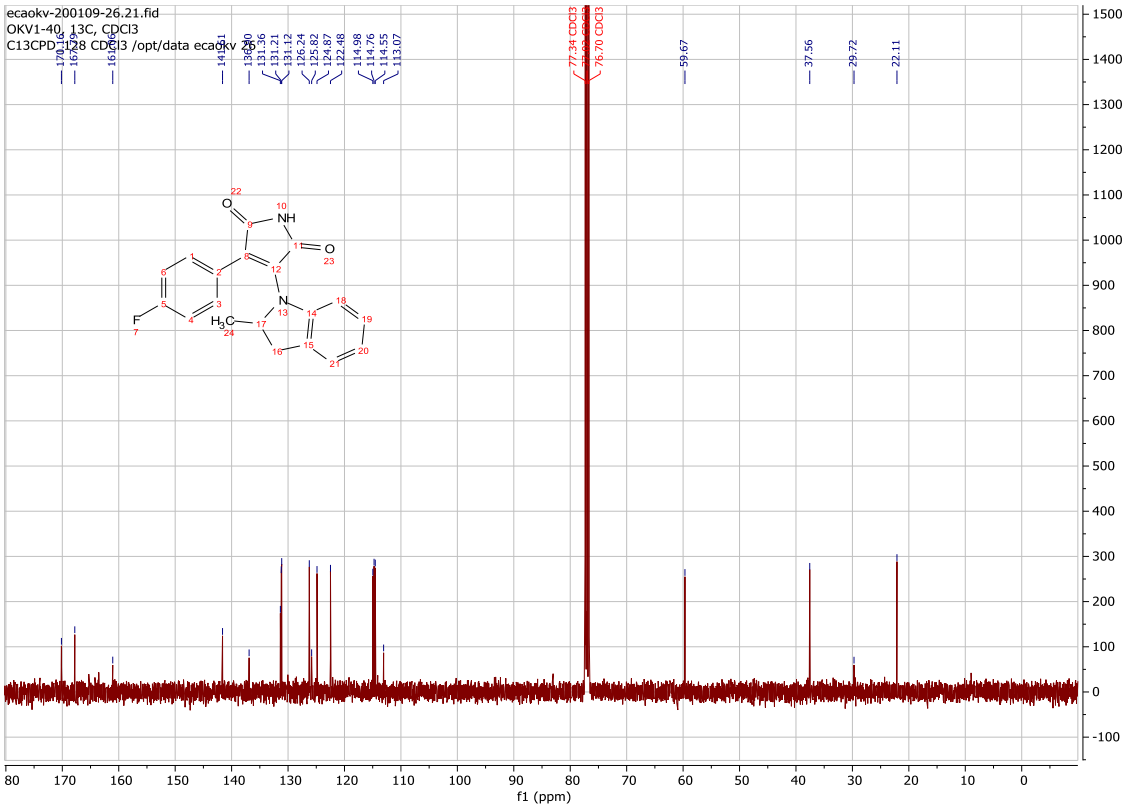
PMI-14


ecaokv-191120-35.10.fid
OKV1-27, 1H, CDCl3
PROTON-CDCl3 /opt/data ecaokv 35
3.46
0.00
0.01
2.96
1.60
1.85
2.00
3.23
f1 (ppm)

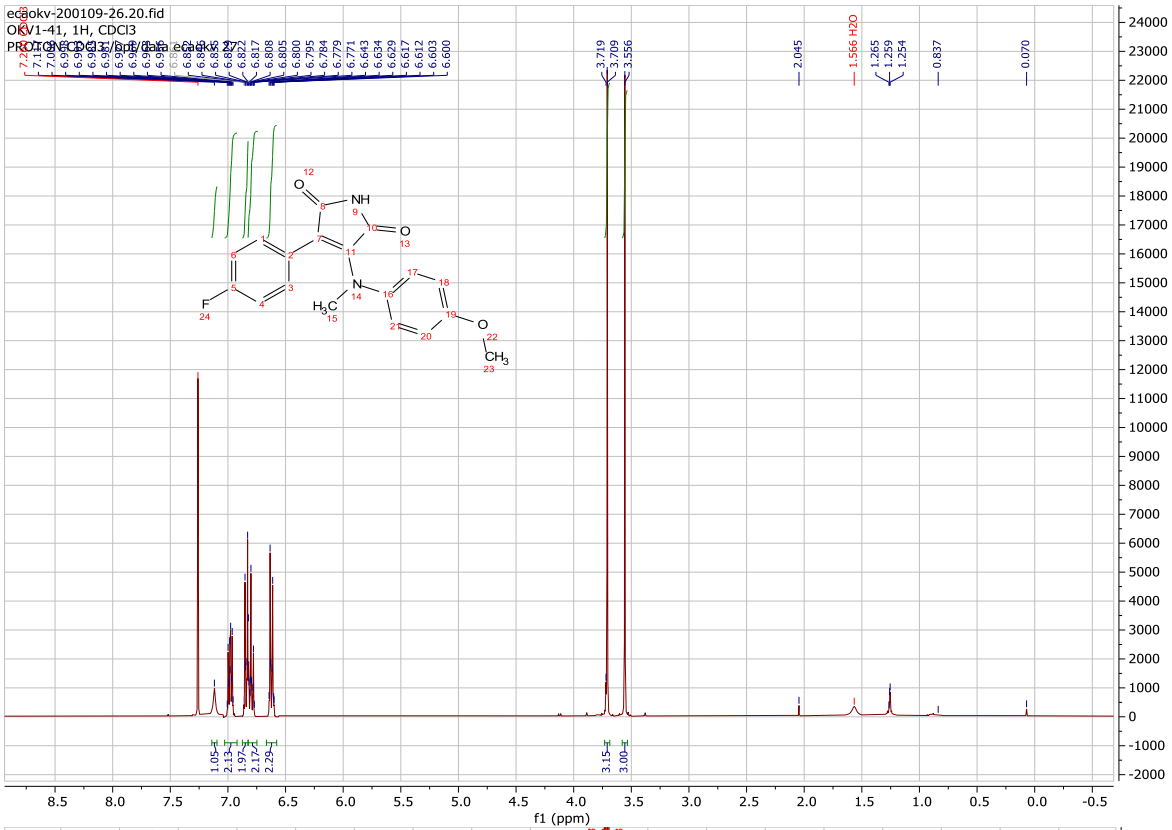

ecaokv-191120-35.11.fid
OKV1-27, 13C, CDCl3
C13CPD-128 CDCl3 /opt/data ecaokv 35
169.12
167.34
164.48
129.34
128.80
128.35
127.80
122.87
77.33 CDCl3
76.70 CDCl3
40.55
0.25
0.00
-0.25
f1 (ppm)

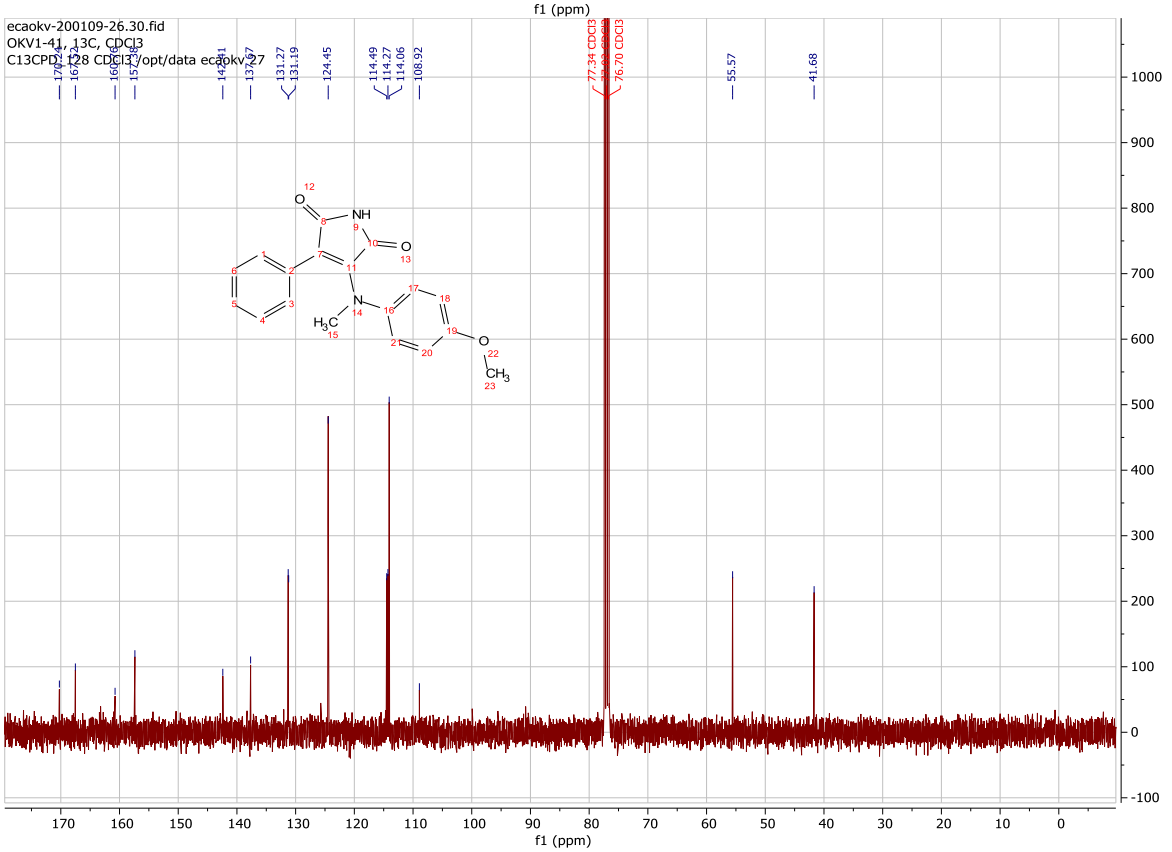
PMI-15


ecaokv-200109-26.10.fid
OKV1-40, 1H, CDCl3
PROTON.CDCL3 /opt/data ecaokv 26
1.56 H2O
0.07
f1 (ppm)

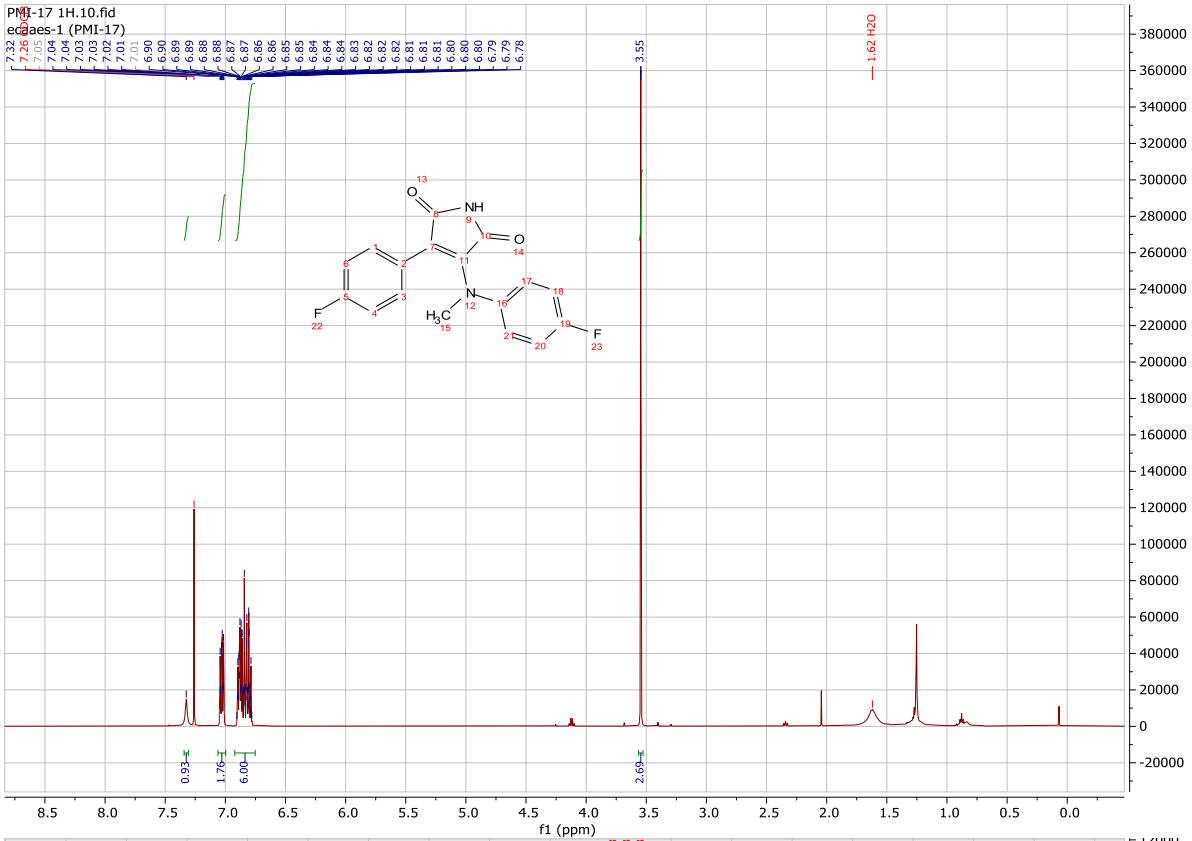

ecaokv-200109-26.21.fid
OKV1-40, 13C, CDCl3
C13CPD-128 CDCl3 /opt/data ecaokv 26
77.34 CDCl3
76.70 CDCl3
59.67
37.56
29.72
22.11
f1 (ppm)

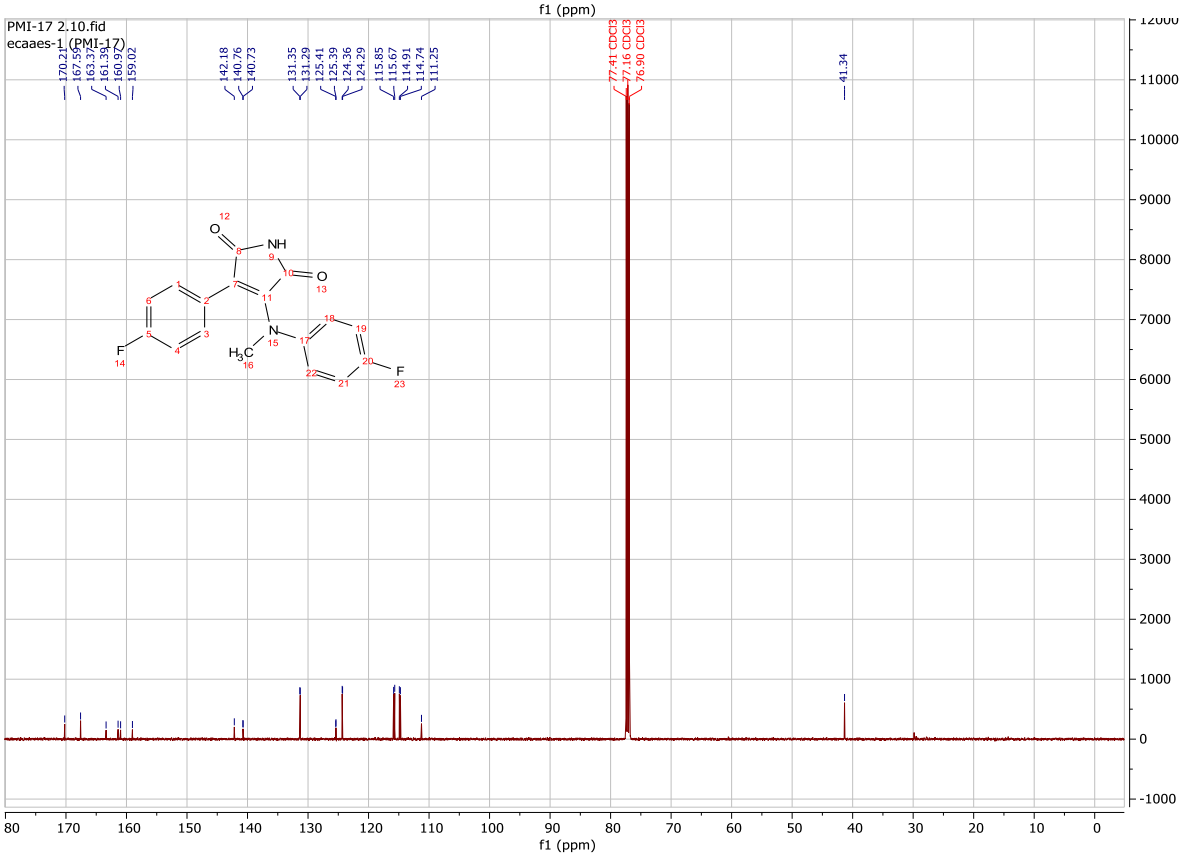
PMI-16


ecaokv-200109-26.20.fid
OKV1-41, 1H, CDCl3
PROTON.CDCL3 /opt/data ecaokv 27
3.719
3.709
3.556
2.045
1.566 H2O
1.265
1.259
1.254
0.837
0.070
f1 (ppm)

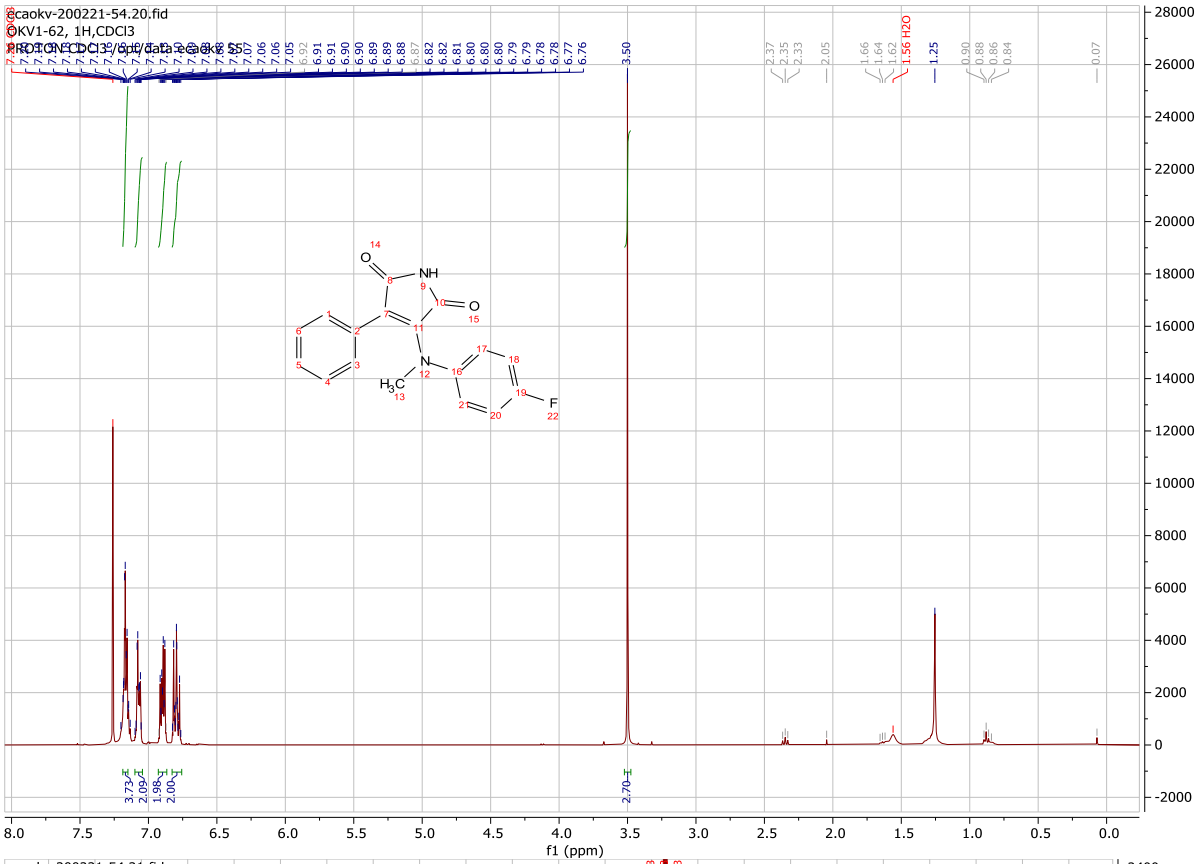

ecaokv-200109-26.30.fid
OKV1-41, 13C, CDCl3
C13CPD 128 CDCL3 /opt/data ecaokv 37
77.34 CDCl3
76.70 CDCl3
55.57
41.68
f1 (ppm)

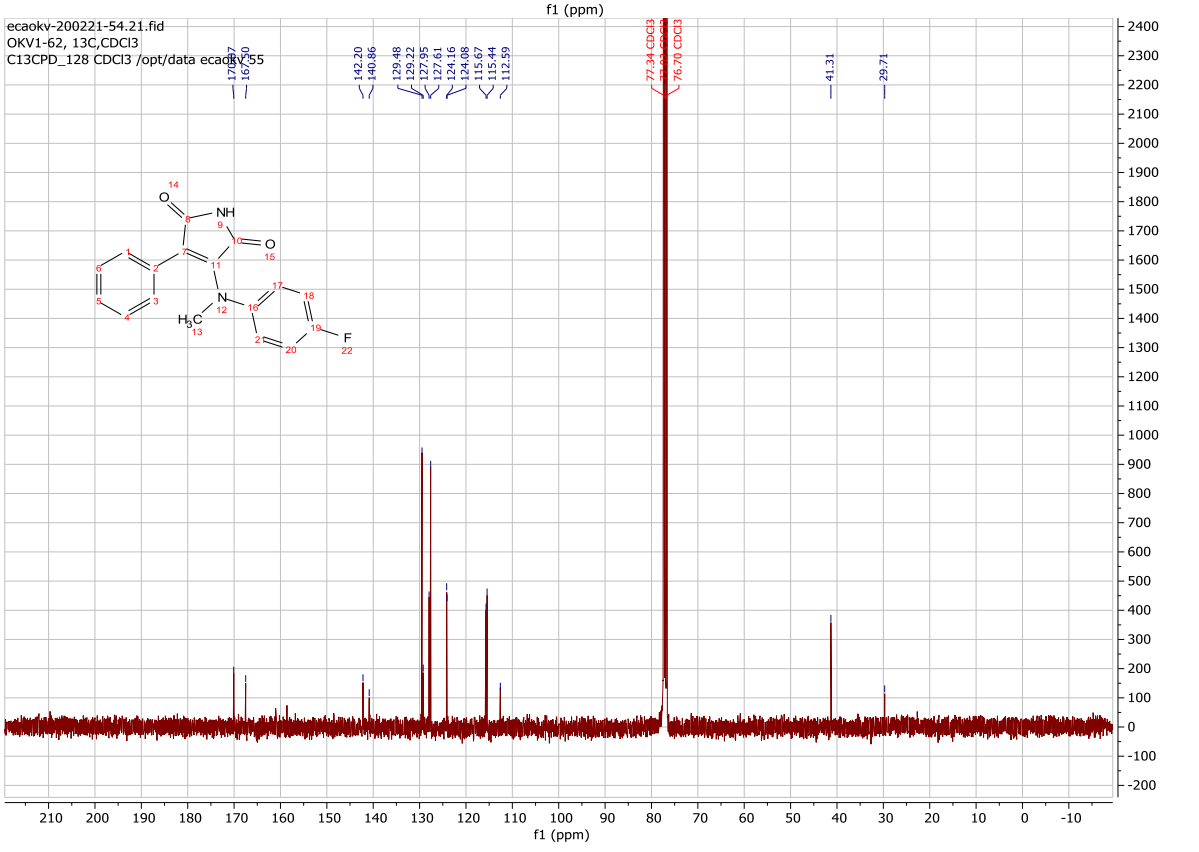
PMI-17


PMI-17 1H.10.fid
ecaaes-1 (PMI-17)
1.62 H2O
3.55
0.93
1.76
6.00
2.69
f1 (ppm)

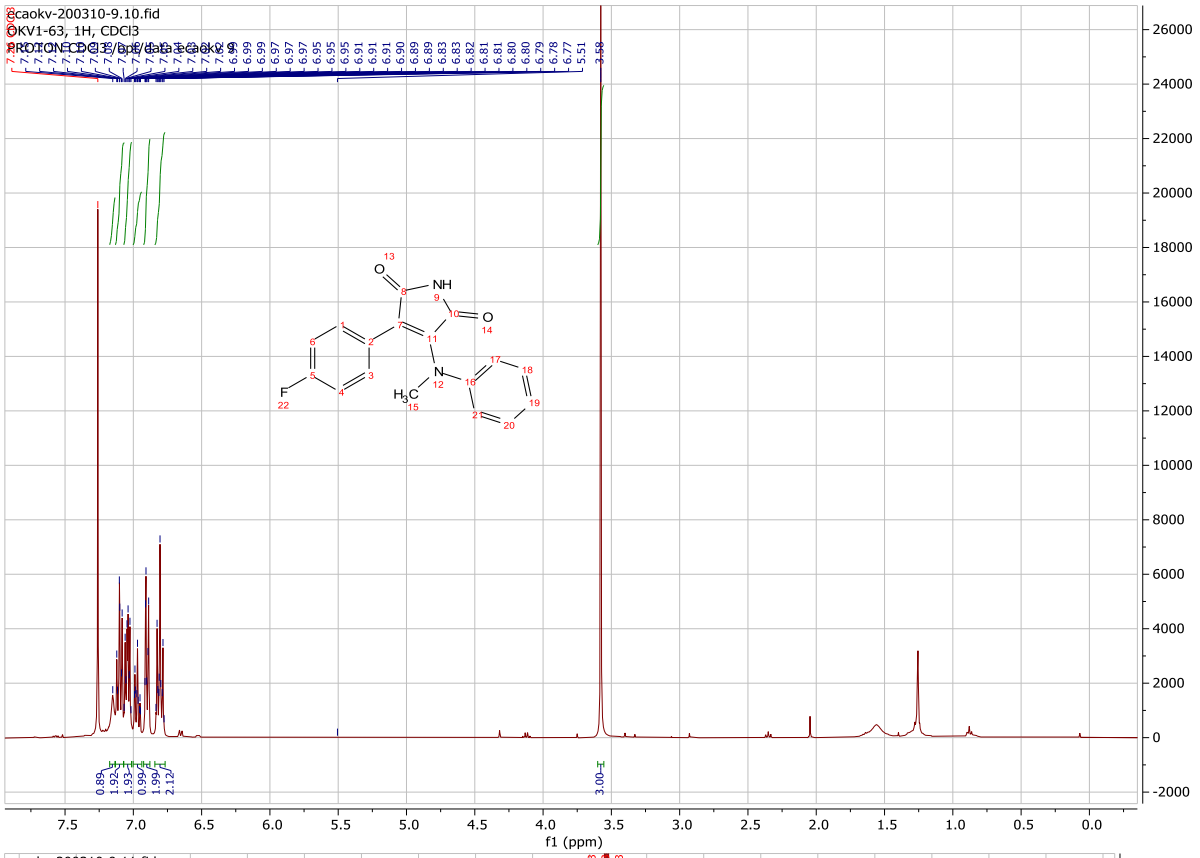

PMI-17 2.10.fid
ecaaes-1 (PMI-17)
170.21
167.55
163.37
161.39
160.97
159.02
142.18
140.76
140.73
131.35
131.29
125.41
125.39
124.36
124.29
115.85
115.67
114.91
114.74
111.25
77.41 CDCl3
77.16 CDCl3
76.90 CDCl3
41.34
f1 (ppm)

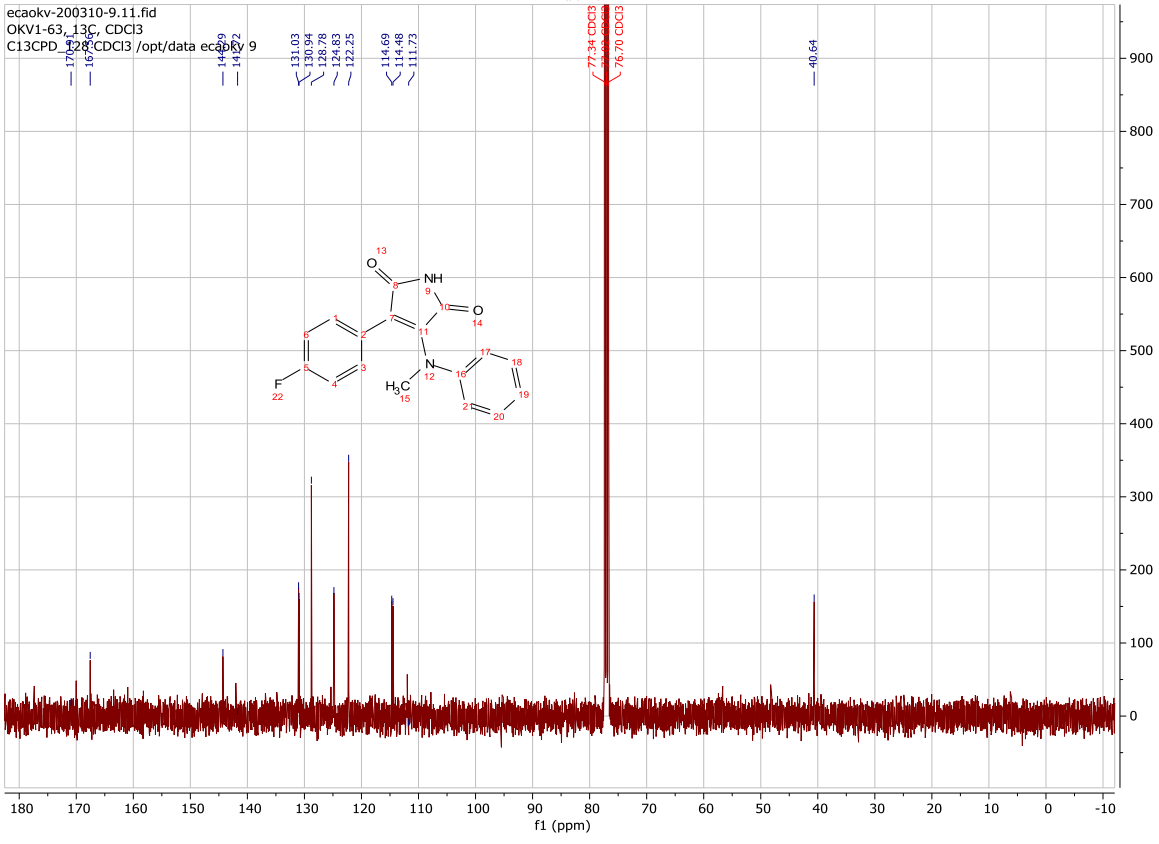
PMI-18


ecaokv-200221-54.20.fid
OKV1-62, 1H,CDCl3
PROTON_CDCl3 /opt/data ecaokv 55
f1 (ppm)

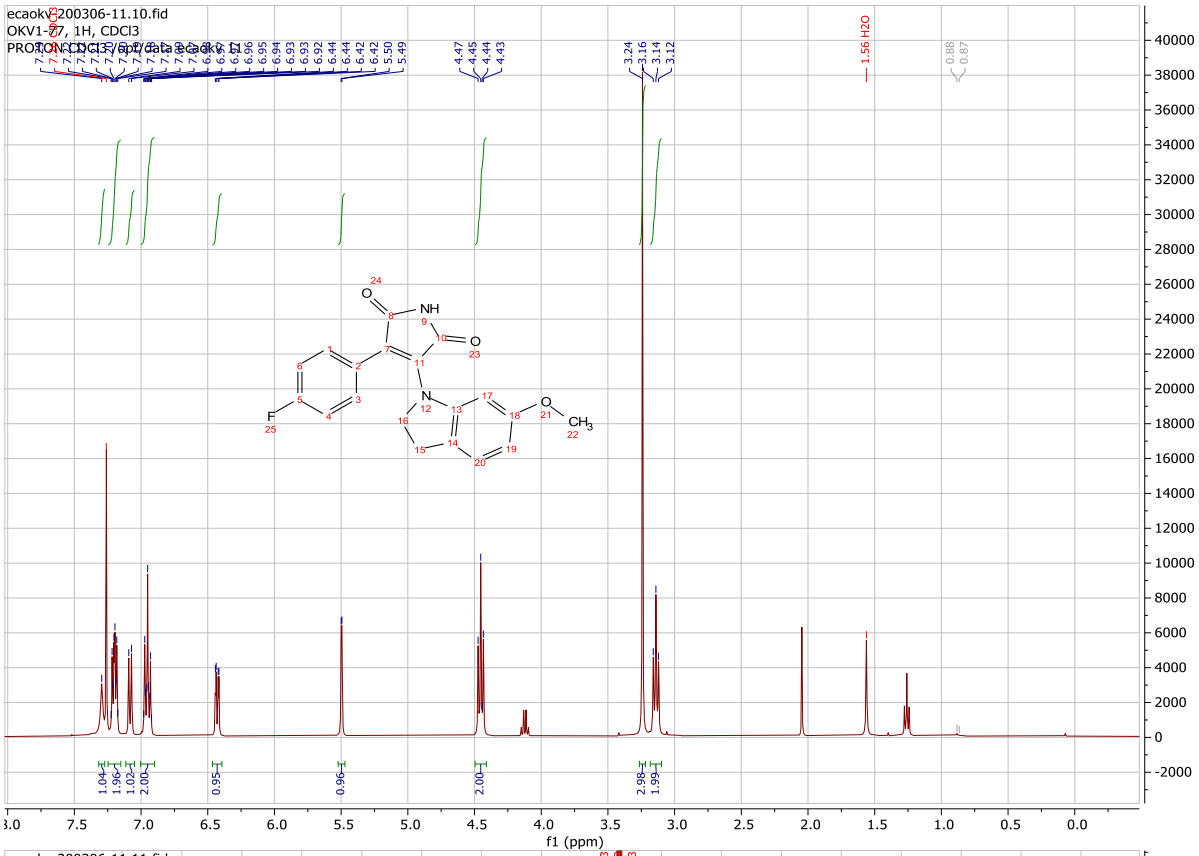

ecaokv-200221-54.21.fid
OKV1-62, 13C,CDCl3
C13CPD_128 CDCl3 /opt/data ecaokv 55
77.34 CDCl3
76.70 CDCl3
f1 (ppm)

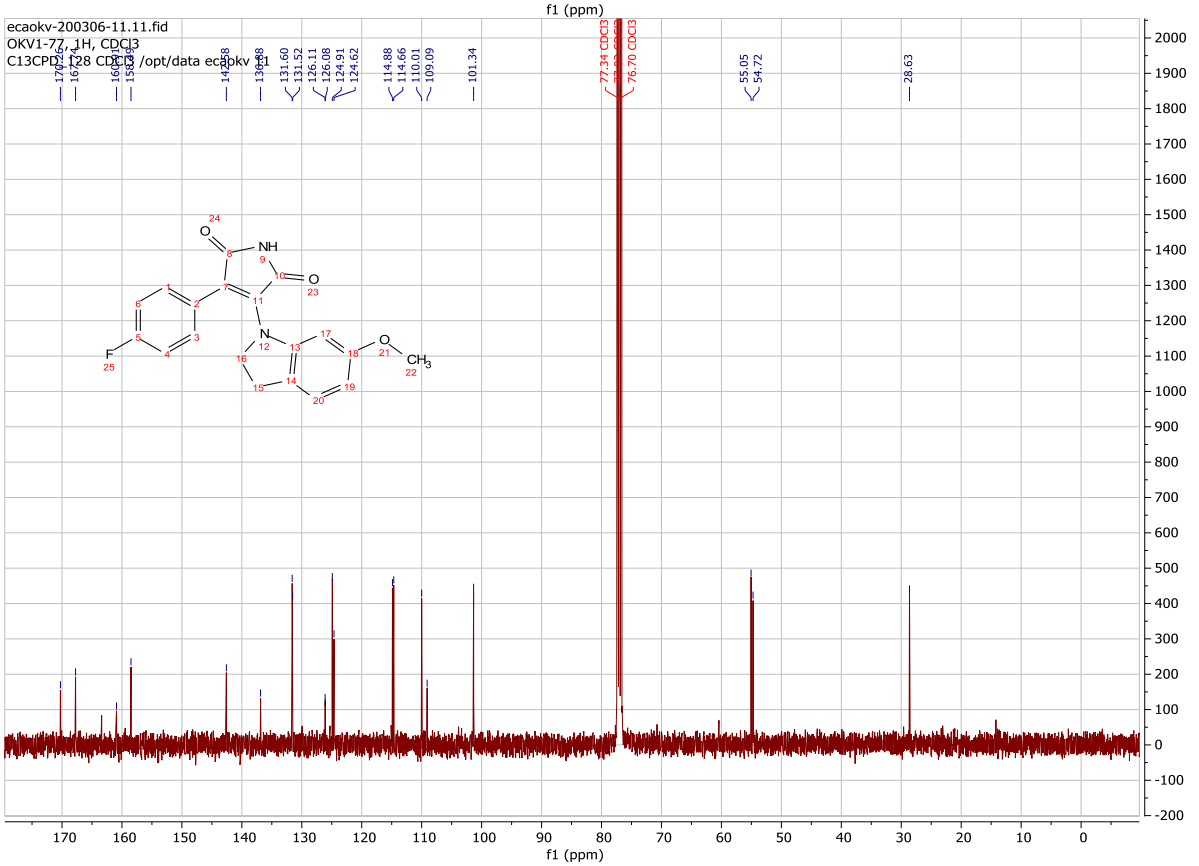
PMI-19


ecaokv-200310-9.10.fid
OKV1-63, 1H, CDCl3
f1 (ppm)


ecaokv-200310-9.11.fid
OKV1-63, 13C, CDCl3
f1 (ppm)

PMI-20


ecaokv-200306-11.10.fid
OKV1-77, 1H, CDCl3
PROTON,CDCl3 /opt/data ecaokv-11
1.56 H2O
f1 (ppm)


ecaokv-200306-11.11.fid
OKV1-77, 1H, CDCl3
C13CPD, 128 CDCl3 /opt/data ecaokv-11
77.34 CDCl3
76.70 CDCl3
f1 (ppm)

References

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Metabolism Protocols and Data

Metabolism Methods and Data

Data obtained from Pharmaron.

Data Summary

Table 1. Metabolic Stability of Test Compounds in Pooled Human and Male Mouse Liver Microsomes (a)

Compound ID	Species	$T_{1/2}$ (min)	CL_{int} (μL/min/mg protein)	Scaled-up CL_{int} (mL/min/Kg)
Diclofenac	Human	6.54	211.98	265.85
	Mouse	22.99	60.28	263.74
ERPM4 (PMI-4)	Human	51.10	27.12	34.02
	Mouse	11.21	123.65	540.97
ERPM5 (PMI-5)	Human	18.23	76.04	95.36
	Mouse	<2.26*	>614.02	>2686.34

* If % remaining at 15 minutes was lower than 1%, then CL_{int} and $t_{1/2}$ will be reported as ">614.02" and "<2.26", respectively.

Table 2. Metabolic Stability of Test Compounds in Pooled Human and Male Mouse Liver Microsomes (b)

Compound ID	Species	Assay Format	Remaining Percentage (%)				
			0 min	15 min	30 min	45 min	60 min
Diclofenac	Human	With NADPH	100.00	9.95	4.16	2.59	1.66
		Without NADPH	100.00	94.81	92.08	93.17	94.54
	Mouse	With NADPH	100.00	49.55	40.48	33.87	32.79
		Without NADPH	100.00	90.58	93.48	95.89	95.17
ERPM4 (PMI-4)	Human	With NADPH	100.00	79.42	62.95	52.86	44.33
		Without NADPH	100.00	90.27	85.79	93.77	90.27
	Mouse	With NADPH	100.00	30.74	10.29	4.55	2.52
		Without NADPH	100.00	88.19	85.78	85.06	81.20
ERPM5 (PMI-5)	Human	With NADPH	100.00	52.67	28.28	15.80	10.55
		Without NADPH	100.00	94.06	97.24	92.78	103.61
	Mouse	With NADPH	100.00	0.95	0.00	0.00	0.00
		Without NADPH	100.00	96.02	93.81	95.35	93.14

Materials

1. Test compounds were provided by the sponsor. The compound information is listed in Table 3.

Table 3. Compound Information

Compound ID	PH ID	MW	FW	PH Lot #
ERPM4	-	359.21	-	-
ERPM5	-	304.35	-	-

2. The liver microsomes were stored at -80°C prior to use. The liver microsomes information is listed in Table 4.

Table 4. Liver Microsomes Information

Species	Cat. No.	Lot. No.	Sponsor
Human	452117	38292	Corning
Mouse	M1000	1710069	Xenotech

Study Design

1. The master solution was prepared according to Table 5.

Table 5. Preparation of Master Solution

Reagent	Stock Concentration	Volume	Final Concentration
Phosphate buffer	200 mM	200 µL	100 mM
Ultra-pure H_2O	-	66 µL	-
$MgCl_2$ solution	50 mM	40 µL	5 mM
Alamethacin	5 mg/mL	2 µL	25 µg/mL
Microsomes	20 mg/mL	10 µL	0.5 mg/mL

2. 40 µL of 10 mM NADPH solution and 40 µL of 20 mM UDPGA solution were added to each well. The final concentrations of NADPH and UDPGA were 1 mM and 2 mM. The mixture was pre-warmed at 37°C for 5 minutes. The negative control samples were prepared by replacing cofactors (NADPH and UDPGA) solution with 80 µL of ultra-pure H_2O. The negative control was used to exclude the misleading factor that resulted from instability of chemical itself. Samples with cofactors were prepared in duplicate. Negative controls were prepared in singlet.

3.The reaction was started with the addition of 2 µL of 400 µM control compound or test compound solutions. Diclofenac was used as positive control in this study. The final concentration of test compound or control compound was 2 µM.

4. Aliquots of 50 µL were taken from the reaction solution at 0, 15, 30, 45 and 60 minutes. The reaction was stopped by the addition of 4 volumes of cold acetonitrile with IS (100 nM alprazolam, 200 nM imipramine, 200 nM labetalol and 2 µM ketoprofen). Samples were centrifuged at 3, 220 g for 40 minutes. Aliquot of 90 µL of the supernatant was mixed with 90 µL of ultra-pure H_2O and then used for LC-MS/MS analysis.

5. Data Analysis

All calculations were carried out using Microsoft Excel.

Peak areas were determined from extracted ion chromatograms. The slope value, k, was determined by linear regression of the natural logarithm of the remaining percentage of the parent drug vs. incubation time curve.

The in vitro half-life (in vitro $t_{1/2}$) was determined from the slope value:

$$in\ vitro\ t_{1/2} = -(0.693 / k)$$

Conversion of the in vitro $t_{1/2}$ (min) into the in vitro intrinsic clearance (in vitro CL_{int}, in µL/min/mg protein) was done using the following equation (mean of duplicate determinations):

$$in\ vitro\ CL_{int} = \left(\frac{0.693}{(t_{1/2})}\right) * \left(\frac{\text{volume of incubation (µL)}}{\text{amount of proteins (mg)}}\right)$$

Conversion of the in vitro $t_{1/2}$ (min) into the scale-up unbound intrinsic clearance (Scale-up CL_{int}, in mL/min/kg) was done using the following equation (mean of duplicate determinations):

$$\text{Scale-up } CL_{int} = \left(\frac{0.693}{(t_{1/2})}\right) * \left(\frac{\text{volume of incubation (mL)}}{\text{amount of proteins (mg)}}\right) * \text{Scaling Factor}$$

Table 6. Scaling Factors for Intrinsic Clearance Prediction in Liver Microsomes

Species	Liver Weight (g liver/kg body weight)[a]	Microsomal Concentration (mg/g liver)[b]	Scaling Factor	Liver blood flow (Q, mL/min/kg)[a]
Human	25.7	48.8	1254.2	20.7
Mouse	87.5	50.0	4375.0	90.0

a. Iwatsubo et al, Davies and Morris, 1993, 10 (7) pp 1093-1095.

b. Barter et al, 2007, Curr Drug Metab, 8(1), pp 33-45; Iwatsubo et al, 1997, JPET, 283 pp 462-469.

Bioanalytical Method

1. Chromatographic conditions

LC system: Shimadzu

MS analysis: API 4000 instrument from AB Inc (Canada) with an ESI interface

Column temperature: RT

Injection volume: 3 µL

Column: Phenomenex Kinetex 2.6 µ EVO C18 100A with preguard column

Mobile phase: 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B)

Time (min)	0	0.3	0.9	1.2	1.5	2.0
% A	5	5	100	100	5	5

Elution rate: 0.6 mL/min

2. MS parameters

Ion source: Turbo spray

Ionization model: ESI

Scan type: MRM

Curtain gas: 30 L/min

Nebulize gas: 50 L/min

Auxiliary gas: 50 L/min

Temperature: 500°C

Ionspray voltage: 5500 v (positive MRM)

Compound ID	Q1 (*m/z*)	Q3 (*m/z*)	DP (v)	EP (v)	CE (v)	CXP (v)
ERPM4	360.0	325.1	100	10	30	12
ERPM5	305.1	260.1	80	10	30	12

Metabolite Identification of ERPM5 in Mouse Liver Microsomes

Pharmaron Study Number: ADME-ITD-190726

Pharmaron Report Number: ADME-ITD-MetID-20190808

Date of Release: August 08, 2019

In vitro ADME Laboratory
Pharmaron

Signature

Quality Control : Chunyan Jin Date: 08/08/2019
Chunyan Jin

Study Director : Dan Wang Date: 08108/2019
Dan Wang

Report Approval : Yu Wang Date: 08/08/2019
Yu Wang

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Content

- **Experimental procedure**
- **Instrumentation & analytical conditions**
- **Flow chart of metabolite identification**
- **Results**
  - ✓Metabolite identification for ERPM5
- **Conclusions**

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Experimental Procedure

Incubation and Sample Preparation Protocol

- Final concentrations in *in vitro* incubations:

Test compounds	**ERPM5**
Test concentration	10 μM
Biological Matrices	Mouse liver microsomes (2 mg/mL)
Cofactor	NADPH (2 mM), UDPGA (5 mM), Alamethicin (0.1mg/mL)
Incubation time	0, 5, 15 min @ 37 °C
Total volume	200 μL

- Liver microsomes information

Species	Strain & Gender	Cat. No.	Lot. No.	Source
Mouse	Male, CD-1	M00501	CBS	BIOIVT

- Incubations were quenched with 3 volumes of acetonitrile followed by centrifugation for 15 min at 16,000 g;
- Supernatant was then analyzed by LC-MS/MS.

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Instrumentation & Analytical Conditions

Instrumentation

- Dionex UltiMate 3000 UHPLC system (Thermo Fisher Scientific, USA)
- Thermo Scientific Q Exactive (Thermo Fisher Scientific, USA)

LC conditions

- Column: Waters XSelect HSS T3, 100 × 2.1 mm, 2.5 μm
- Solvents: A, water (0.1% formic acid); D, acetonitrile (0.1% formic acid)
- Flow rate: 500 μL/min
- Program: 0-1.5 min, 10%D, 1.5-9 min, 10%-75%D, 9-12 min, 75%-100%D, 12-14min, 100%D, 14-14.3 min, 100%-10%D, 14.3-15 min, 10%D;

MS conditions

- Ionisation mode: Positive mode
- Spray Voltage: 3.5 kV
- Aux gas flow rate: 15
- Aux gas heater temp (°C): 350 °C
- Scan type: Full MS/ddMS^2
- Resolution: 70,000
- AGC Target: 3 × e^6
- NCE/stepped NCE: 25, 35, 45

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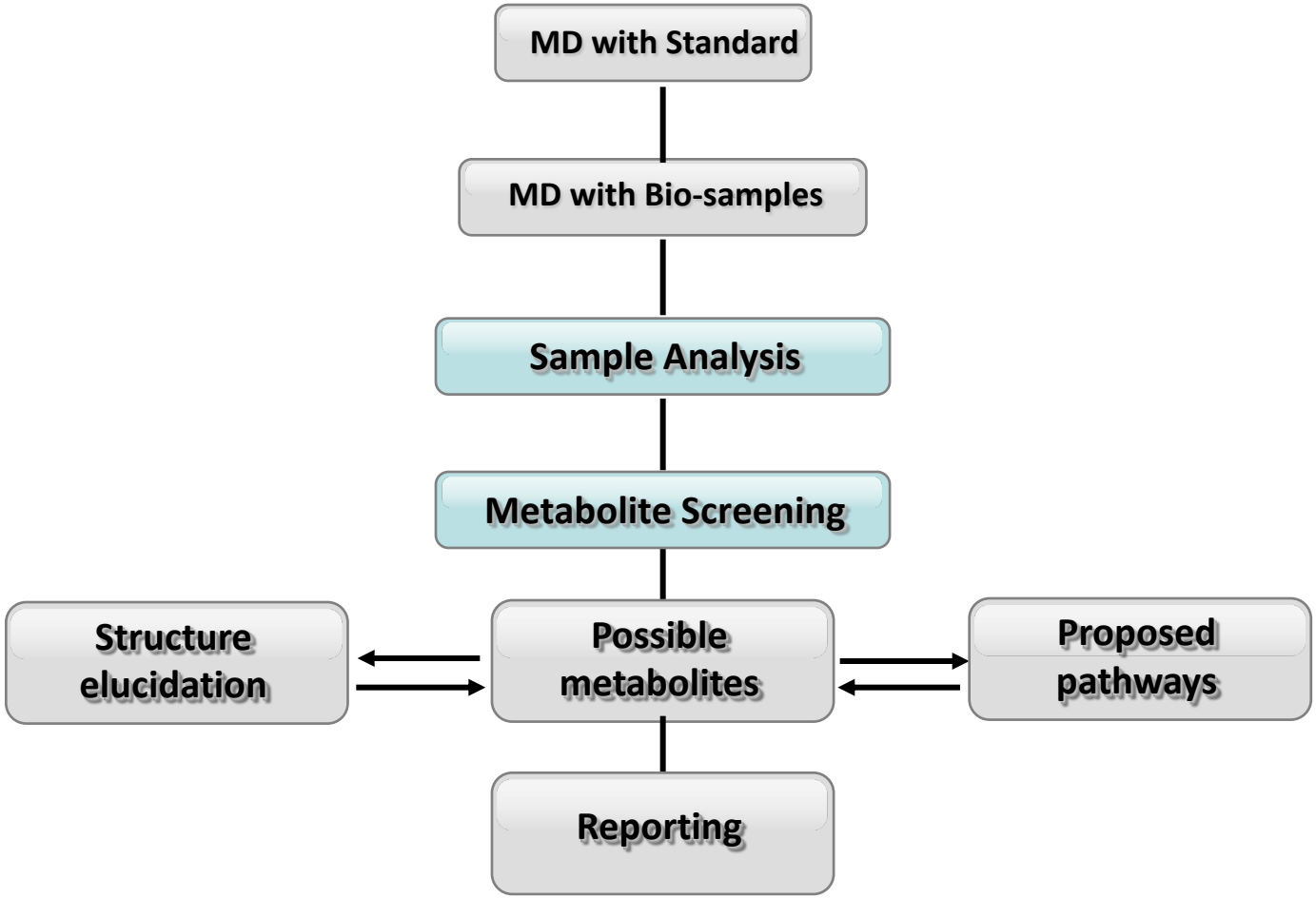
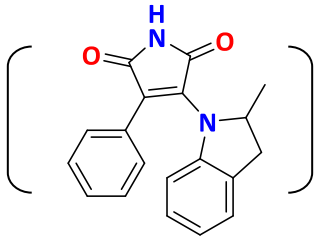
Flow Chart of Metabolite Identification



Results – ERPM5

Oxidation
Hydrogenation
De-hydrogenation

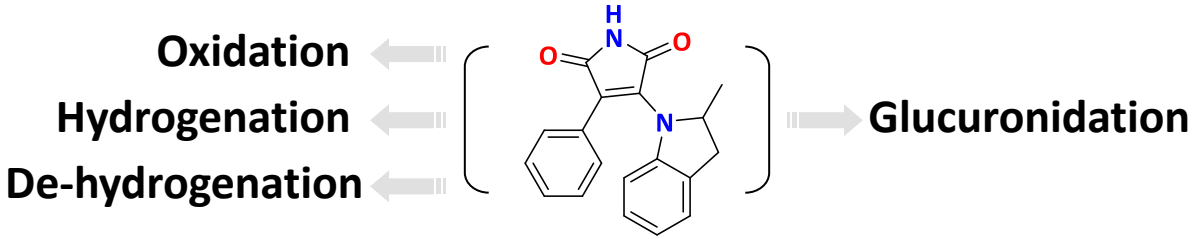


Glucuronidation

Peak No.	R.T. (min)	Meas. *m/z*	Mass error (ppm)	Mass shift	Biotransformation	Metabolites peak area @ 15 min
1	4.47	495.13907	-1.5	190.01136	Oxidation + De-hydrogenation + Glucuronidation	++
2	4.75	513.14960	-1.5	208.02192	Oxidation + Glucuronidation	+
3	4.95	515.16528	-1.4	210.03757	Oxidation + Hydrogenation + Glucuronidation	+
4	5.15	497.15454	-1.9	192.02701	Oxidation+ Glucuronidation	+++
5	5.51	497.15460	-1.7	192.02701	Oxidation+ Glucuronidation	+
6	5.55	335.10199	-1.9	29.97418	Oxidation + De-hydrogenation	++
7	5.56	495.13840	-2.8	190.01136	Oxidation + De-hydrogenation + Glucuronidation	++
8	5.62	337.11752	-2.3	31.98983	Oxidation	++
9	5.71	337.11752	-2.3	31.98983	Oxidation	+
10	5.71	303.11212	-2.2	-2.01565	De-hydrogenation	++

[1]: molecular ion with losing H_2O;
"-": not observed; "+": trace (< 1% relative peak area); "++": minor (1-10% relative peak area); "+++": major (> 10% relative peak area);
Relative peak area determined from extracted ion chromatograms of liver microsomal samples at 15 min.

Results – ERPM5



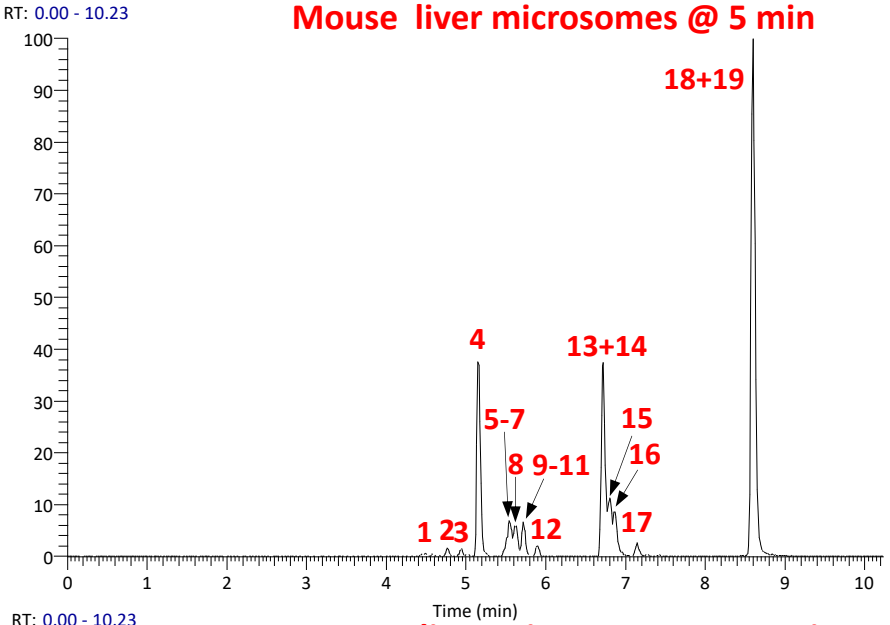
Peak No.	R.T. (min)	Meas. *m/z*	Mass error (ppm)	Mass shift	Biotransformation	Metabolites peak area @ 15 min
11	5.72	335.10196	-2.0	29.97418	Oxidation + De-hydrogenation	+
12	5.89	339.13312	-2.4	34.00548	Oxidation + Hydrogenation	+
13	6.71	337.11783[1]	-1.3	31.98983	Oxidation	++
14	6.72	321.12262	-2.3	15.99492	Oxidation	++
15	6.80	303.11206	-2.4	-2.01565	De-hydrogenation	++
16	6.86	321.12280	-1.8	15.99492	Oxidation	+
17	7.14	321.12262	-2.3	15.99492	Oxidation	+
18	8.56	303.11209	-2.3	-2.01565	De-hydrogenation	+
19	8.59	305.12775	-2.3	0.00000	Parent drug	++

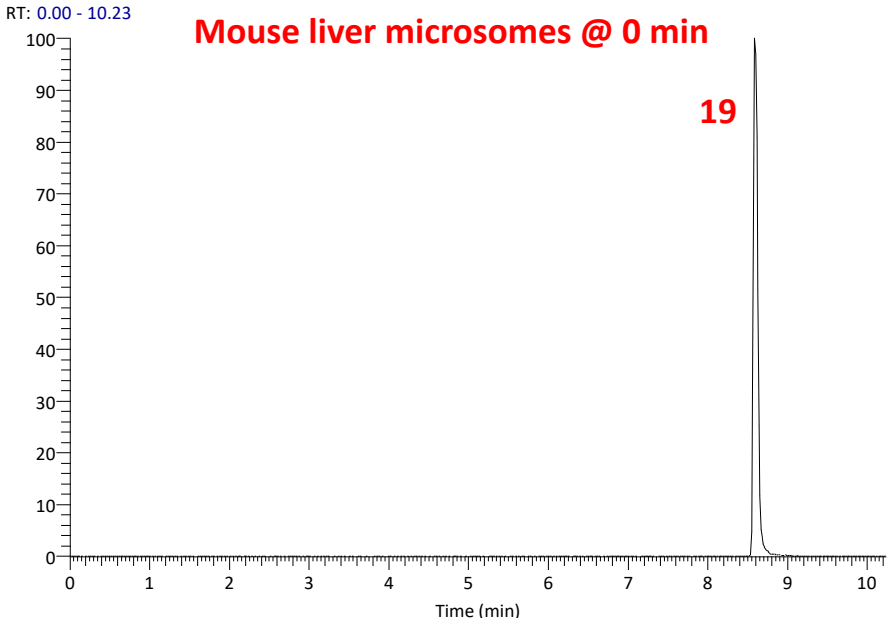
[1]: molecular ion with losing H_2O;
"-": not observed; "+": trace (< 1% relative peak area); "++": minor (1-10% relative peak area); "+++": major (> 10% relative peak area);
Relative peak area determined from extracted ion chromatograms of liver microsomal samples at 15 min.

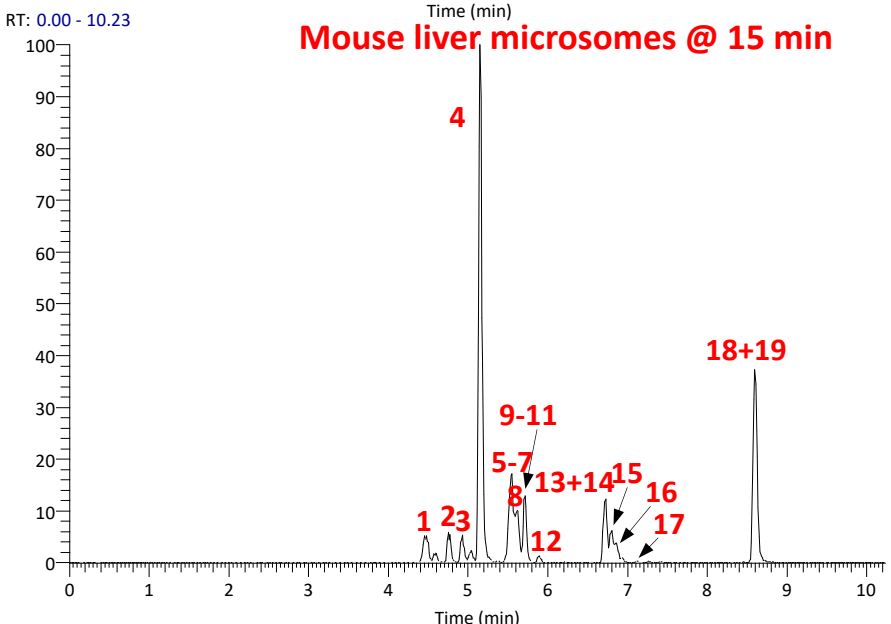
XIC of Full MS/ddMS^2 scan

- Mouse Liver Microsomes

- 18 metabolites were detected in mouse liver microsomal samples.



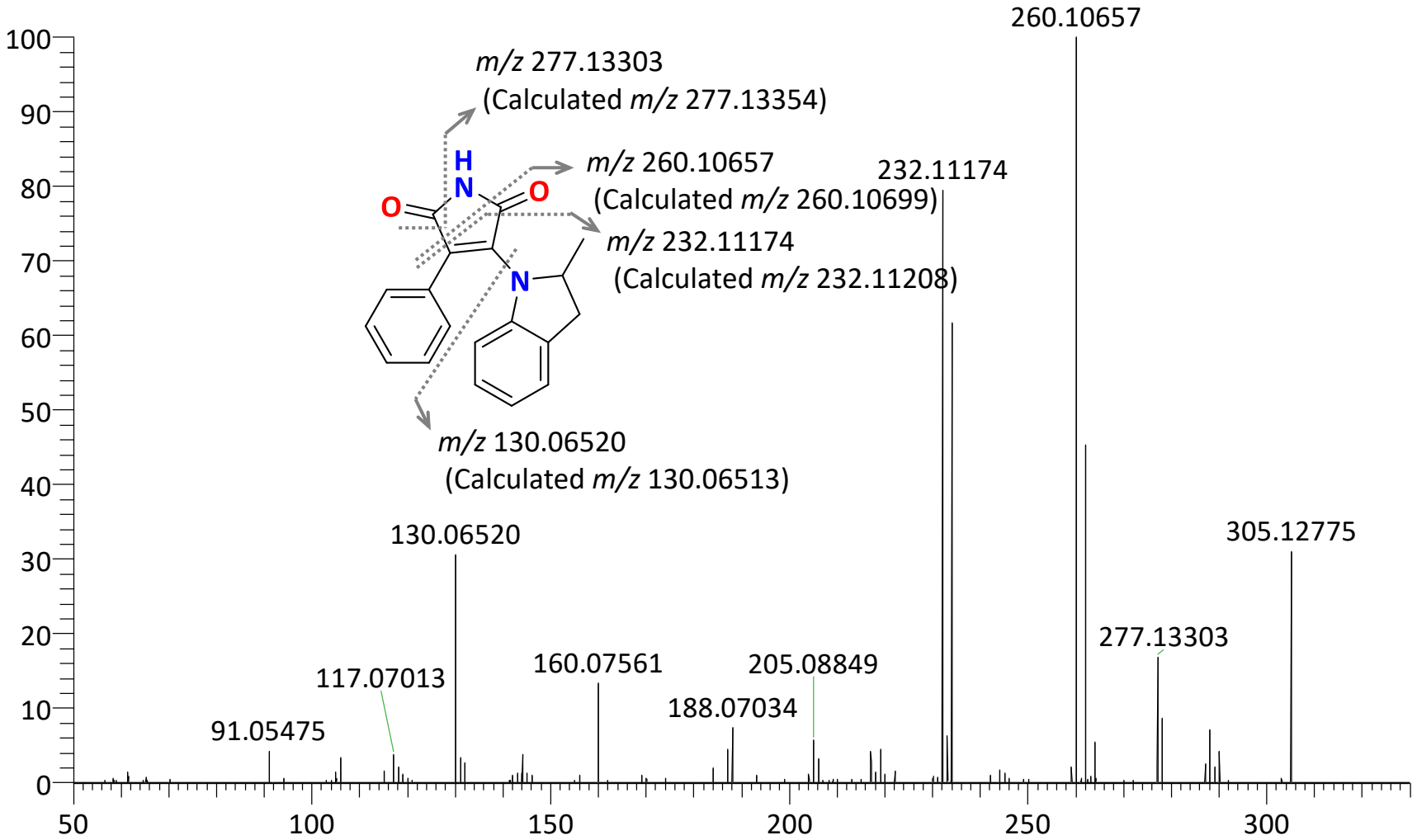




MS^2 fragmentation of ERPM5

RT: 8.59 min;
MS^2: 277.13303, 260.10657, 232.11174, 130.06520;

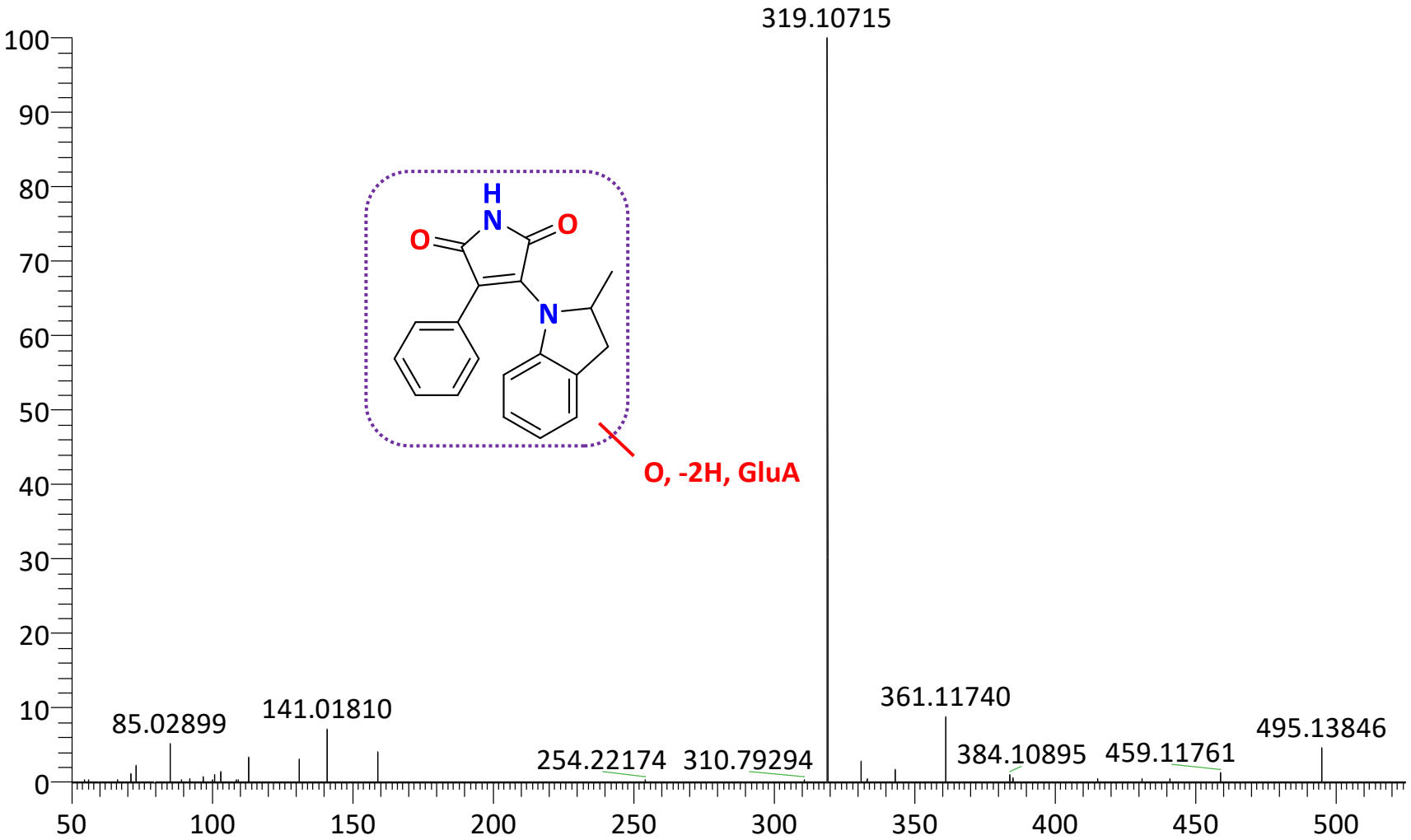
H^+ *m/z* 305.12775 (Calculated *m/z* 305.12845)



MS^2 fragmentation of M1

RT: 4.47 min;
MS^2: 319.10715;

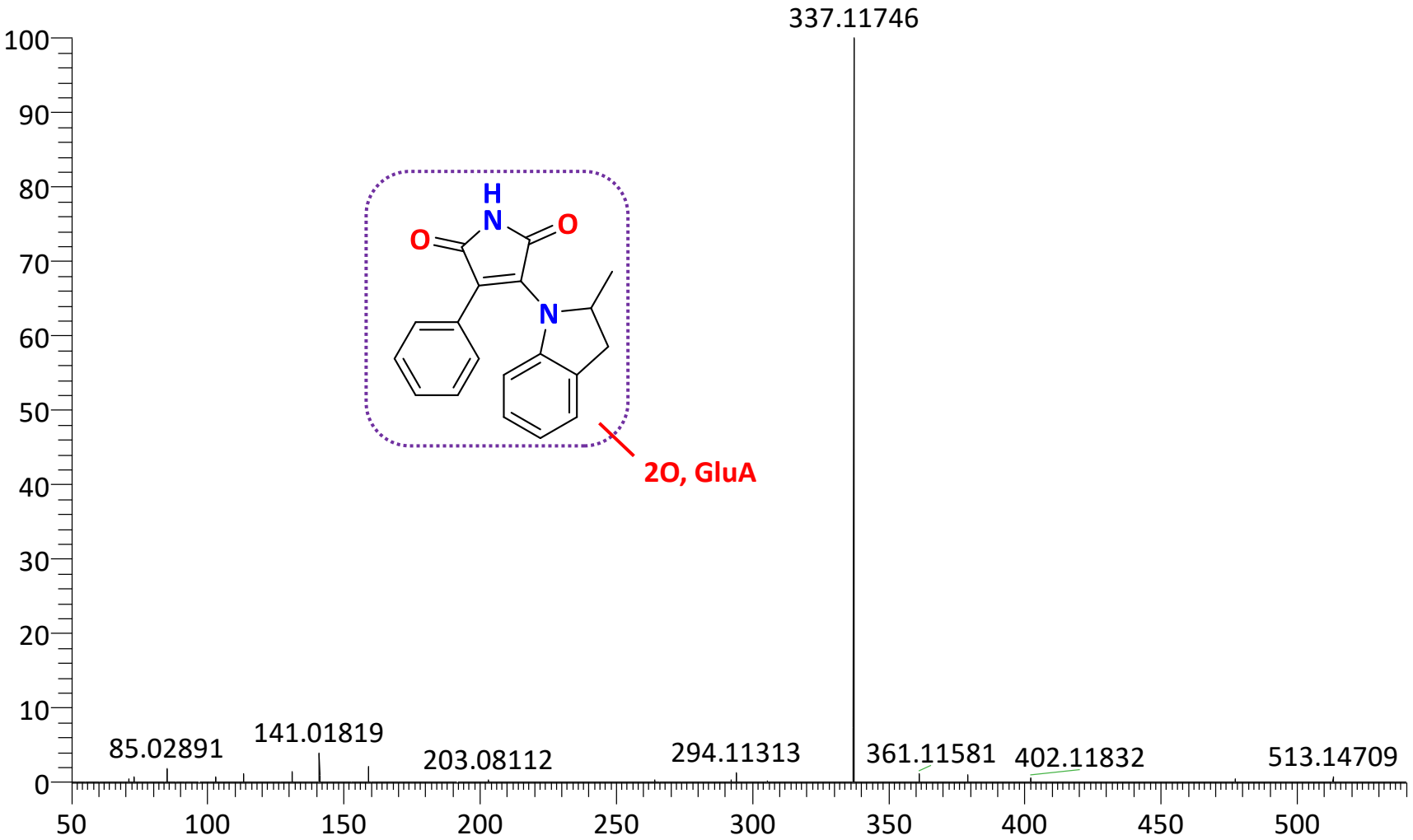
H^+ *m/z* 495.13846 (Calculated *m/z* 495.13981)
-GluA *m/z* 319.10715 (Calculated *m/z* 319.10772)



MS^2 fragmentation of M2

RT: 4.75 min;
MS^2: 337.11746;

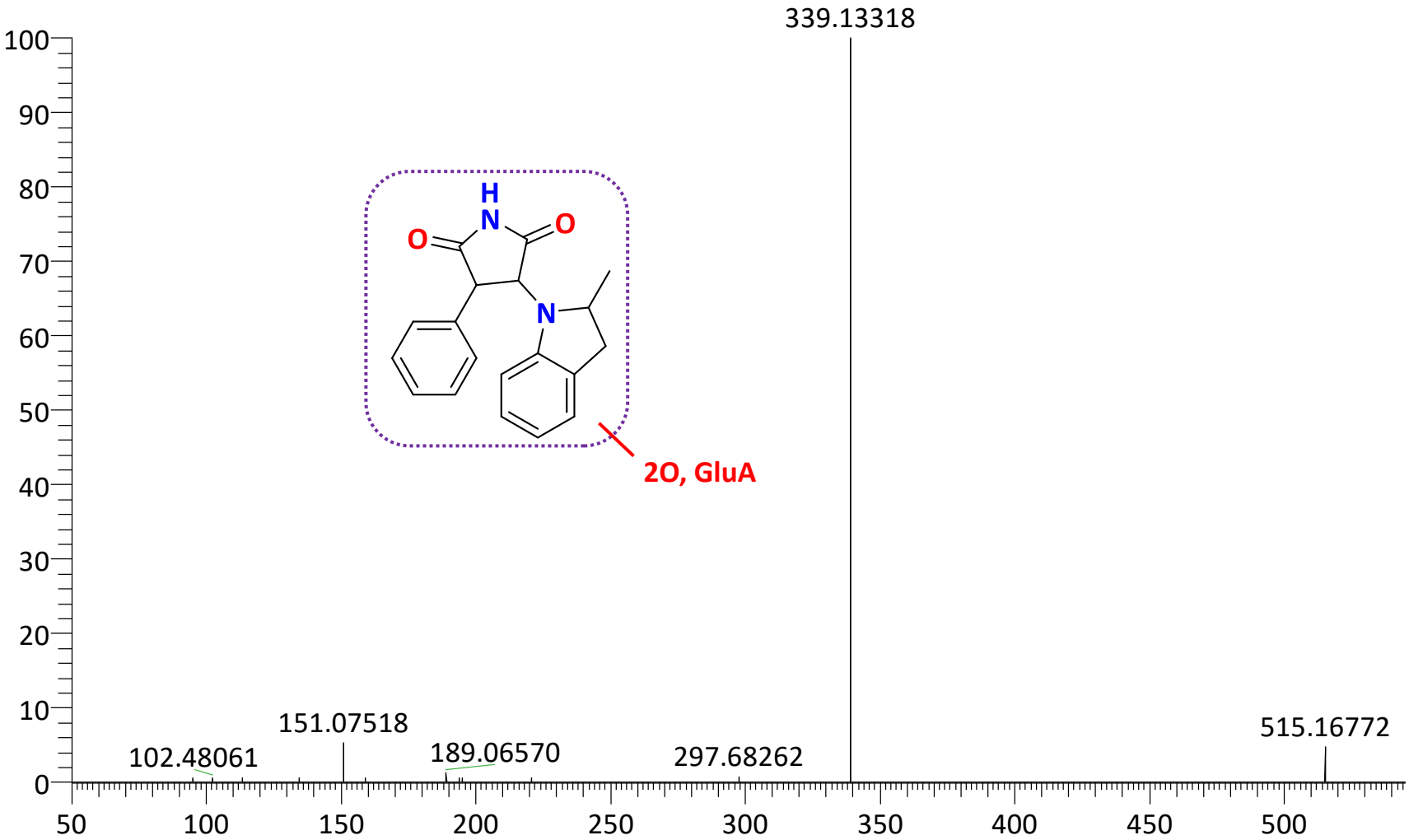
H^+ *m/z* 513.14709 (Calculated *m/z* 513.15037)
-GluA *m/z* 337.11746 (Calculated *m/z* 337.11828)



MS^2 fragmentation of M3

RT: 4.95 min;
MS^2: 339.13318;

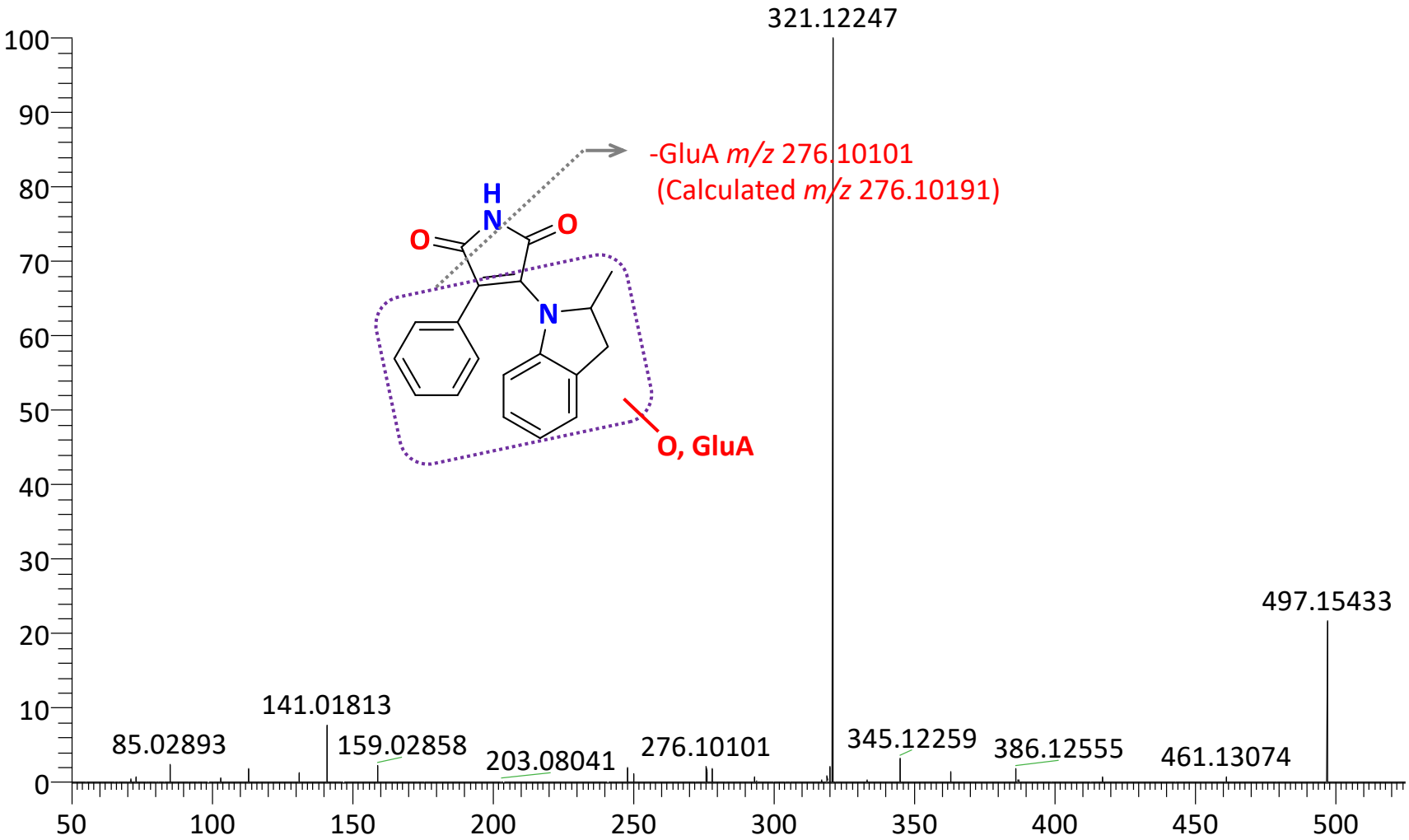
H^+ *m/z* 515.16772 (Calculated *m/z* 515.16602)
-GluA *m/z* 339.13318 (Calculated *m/z* 339.13393)



MS^2 fragmentation of M4

RT: 5.15 min;
MS^2: 321.12247, 276.10101;

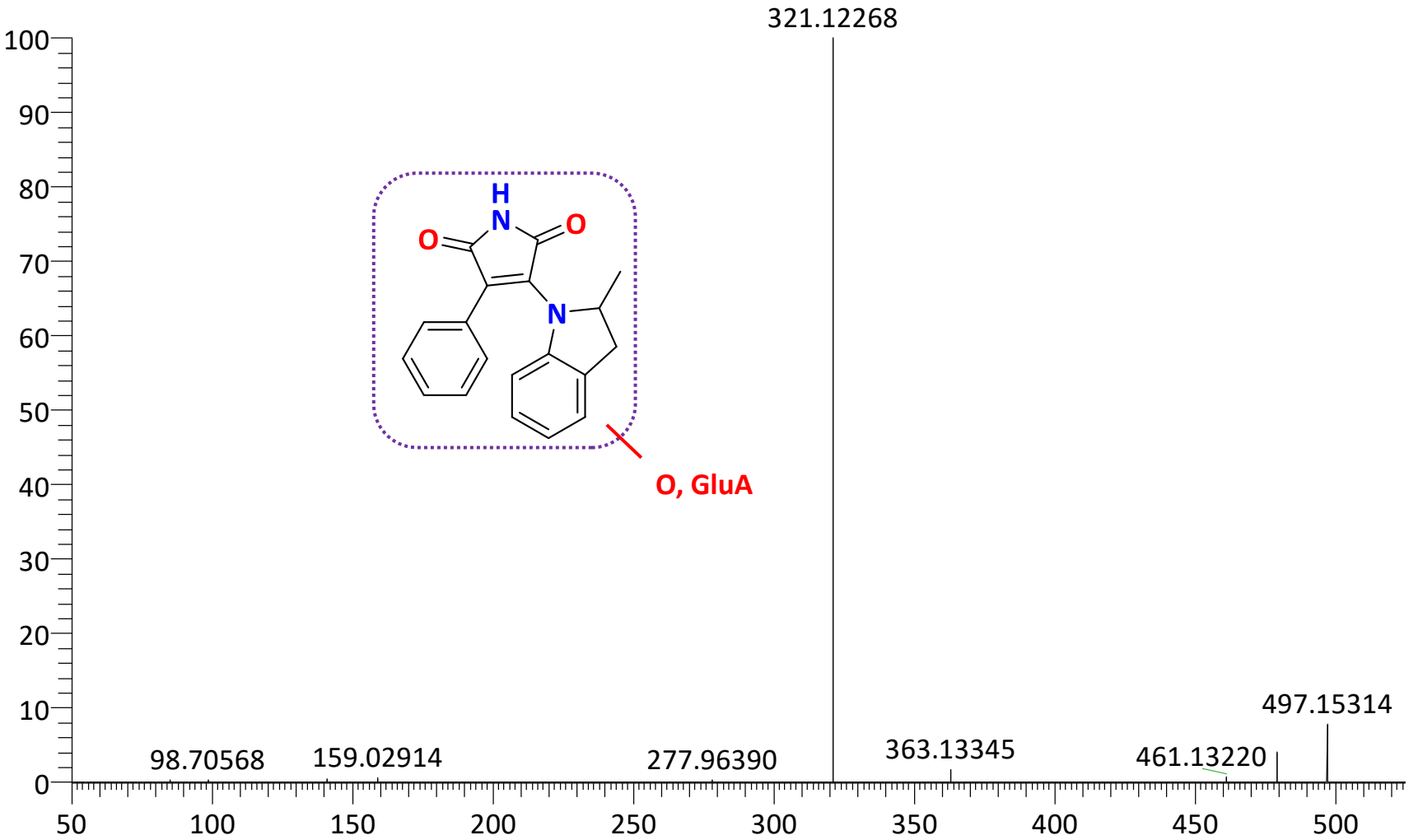
H^+ *m/z* 497.15433 (Calculated *m/z* 497.15546)
-GluA *m/z* 321.12247 (Calculated *m/z* 321.12337)



MS^2 fragmentation of M5

RT: 5.51 min;
MS^2: 321.12268;

H^+ *m/z* 497.15314 (Calculated *m/z* 497.15546)
-GluA *m/z* 321.12268 (Calculated *m/z* 321.12337)



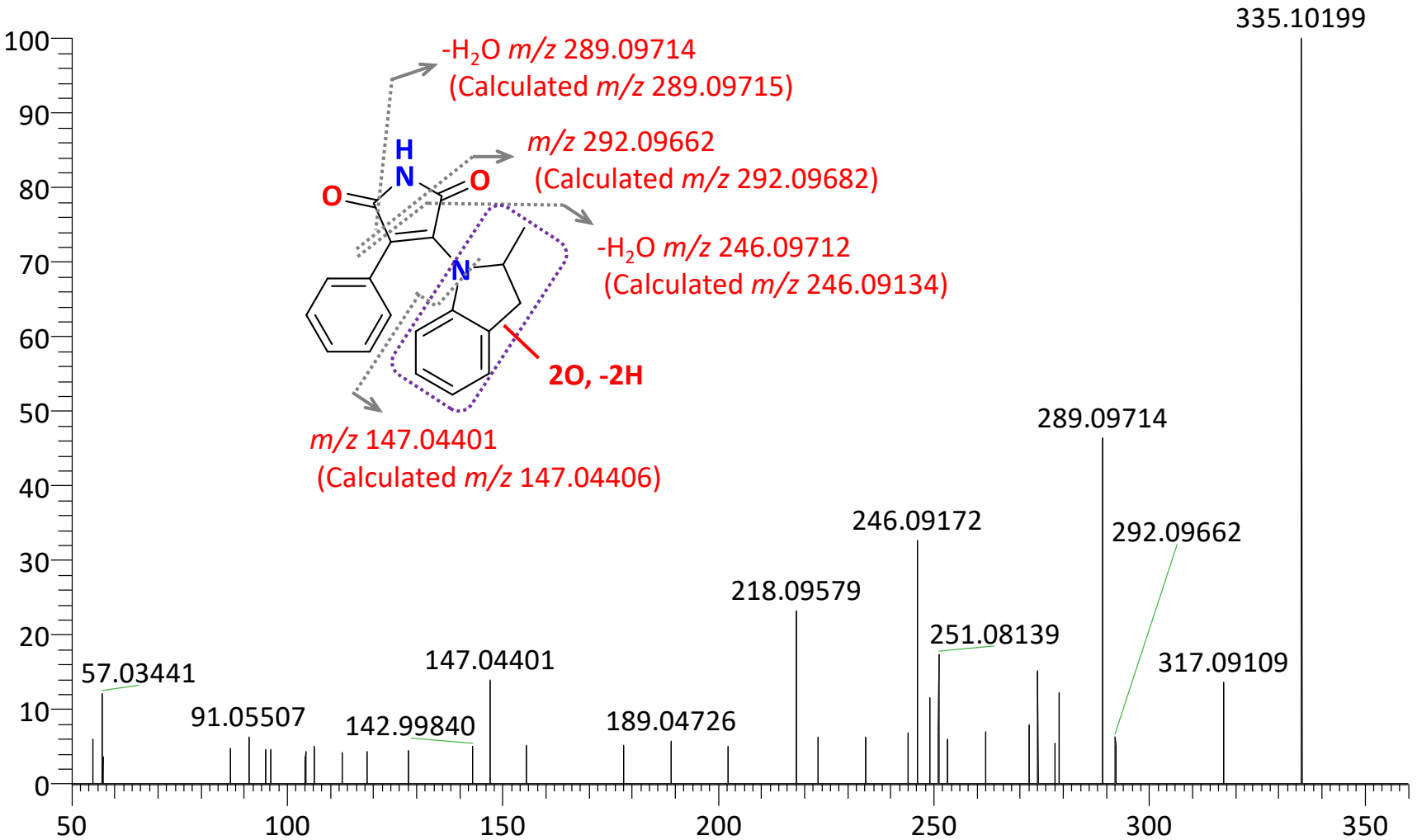
MS^2 fragmentation of M6

RT: 5.55 min;

MS^2: 317.09109, 292.09662, 289.09714, 246.09712, 147.04401;

H^+ *m/z* 335.10199 (Calculated *m/z* 335.10263)

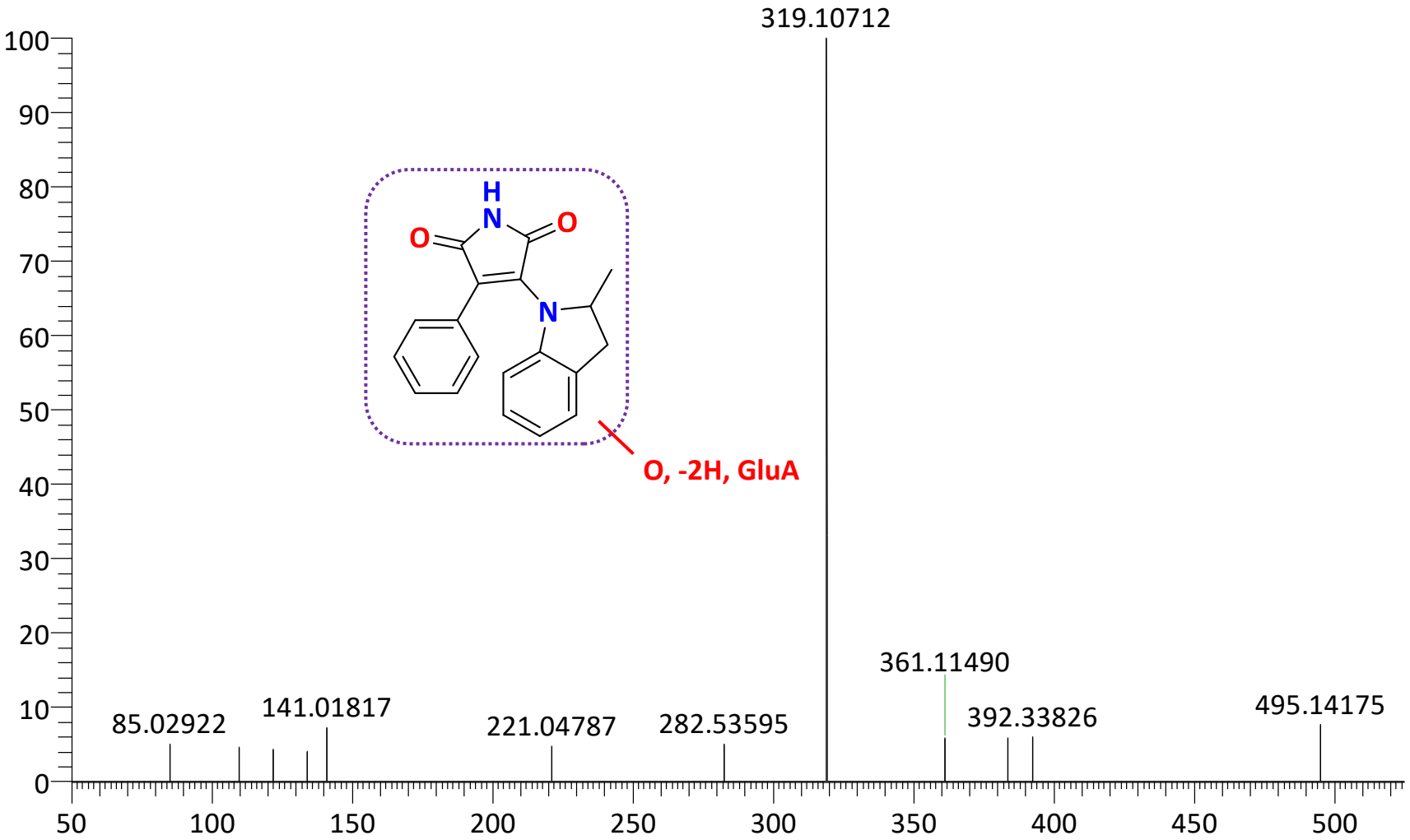
$-H_2O$ *m/z* 317.09109 (Calculated *m/z* 317.09207)



MS^2 fragmentation of M7

RT: 5.56 min;
MS^2: 319.10712;

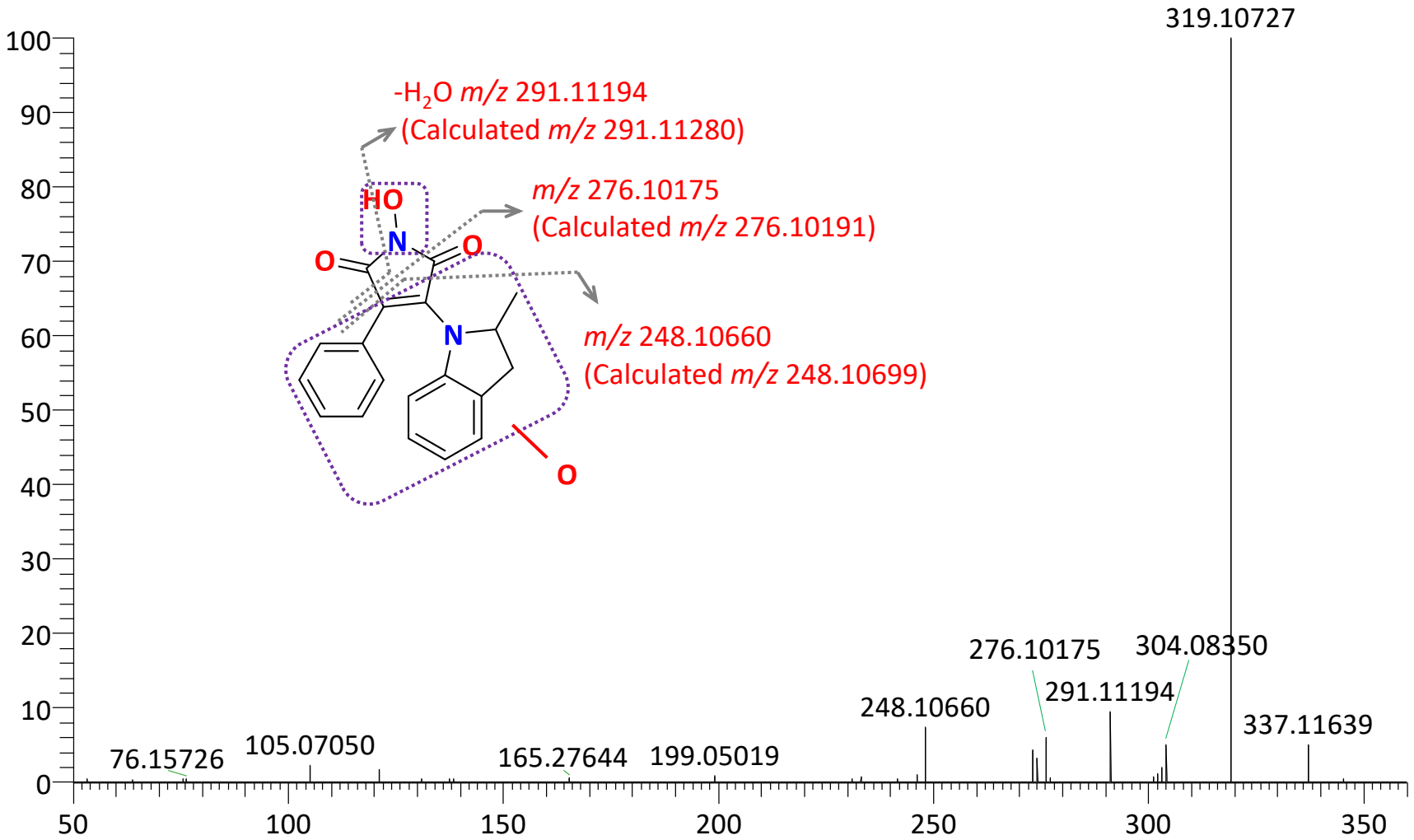
H^+ *m/z* 495.14175 (Calculated *m/z* 495.13981)
-GluA *m/z* 319.10712 (Calculated *m/z* 319.10772)



MS^2 fragmentation of M8

RT: 5.62 min;
MS^2: 319.10727, 291.11194, 276.10175, 248.10660;

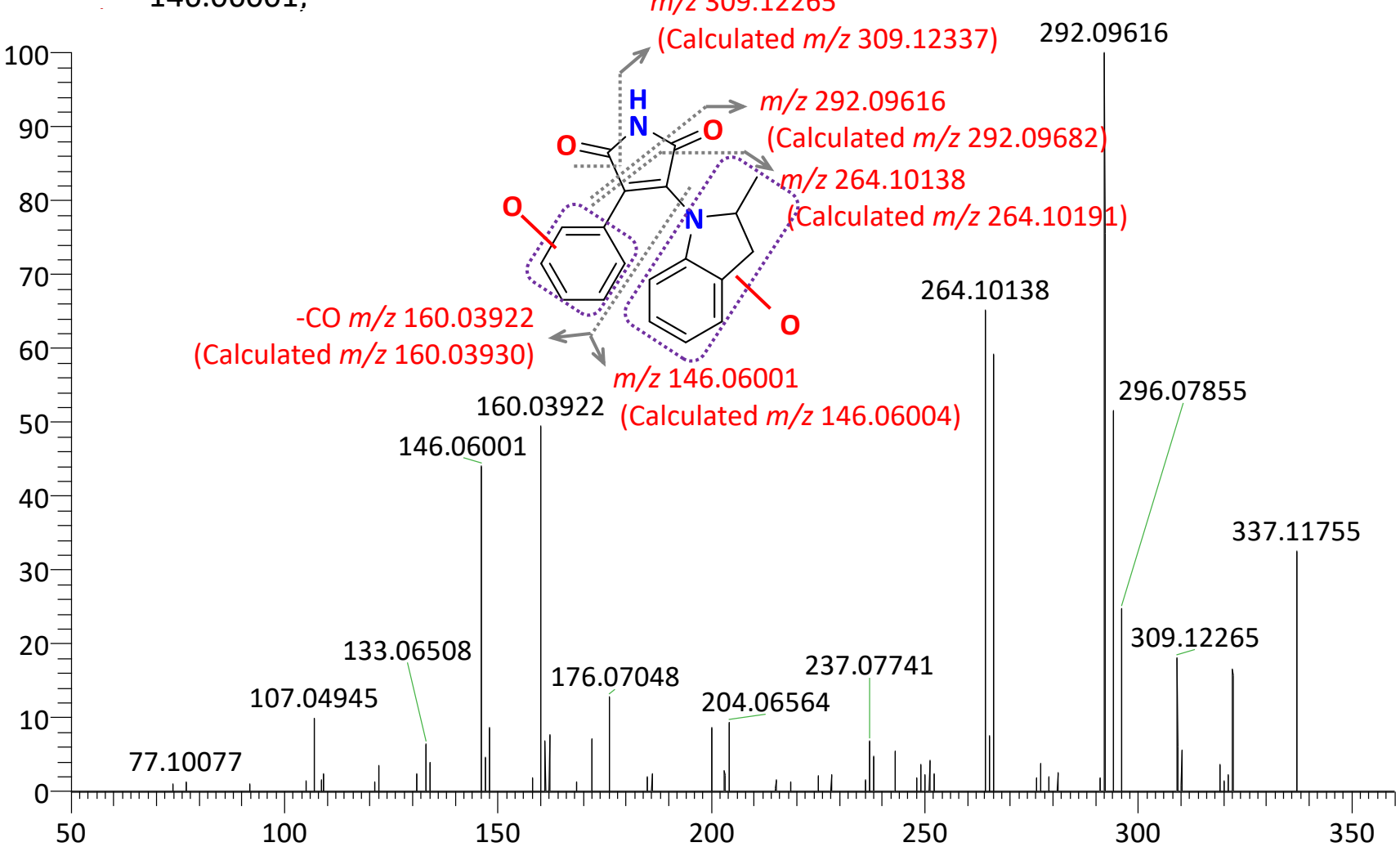
H^+ *m/z* 337.11639 (Calculated *m/z* 337.11828)
$-H_2O$ *m/z* 319.10727 (Calculated *m/z* 319.10772)



MS^2 fragmentation of M9

RT: 5.71 min;
MS^2: 309.12265, 292.09616, 264.10138, 160.03922, 146.06001;

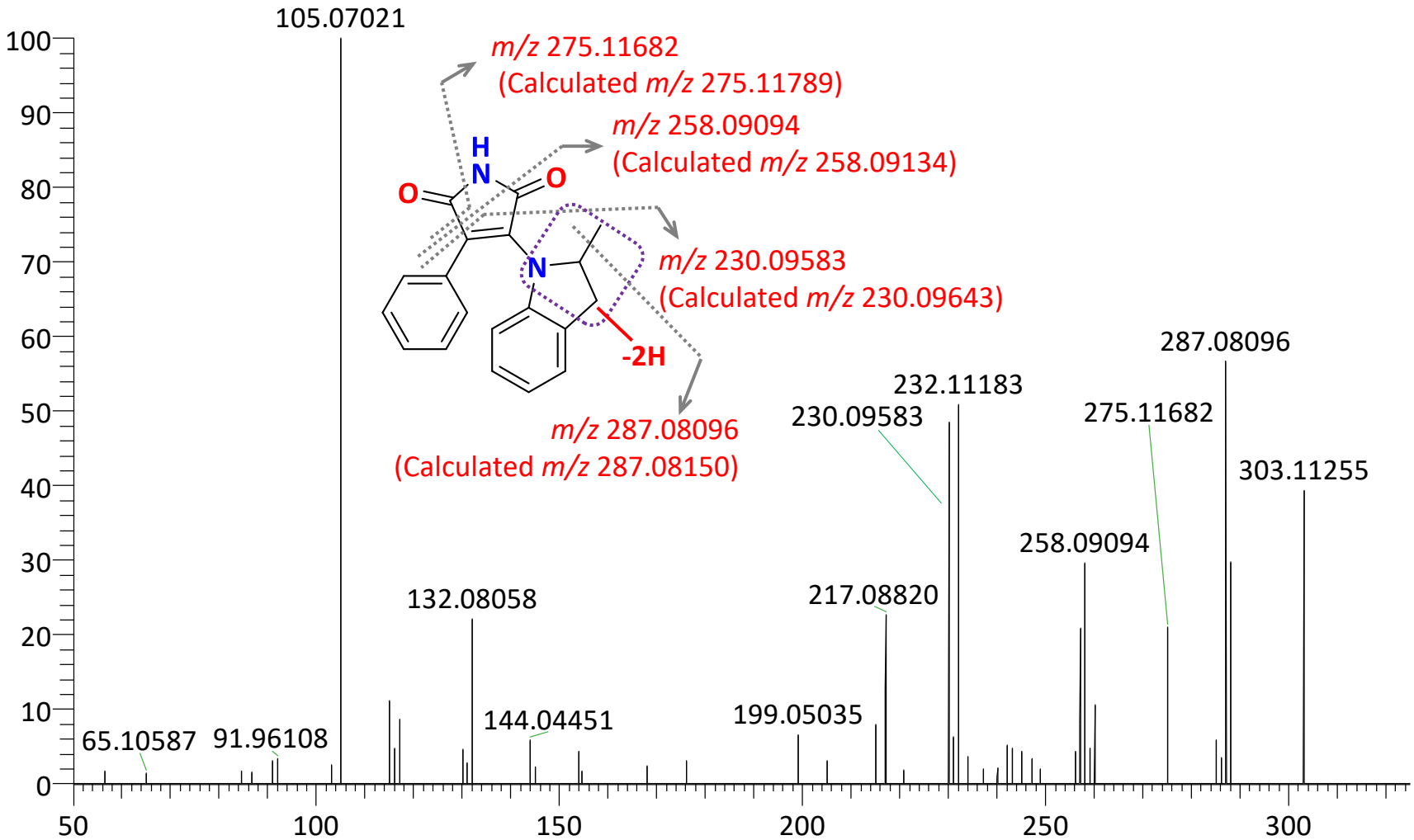
H^+ *m/z* 337.11755 (Calculated *m/z* 337.11828)



MS^2 fragmentation of M10

RT: 5.71 min;
MS^2: 287.08096, 275.11682, 258.09094, 230.09583;

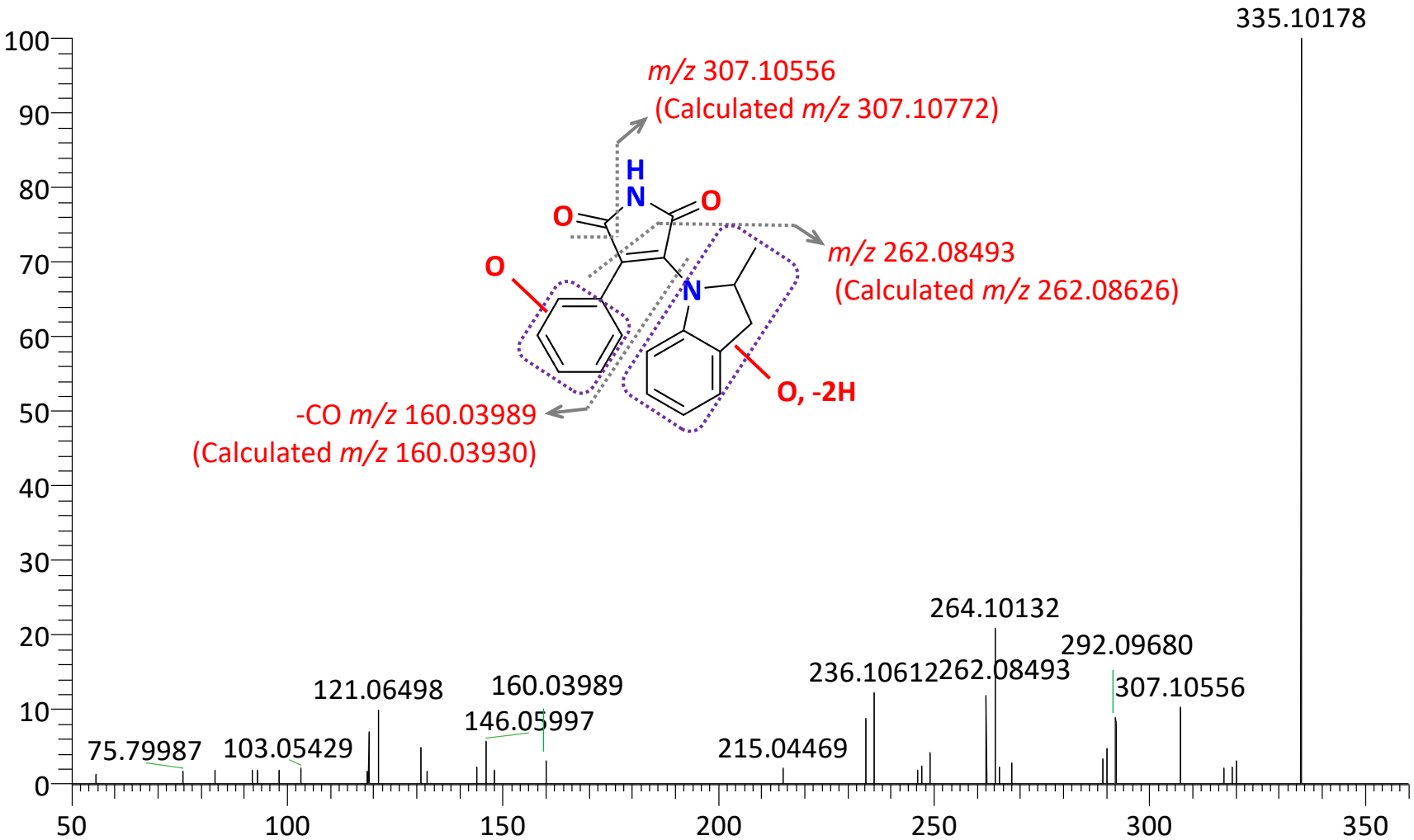
H^+ *m/z* 303.11255 (Calculated *m/z* 303.11280)



MS^2 fragmentation of M11

RT: 5.72 min;
MS^2: 307.10556, 262.08493, 160.03989;

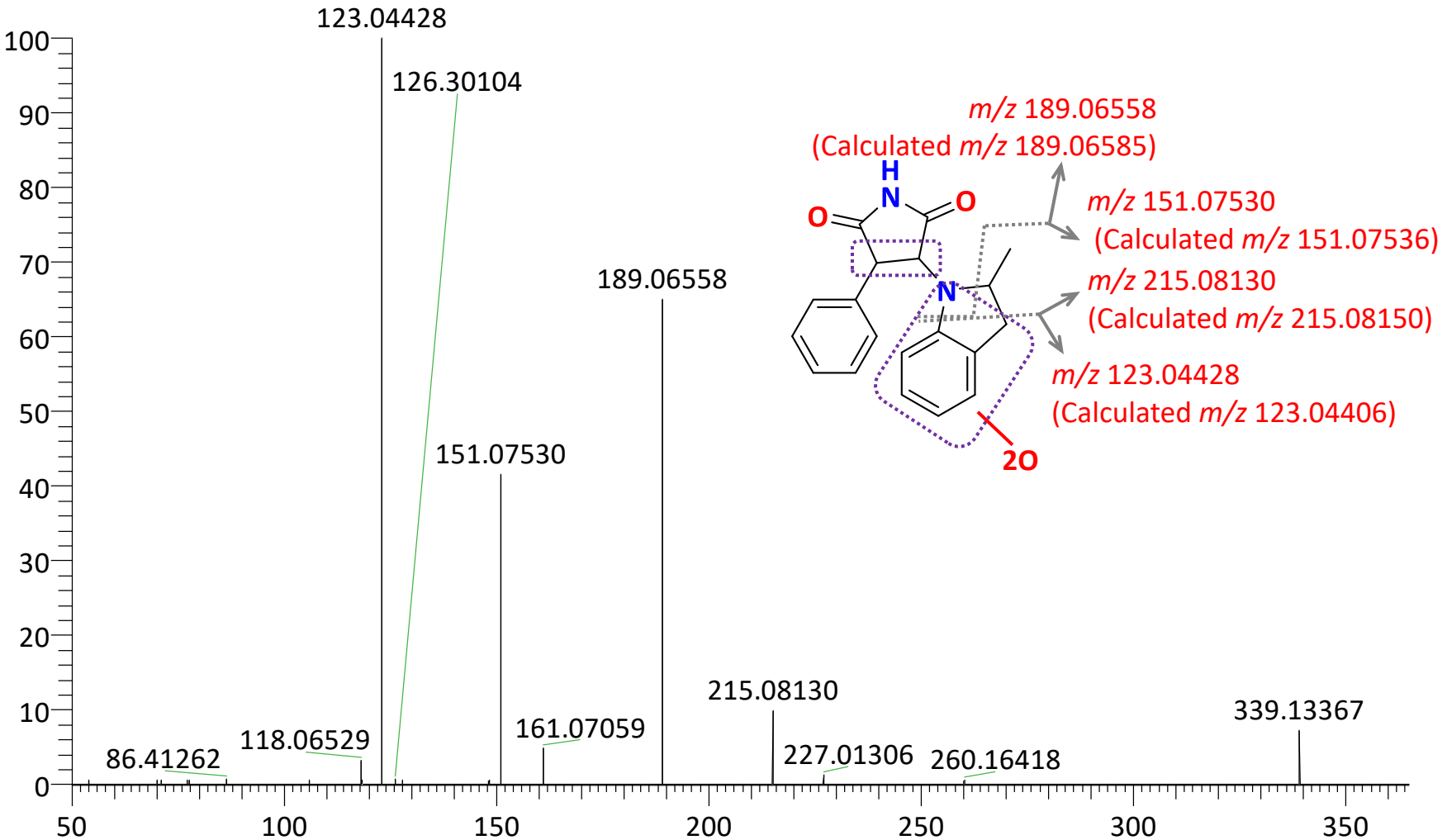
H^+ *m/z* 335.10178 (Calculated *m/z* 335.10263)



MS^2 fragmentation of M12

RT: 5.89 min;
MS^2: 215.08130, 189.06558, 151.07530, 123.04428;

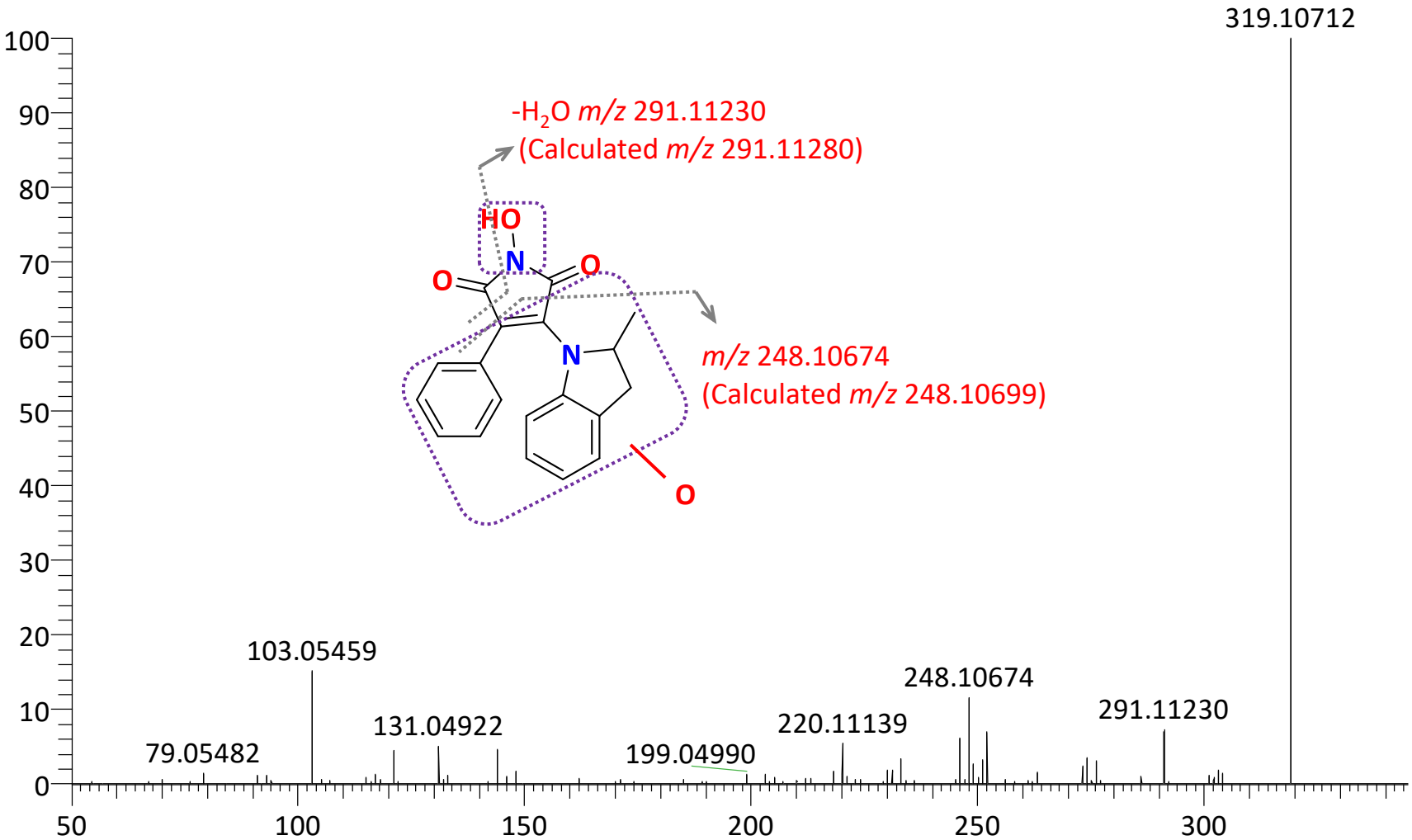
H^+ *m/z* 339.13367 (Calculated *m/z* 339.13393)



MS^2 fragmentation of M13

RT: 6.71 min;
MS^2: 291.11230, 248.10674;

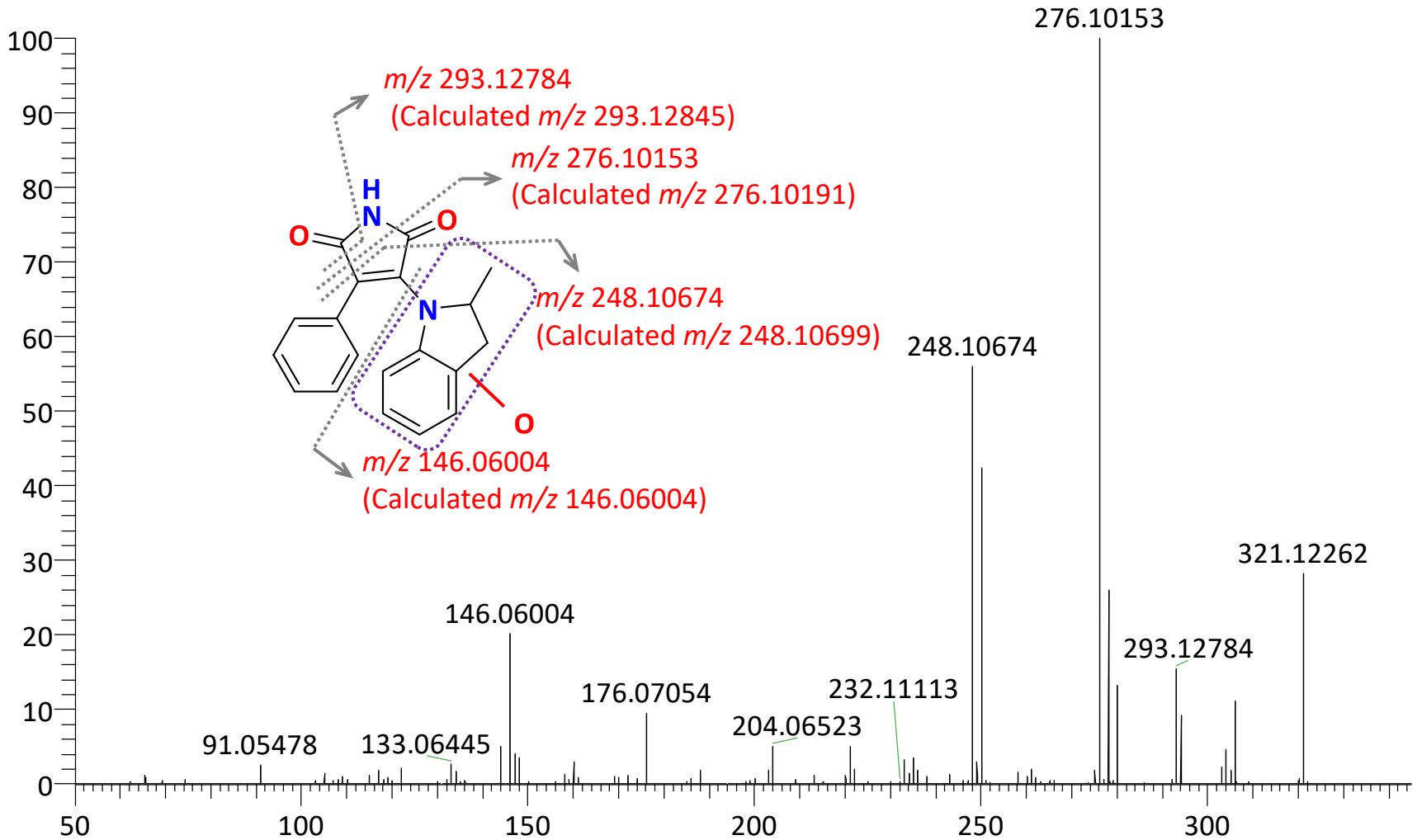
$-H_2O$ H^+ *m/z* 319.10712 (Calculated *m/z* 319.10772)



MS^2 fragmentation of M14

RT: 6.72 min;
MS^2: 293.12784, 276.10153, 248.10674, 146.06004;

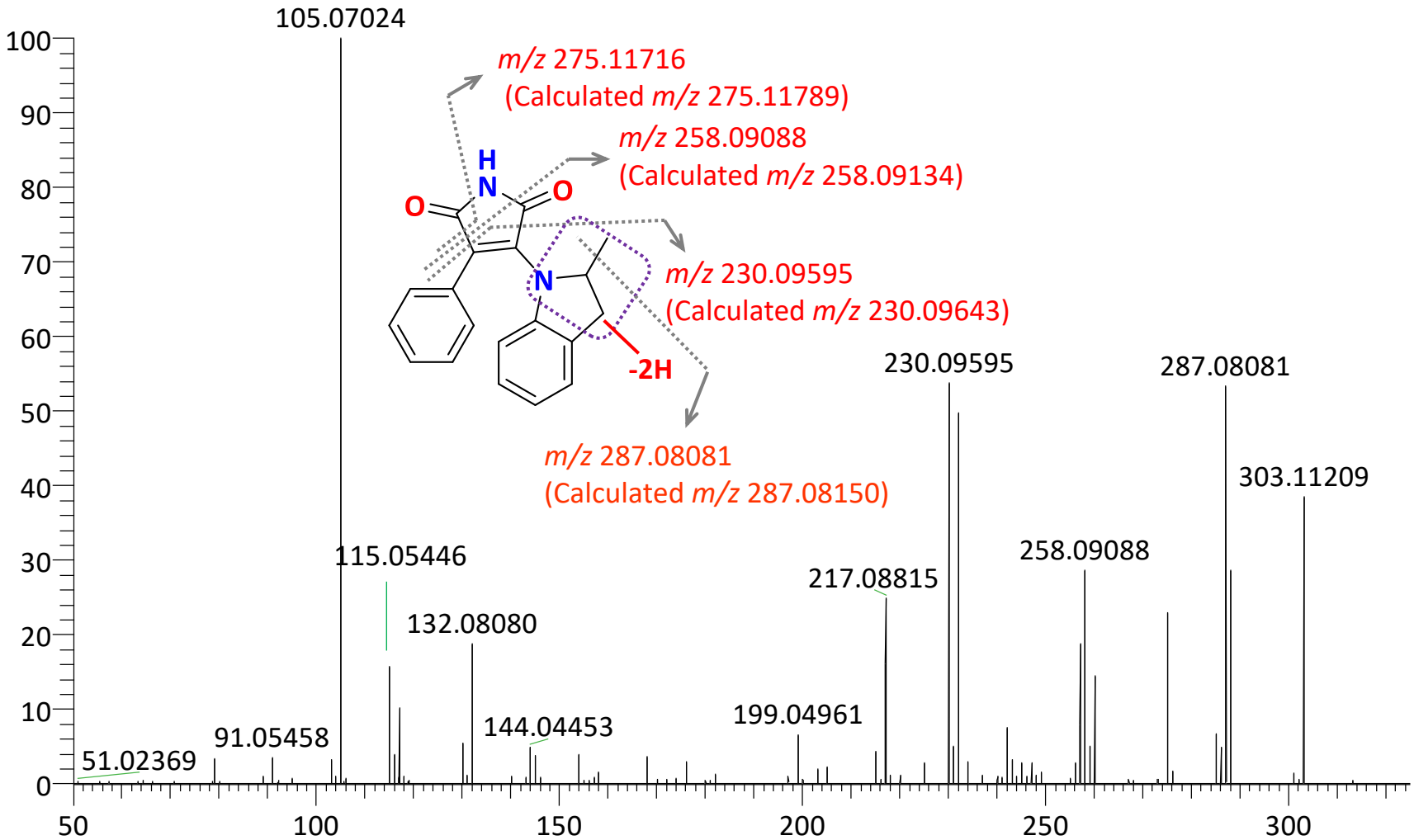
H^+ *m/z* 321.12262 (Calculated *m/z* 321.12337)



MS^2 fragmentation of M15

RT: 6.80 min;
MS^2: 287.08081, 258.09088, 230.09595;

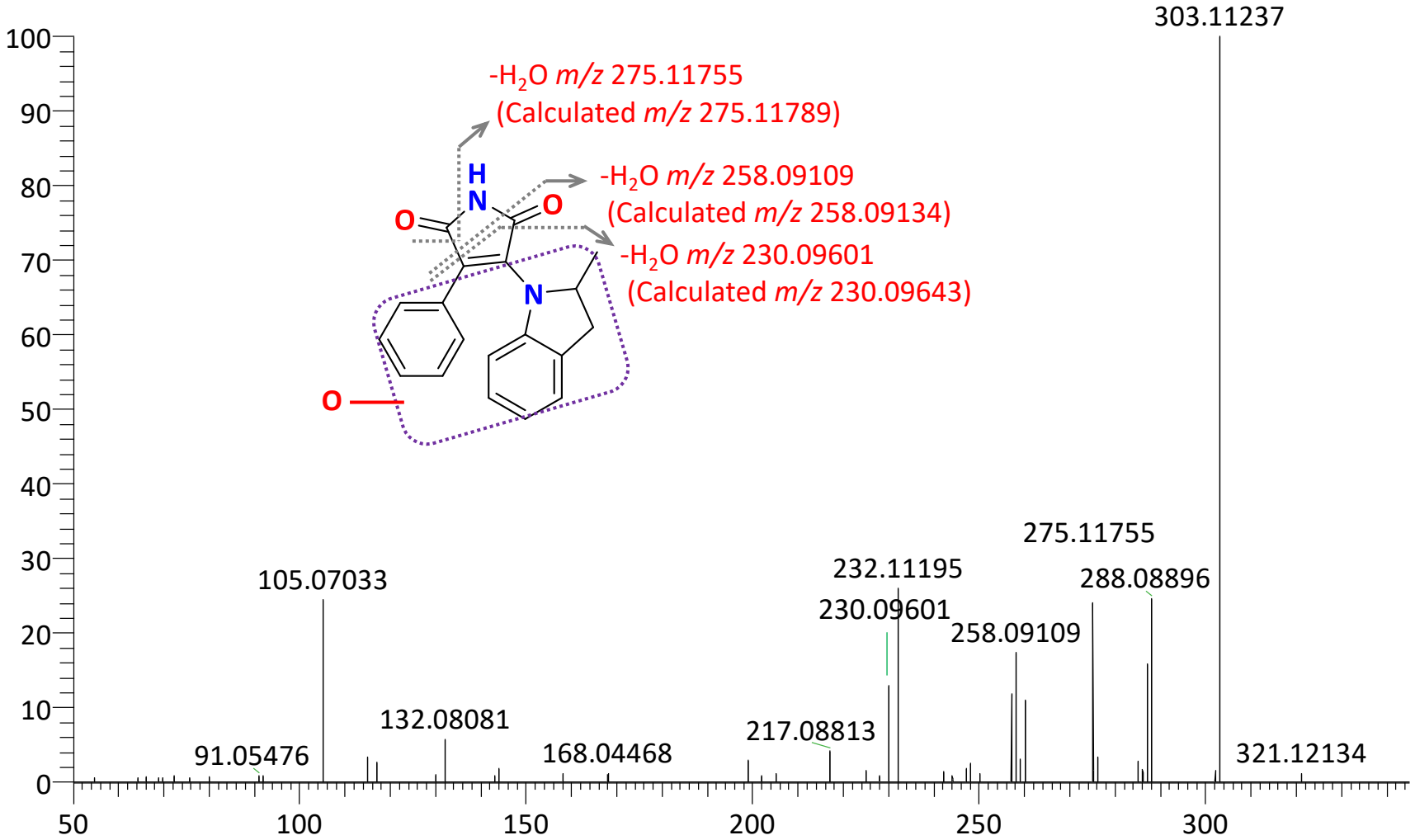
H^+ *m/z* 303.11209 (Calculated *m/z* 303.11280)



MS^2 fragmentation of M16

RT: 6.86 min;
MS^2: 303.11237, 275.11755, 258.09109, 230.09601;

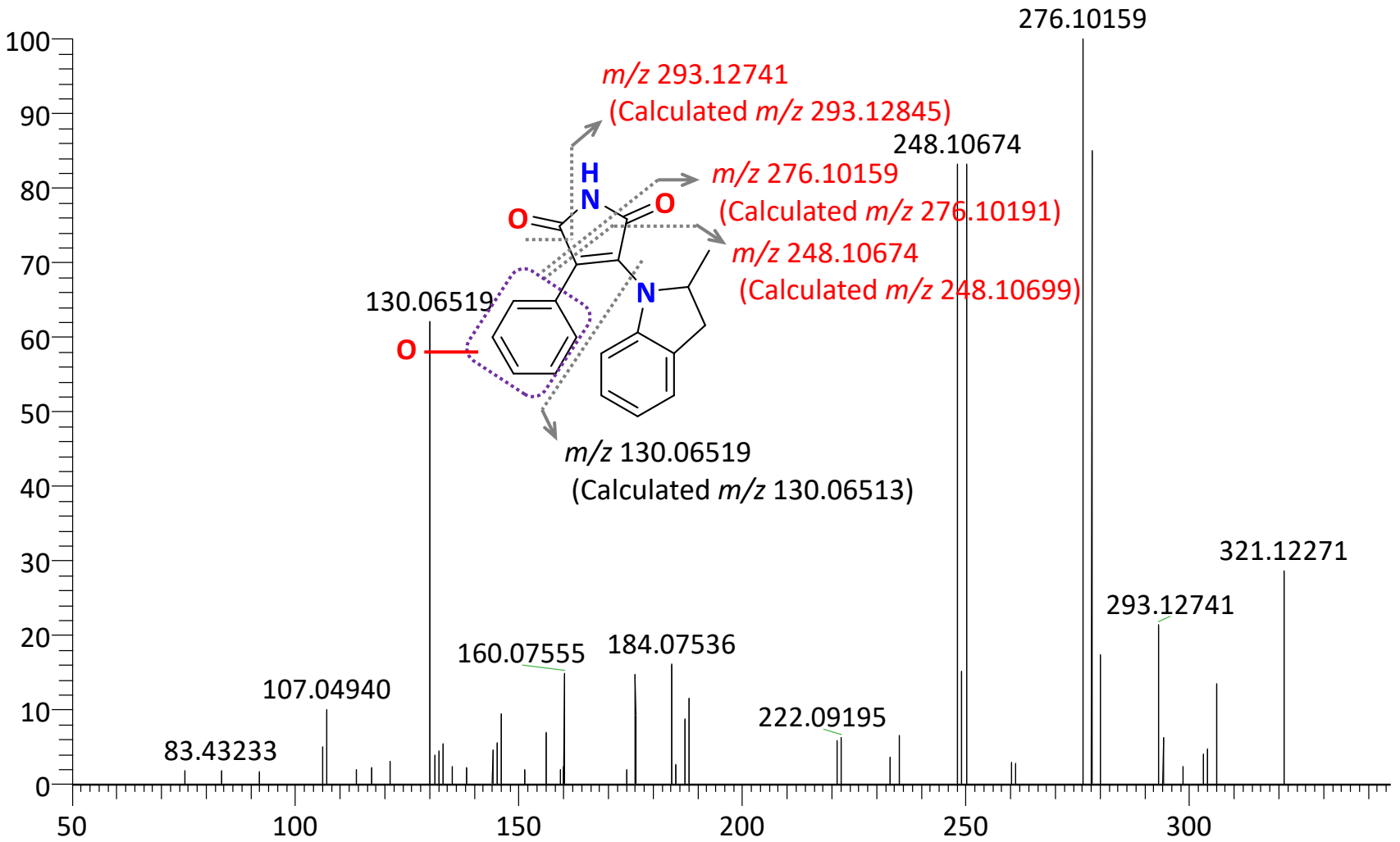
H^+ *m/z* 321.12134 (Calculated *m/z* 321.12377)
$-H_2O$ *m/z* 303.11237 (Calculated *m/z* 303.11280)



MS^2 fragmentation of M17

RT: 7.14 min;
MS^2: 293.12741, 276.10159, 248.10674, 130.06519;

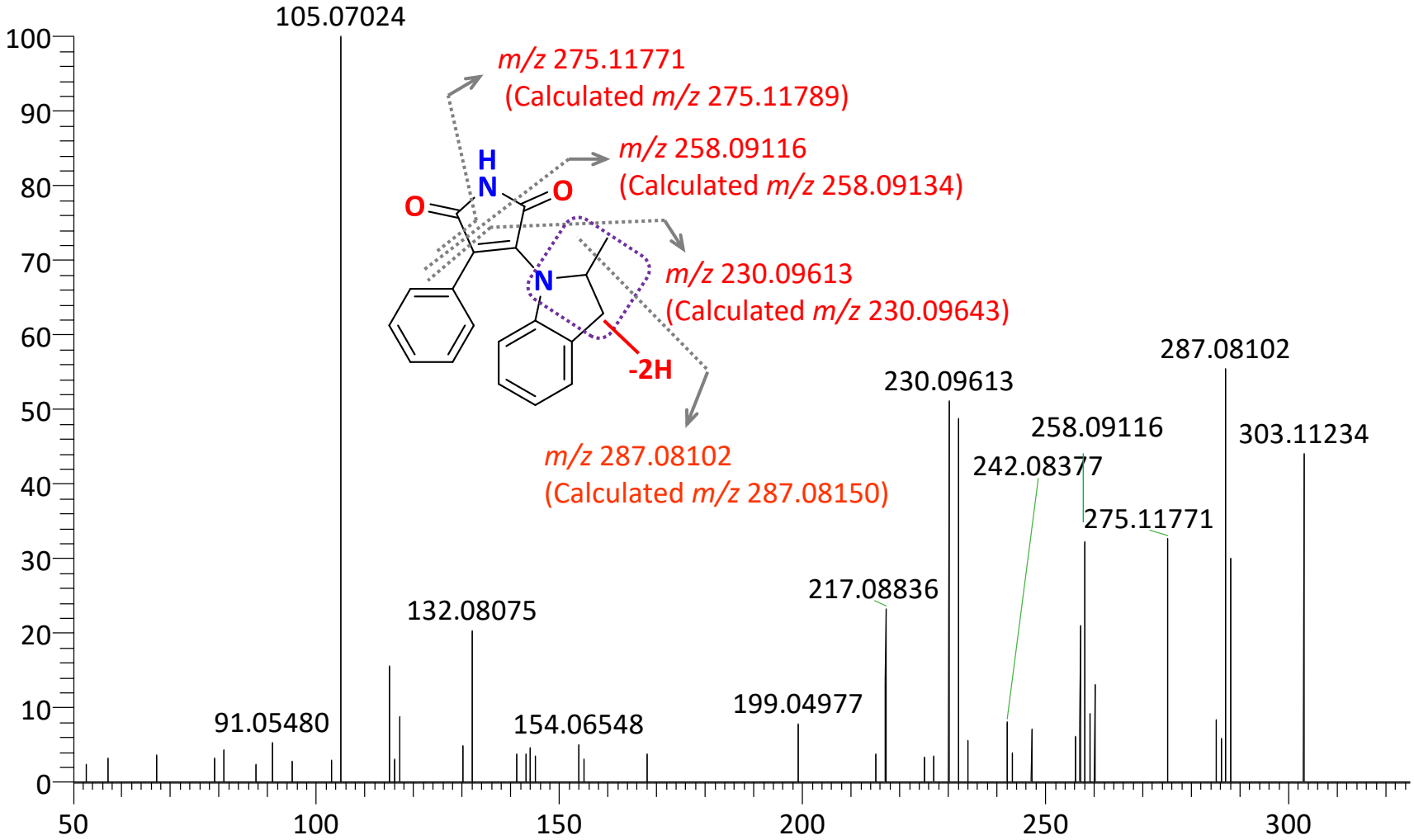
H^+ *m/z* 321.12271 (Calculated *m/z* 321.12337)



MS^2 fragmentation of M18

RT: 8.56 min;
MS^2: 287.08102, 275.11771, 258.09116, 230.09613;

H^+ *m/z* 303.11234 (Calculated *m/z* 303.11280)



Conclusions – ERPM5

- Under the experimental conditions, 18 metabolites were detected in mouse liver microsomal samples.
- **Proposed metabolic pathways**