SUPPORTING INFORMATION

Isatin derived spirocyclic analogs with α-methylene-γ-butyrolactone as anticancer agents: A structure activity relationship study

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Abbreviations

SAR Structure Activity Relationship

TNFα Tumor Necrosis Factor Alpha

NF-κB Nuclear factor kappa B

IκBα inhibitor of nuclear factor κB

IKKβ IκB kinase β

Cys Cysteine

PARP poly (ADP-ribose) polymerase

FBS fetal bovine serum

HEK Human embryonic kidney cells

DMEM Dulbecco's modified Eagle's medium

RPMI Roswell Park Memorial Institute-1640 medium

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

EDTA Ethylenediaminetetraacetic acid

DTT Dithiothreitol

SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

HNTG HEPES, NaCl, Triton X-100, Glycerol

PBS Phosphate-buffered saline

PVDF polyvinylidene difluoride

GAPDH Glyceraldehyde 3-phosphate dehydrogenase

TCEP tris(2-carboxyethyl)-phosphine hydrochloride

TBTA tris[(1-benzyl-1-H-1,2,3-triazol-4-yl)methyl]-amine

TEA Triethanolamine

RIPA radioimmunoprecipitation assay

DMSO Dimethyl sulfoxide

General Experimental

Cell lines and culture conditions

Human pancreatic cancer cell lines MiaPaCa2, PANC1 and SUIT2, cervical cancer cell line HeLa and breast cancer cell line MDA-MB-231 were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose supplemented with 10% fetal bovine serum (FBS). Human embryonic kidney cells (HEK cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) low glucose supplemented with 10% fetal FBS. Human ovarian cancer cell line A2780 was cultured in Roswell Park Memorial Institute-1640 medium (RPMI) supplemented with 10% FBS. Human lung cancer cell line luciferase A549 was cultured in DMEM high supplemented with 10% FBS and 100 µg/mL hygromycin B.

Cell Lysis

Cytoplasmic fractions were generated by harvesting cells on ice in buffer A (10 mmol/L HEPES, 10 mmol/L KCl, 0.1 mmol/L EDTA, and 2 mmol/L DTT) containing protease and phosphatase inhibitors (MDA-MB-231 SAR Figure 3B). The cells were allowed to swell on ice for 30 minutes and 1.25% Nonidet P-40 was added and the samples were centrifuged in the cold. The resulting supernatant was transferred to a new tube and designated as the cytoplasmic fraction. All samples were stored at -80°C until needed.

Whole cell lysates (MiaPaCa2 dose response) were generated by harvesting cells on ice in HNTG lysis buffer (20 mM HEPES, pH 7.5, 25 mM NaCl, 0.1% Triton X-100, 10% glycerol) containing protease and phosphatase inhibitors. The cells were allowed to swell on ice for 30 minutes and the samples were centrifuged in the cold. The resulting supernatant was transferred to a new tube and designated as a whole cell lysate. All samples were stored at -80°C until needed.

For click chemistry cells were harvested via scraping in 1X PBS with protease and phosphatase inhibitors and sonicated for 15 m. Lysates were spun and the soluble fraction (supernatant) was collected and transferred to a new tube. All samples were stored at -80°C until needed.

Western Blotting

Western blot analyses were conducted as described previously with minor modifications. Briefly, cytoplasmic fractions or whole cell lysates were prepared from control and treated cells. The cell lysates (25 mg) were resolved in 4% to 20% denaturing polyacrylamide gel (Bio-Rad) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The blotted membranes were incubated with the following antibodies: rabbit anti-IkBa (Cell Signaling 4812), mouse antiphospho-IkBa (Cell Signaling 9246) or mouse anti-PARP antibody (Cell Signaling 9542). Internal loading control for cytoplasmic fractions and whole cell lysates used mouse anti-tubulin (Cell Signaling 3873) or GAPDH (Santa Cruz Biotechnology, Inc. sc-25778) as noted. These primary antibodies were incubated with horseradish peroxidase–conjugated specific secondary antibodies. The signals were developed by using Amersham ECL Prime Western Blotting Detection Reagent (General Electric Healthcare Life Sciences).

Cell viability assay

Cancer cells were plated at 4,000 cells/well in a 96-well plate and incubated overnight. On the following day, cells were treated with 40, 10, 2.5, 0.625, 0.156, 0.039 or 0 (vehicle control) µM

of the indicated compounds and incubated at 37°C at 5% CO_2 for 24, 48 and 72 hours. Cells were then treated with Presto Blue (Life Technologies) for 15 minutes and read on a Spectramax M5^e plate reader at $560_{ex}/590_{em}$. Dose–response curves were evaluated using the National Cancer Institute (NCI) algorithm: Tz = number of control cells at time t0, C = number of control cells at time t, and T = number of treated cells at time t; 100 × [(T - Tz)/(C - Tz)].

kB-luciferase assay

A549 luciferase cells (generous gift from Prof. Brasier, University of Texas Medical Branch, Galveston, TX) were seeded in white 96-well plates at a density of 50,000 cells per well and incubated overnight. Cells were then treated with the indicated concentrations of drugs for 2 hours. Cells were then stimulated with 20 ng/mL TNFα (Panomics) for 3 hours. AlamarBlue (abSerotec) was added (which served as a control for seeding and viability) and the cells were incubated for 3 additional hours. ONE-Glo Luciferase reagent (Promega) was added to each well and cells were incubated at room temperature for an additional 10 minutes before luminescence was measured at 1000 ms integration on a SpectraMax M5^e plate reader.

For the focused library of of α-methylene-γ-butyrolactone analogs and the IKKβ inhibitor ML-120B, A549 luciferase cells were grown in the absence of TNFα for 5 passages and then in the absence or presence of 5 ng/mL TNFα for 2 passages. Cells grown in the absence or presence of 5 ng/mL TNFα were plated in a 96-well clear-bottom, white-walled plate at 50,000 cells per well (or 12,500 cells per well for a 384-well plate) and incubated overnight. The following day, cells were treated with compound at a final concentration of 10 μM or appropriate controls and incubated for 2h. After 2h, TNFα pretreated cells were treated with TNFα at a final concentration of 20 ng/mL and incubated for 7 hours. PrestoBlue cell viability reagent was added and the cells were incubated for 10 minutes and fluorescence ($560_{ex}/590_{em}$) was measured on the bottom-read setting on SpectramaxM5^e plate reader. Then 110 μL of ONE-Glo Luciferase reagent was added to each well and cells were incubated at room temperature for an additional 10 minutes and read for luminescence at 1000 ms integration on Spectramax M5^e plate reader.

Click Chemistry

For click chemistry with purified proteins, purified IKKβ (Cell Signaling 7584) or p65 (Active Motif 31102) (250 ng in a total volume of 200 μL) was incubated with 10 μM **20** for 1 hour. 5-TAMRA azide (Lumiprobe) (0.1 mM from a 1 mM DMSO stock) and click chemistry reagents (1 mM tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) from 50 mM stock in water, 1 mM CuSO4·5H2O from a freshly prepared 50 mM stock in water, and 0.1 mM tris[(1-benzyl-1-H-1,2,3-triazol-4-yl)methyl]-amine (TBTA) from 10 mM stock in DMSO) at a total volume of 48 μL were added for 1 hour and then the reaction was quenched with ice cold methanol and stored at -20°C for 1 hour to precipitate. The resulting precipitate was spun and the methanol was removed. The pellet was resuspended in 4% SDS buffer (4% SDS, 150 mM NaCl, 50 mM TEA pH 7.4) and 2X sample buffer (20% glycerol, 0.2% bromophenol blue, 1.4% β mercaptoethanol) to 1X. Samples were resolved on 4% to 20% denaturing polyacrylamide gel (Bio-Rad) and visualized using Typhoon 9410 Variable Mode Imager.

PARP Cleavage

Cells were treated as indicated and harvested by collecting media, trypsinizing cells, and centrifuging to obtain a combined cell pellet from all steps. Cells were lysed in

radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris, pH 8.0) and protein content was resolved on 4% to 20% denaturing polyacrylamide gel (Bio-Rad) by SDS-PAGE and PARP cleavage was determined via Western blotting using anti-PARP antibody following transfer.

Caspase 3/7 assay

Plated 3,000 SKOV3 cells/well in a white, clear bottom sterile 96 well dish in 95 µL and incubated cells overnight. The next day, cells were treated with 5 µL compounds to yield final drug concentration as indicated. Cells were incubated with compound for 24 hr. alamarBlue was added to each well and cells were incubated for another 3 hours. Fluorescence ($560_{ex}/590_{em}$) was measured on the SpectraMax M5^e plate reader. 110 µL Caspase Glo-3/7 reagent was added to each well and mixed with the plate reader at 300-500 rpm for 30 seconds. The plate was incubated at room temperature for 1 hour. Luminescence was measured at 1000 ms integration on the SpectraMax M5^e plate reader.

Colony formation assay

HeLa cells were plated in 6-well plates at a density of 500-1000 cells per well and cultured for 24 h, followed by drug treatment for 48 h at 37°C. DMSO was used as a control. Then, the supernatant fluid was replaced with fresh medium and the cells were cultured for 7 days. Cells were washed twice with PBS and then fixed with methanol for 15 min, followed by 0.1% crystal violet staining for 30 min at room temperature. Then, crystal violet was carefully removed by immersing the plates in tap water. The plates were then air-dried at room temperature for colony counting, where colonies with more than 50 cells were counted.

Mouse Studies

All animal experiments were performed according to an Institutional Animal Care and Use Committee of Henry Ford Health Systems approved protocol, and institutional guidelines for the proper and humane use of animals in research were followed. Our facility is approved by the Association for Assessment and Accreditation of Laboratory Animal Care. C57B6 mice were purchased from Jackson Laboratories (Bar Harbor, ME).Nude mice were injected with 2 million A2780 ovarian cancer cells. On day 3, mice were divided into 7 groups and treatments were started. Mice were treated with vehicle control, 1, 2.5, or 5 mg/kg **19** (given 5 days/week until day 30), 2 or 4 mg/kg cisplatin (given on days 5, 9, 14, and 21) or 2.5 mg/kg **19** and 2 mg/kg cisplatin in combination. On day 30, the mice were sacrificed and tumors were removed, weighed, and frozen for future immunohistochemistry studies.

Immunohistochemistry

Hematoxylin and eosin staining of ovarian tumor tissues was performed to identify tissue areas containing tumor. Tumor cores were punched, arranged in a tumor microarray, and sliced for staining. Tumor tissues were stained with p65 and Mcl-1 using Leica bond polymer refine detection kit according to manufacturer's instructions (Leica Microsystems Inc.). Briefly, the ovarian tumor tissue sections were incubated with the following primary antibodies rabbit anti-p65 antibody (Santa Cruz Biotechnology, Inc. sc-372), rabbit anti-Mcl-1 antibody (Santa Cruz Biotechnology, Inc. sc-819). A different slice was used for each antigen. Pictures were captured by using Coreo Au slide scanner (Ventana Medical Systems Inc.). The images were scored on a

scale of 0-3 to indicate percentage of positive cells (0 = no detectable expression and 3 = ≥ 67% cells express the protein).

Synthetic Procedures

General methods

All reagents were purchased from commercial sources and were used without further purification. Flash chromatography was carried out on silica gel (200–400 mesh). Thin layer chromatography (TLC) were run on pre-coated EMD silica gel 60 F254 plates and observed under UV light at 254 nm and with basic potassium permanganate dip. Column chromatography was performed with silica gel (230-400 mesh, grade 60, Fisher scientific, USA). 1H NMR (500 MHz) and 13C NMR (125 MHz) spectra were recorded in chloroform-d or DMSO-d6 on a Varian-500, Varian-600 and Bruker-500 spectrometer (DMSO-d_6 was 2.50 ppm for ^{1}H and 39.55 ppm for ^{13}C and CDCl3 was 7.27 ppm for ^{1}H and 77.23 ppm for ^{13}C. Proton and carbon chemical shifts were reported in ppm relative to the signal from residual solvent proton and carbon. The purity of all final compounds was ≥95% as determined by analytical HPLC on a reverse-phase column (Zorbax 300SB C18, 2.1 × 150 mm, 5 μm particle size) using an Agilent 1200 series system with UV detector (214 nm and 254 nm) with the binary system water/acetonitrile containing 0.1% trifluoracetic acid (TFA) as eluent. HRMS was carried out University of Kansas at Lawrence Mass Spectrometry facility.

General Procedure for Barbier-type reaction

In a dry round bottom flask equipped with a reflux condenser was taken indium metal powder (4.06 mmol, 2 eq) and ammonium chloride (4.06 mmol, 2 eq). To it was added a solution of methyl 2-(bromomethyl) acrylate (2.45 mmol, 1.2 eq) in dry methanol (5 mL). The reaction mixture was heated at 50 °C for 15 minutes followed by addition of a solution of isatin/isatin derivatives (2.03 mmol, 1 eq) in 5 mL methanol. Reaction mixture was heated at 50 °C with constant stirring for 30 minutes and reaction completion (disappearance of isatin) was monitored by thin layer chromatography using 50% ethyl acetate in hexane solvent. Following reaction completion, reaction mixture was cooled to room temperature and passed through silica gel to remove ammonium chloride and indium metal. Reaction solvent was evaporated under reduced pressure using rotavapor. Crude reaction mixture was dissolved in 0.1% HCl and extracted using ethyl acetate followed by washing with brine, and dried using magnesium sulfate. Crude mixture was column chromatographed using hexane and ethyl acetate gradient to obtain spirocyclic compound and acyclic compound respectively.

General procedure for the cyclization reaction of acyclic compounds: Taken acyclic compound (0.25 mmol, 1 eq.) in round bottom flask and added 5 mL of dichloromethane via syringe followed by addition of p-toluenesulfonic acid monohydrate salt (0.25 mmol, 1 eq.). Reaction mixture was stirred at room temperature for 12 h under nitrogen atmosphere. Completion of the reaction was monitored by thin layer chromatography. Crude mixture was washed with brine and extracted using dichloromethane, dried under magnesium sulfate and column chromatographed using hexane and ethyl acetate gradient to obtain desired product as white solid.

NMR spectra

1. 5-hexyl-3-methylenedihydrofuran-2(3H)-one:[i] ^{1}H NMR ($CDCl_3$) δ 6.22 (s, 1H), 5.61 (s, 1H), 4.51 (p, *J* = 6.5 Hz, 1H), 3.07 – 3.02 (m, 1H), 2.60 – 2.55 (m, 1H), 1.77 – 1.70 (m, 1H), 1.64 – 1.57 (m, 2H), 1.48 – 1.42 (m, 2H), 1.39 – 1.28 (m, 10H), 0.88 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR δ 170.59, 135.02, 122.08, 77.80, 36.51, 33.79, 31.93, 29.47, 29.33, 25.08, 22.82, 14.28.

2. 3-methylene-5-phenyldihydrofuran-2(3H)-one:[i] ^{1}H NMR ($CDCl_3$) δ 7.38 – 7.29 (m, 5H), 6.27 (t, *J* = 2.5 Hz, 1H), 5.66 (t, *J* = 2.5 Hz, 1H), 5.49 (t, *J* = 8.0 Hz, 1H), 3.41 – 3.35 (m, 1H), 2.90 – 2.85 (m, 1H), 1.77 – 1.70 (m, 1H), 1.64 – 1.57 (m, 2H), 1.48 – 1.42 (m, 2H), 1.39 – 1.28 (m, 10H), 0.88 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR δ 170.19, 139.88, 134.29, 128.86, 128.58, 125.54, 125.48, 122.42, 78.02, 36.23.

3. 5-(4-fluorophenyl)-3-methylenedihydrofuran-2(3H)-one:[i] ^{1}H NMR ($CDCl_3$) δ 7.36 – 7.33 (m, 2H), 7.06 – 7.02 (m, 2H), 6.39 (s, 1H), 5.70 (s, 1H), 4.94 – 4.91 (m, 1H), 2.79 – 2.75 (m, 1H), 2.70 – 2.65 (m, 1H); ^{13}C NMR δ 163.40, 161.44, 139.54, 136.23, 131.12, 127.58, 115.55, 115.38, 72.74, 42.31.

4. 5-(4-methoxyphenyl)-3-methylenedihydrofuran-2(3H)-one:[i] ^{1}H NMR ($CDCl_3$) δ 7.26 – 7.24 (m, 2H), 6.92 – 6.90 (m, 2H), 6.30 (t, *J* = 2.5 Hz, 1H), 5.68 (t, *J* = 2.5 Hz, 1H), 5.47 (t, *J* = 7.0 Hz, 1H), 3.81 (s, 3H), 3.38 – 3.32 (m, 1H), 2.94 – 2.88 (m, 1H); ^{13}C NMR δ 170.39, 160.06, 134.77, 131.86, 127.27, 122.41, 114.41, 78.25, 55.54, 36.42.

5. 5-(4-(benzyloxy)phenyl)-3-methylenedihydrofuran-2(3H)-one: ^{1}H NMR ($CDCl_3$) δ 7.43 – 7.24 (m, 7H), 6.99 – 6.97 (m, 2H), 6.30 (t, *J* = 2.5 Hz, 1H), 5.68 (t, *J* = 2.5 Hz, 1H), 5.47 (t, *J* = 7.0 Hz, 1H), 5.07 (s, 2H), 3.38 – 3.32 (m, 1H), 2.94 – 2.89 (m, 1H); ^{13}C NMR δ 170.36, 159.27, 136.92, 134.76, 132.17, 128.85, 128.29, 127.67, 127.31, 122.44, 115.38, 78.21, 70.32, 36.44.

6. 3-methylene-5-(3-(trifluoromethyl)phenyl)dihydrofuran-2(3H)-on: δ 7.62 – 7.53 (m, 4H), 6.35 (t, *J* = 2.5 Hz, 1H), 5.74 (t, *J* = 2.5 Hz, 1H), 5.58 (t, *J* = 7.5 Hz, 1H), 3.50 – 3.44 (m, 1H), 2.93 – 2.87 (m, 1H); ^{13}C NMR δ 169.90, 141.12, 133.66, 129.71, 128.84, 125.64, 123.40, 36.40.

7. 5-(3-methoxyphenyl)-3-methylenedihydrofuran-2(3H)-one:[i] ^{1}H NMR ($CDCl_3$) δ 7.32 – 7.26 (m, 1H), 6.90 – 6.86 (m, 3H), 6.31 (t, *J* = 2.5 Hz, 1H), 5.69 (t, *J* = 2.5 Hz, 1H), 5.50 (t, *J* = 8.0 Hz, 1H), 3.81 (s, 3H), 3.42 – 3.37 (m, 1H), 2.93 – 2.88 (m, 1H); ^{13}C NMR δ 170.33, 160.19, 141.67, 134.34, 130.19, 122.70, 117.68, 114.20, 111.08, 77.98, 55.54, 36.49.

8. 5-(3-(benzyloxy)phenyl)-3-methylenedihydrofuran-2(3H)-one: ^{1}H NMR ($CDCl_3$) δ 7.42 – 7.24 (m, 6H), 6.94 – 6.88 (m, 3H), 6.28 (t, *J* = 2.5 Hz, 1H), 5.66 (t, *J* = 2.5 Hz, 1H), 5.46 (t, *J* = 7.0 Hz, 1H), 5.04 (s, 2H), 3.39 – 3.33 (m, 1H), 2.89 – 2.83 (m, 1H); ^{13}C NMR δ 170.24, 159.29, 141.66, 136.80, 134.23, 130.16, 128.75, 128.19, 127.65, 122.64, 117.93, 114.91, 112.08, 77.87, 70.20, 36.33. HRMS (TOF MS EI+) calcd for C18H16O3Na m/z 303.0997, found: 303.0999.

9. 5-(3,4-dimethoxyphenyl)-3-methylenedihydrofuran-2(3H)-one:[ii] ^{1}H NMR ($CDCl_3$) δ 6.87 (s, 2H), 6.84 (s, 1H), 6.31 (t, *J* = 2.5 Hz, 1H), 5.70 (t, *J* = 2.5 Hz, 1H), 5.47 (t, *J* = 7.5 Hz, 1H), 3.89 (s, 6H), 3.40 – 3.34 (m, 1H), 2.96 – 2.90 (m, 1H); ^{13}C NMR δ 170.37, 149.58, 149.51, 134.69, 132.31, 122.54, 118.26, 111.35, 108.78, 78.34, 56.19, 36.51.

10. 5-(3-(benzyloxy)-4-methoxyphenyl)-3-methylenedihydrofuran-2(3H)-one: ^{1}H NMR ($CDCl_3$) δ 7.44 – 7.29 (m, 5H), 6.88 – 6.85 (m, 3H), 6.28 (t, *J* = 3.0 Hz, 1H), 5.65 (t, *J* = 2.5 Hz, 1H), 5.41 (dd, *J* = 8.0 Hz, 6.5 Hz, 1H), 5.13 (dd, *J* = 17 Hz, 12.5 Hz, 2H) 3.88 (s, 3H), 3.34 – 3.28 (m, 1H), 2.85 – 2.79 (m, 1H); ^{13}C NMR δ 170.32, 150.25, 148.67, 136.98, 134.59, 132.24, 128.78, 128.17, 127.64, 122.49, 118.94, 111.96, 111.78, 78.18, 71.43, 56.30, 36.38. HRMS (TOF MS EI+) calcd for C19H18O4Na m/z 333.1103, found: 333.1101.

11. 3-methylene-5-(pyridin-2-yl)dihydrofuran-2(3H)-one: ^{1}H NMR ($CDCl_3$) δ 8.61 – 8.60 (m, 1H), 7.75 – 7.72 (m, 1H), 7.44 (d, *J* = 3 Hz, 1H), 7.28 – 7.25 (m, 1H), 6.31 (t, *J* = 2.5 Hz, 1H), 5.71 (t, *J* = 2.5 Hz, 1H), 5.61 (dd, *J* = 8.0 Hz, 5.5 Hz, 1H), 3.49 – 3.43 (m, 1H), 3.21 – 3.15 (m, 1H); ^{13}C NMR δ 170.30, 158.93, 149.81, 137.27, 133.86, 123.46, 123.01, 120.45, 34.20.

12. 5-(6-bromopyridin-2-yl)-3-methylenedihydrofuran-2(3H)-one 31-063: ^{1}H NMR ($CDCl_3$) d 7.60 (t, *J* = 8.0 Hz, 1H), 7.44 (d, *J* = 17.0 Hz, 1H), 7.42 (d, *J* = 17.0 Hz, 1H), 6.31 (d, *J* = 2.5 Hz, 1H), 5.72 (s, 1H), 5.59 – 5.56 (m, 1H), 3.50 – 3.45 (m, 1H), 3.16 – 3.11 (m, 1H); ^{13}C NMR d 169.99, 160.52, 142.17, 139.59, 133.26, 127.93, 123.54, 119.06, 76.97, 34.25.

13. 5,5'-(1,4-phenylene)bis(3-methylenedihydrofuran-2(3H)-one): ^{1}H NMR ($CDCl_3$) δ 7.35 (s, 4H), 6.31 (s, 2H), 5.71 (s, 2H), 5.53 (t, *J* = 7.0 Hz, 2H), 3.35 – 3.40 (m, 2H), 2.91 – 2.86 (m, 2H); ^{13}C NMR δ 170.14, 140.47, 134.07, 126.10, 122.91, 77.63, 36.34.

14. 3-methylene-5-(naphthalen-1-yl)dihydrofuran-2(3H)-one:[iii] ^{1}H NMR ($CDCl_3$) δ 7.91 (d, *J* = 7.5 Hz, 1H), 7.83 (d, *J* = 7.5 Hz, 1H), 7.79 (d, *J* = 8.5 Hz, 1H), 7.57 – 7.45 (m, 4H), 6.35 – 6.34 (m, 1H), 6.28 – 6.25 (m, 1H), 5.67 – 5.66 (m, 1H), 3.66 – 3.60 (m, 1H), 2.99 – 2.94 (m, 1H); ^{13}C NMR δ 170.55, 135.89, 134.09, 134.05, 129.61, 129.48, 129.06, 126.89, 126.25, 125.70, 123.34, 122.62, 122.03, 75.72, 36.17.

15. 3-methylene-5-(quinolin-4-yl)dihydrofuran-2(3H)-one: ^{1}H NMR ($CDCl_3$) δ 9.06 – 9.04 (m, 2H), 8.15 – 7.98 (m, 3H), 6.47 – 6.46 (m, 1H), 6.41 – 6.38 (m, 1H), 5.83 – 5.82 (m, 1H), 3.90 – 3.85 (m, 1H), 2.98 – 2.93 (m, 1H); ^{13}C NMR δ 168.41, 156.93, 143.15, 138.45, 134.93, 131.27, 131.08, 125.81, 125.18, 123.99, 123.15, 116.70, 73.33, 35.61. HRMS (TOF MS EI+) calcd for C14H12NO2 m/z 226.0868, found: 226.0850.

16. 5-(2-methoxynaphthalen-1-yl)-3-methylenedihydrofuran-2(3H)-one: ^{1}H NMR ($CDCl_3$) δ 7.94 – 7.81 (m, 3H), 7.50 (t, *J* = 7.5 Hz, 1H), 7.37 (t, *J* = 7.5 Hz, 1H), 7.29 (d, *J* = 9.5 Hz, 1H), 6.51 – 6.48 (m, 1H), 6.36 (t, *J* = 2.5 Hz, 1H), 5.68 (t, *J* = 2.5 Hz, 1H), 3.90 (s, 3H), 3.40 – 3.34 (m, 1H), 3.26 – 3.20 (m, 1H); ^{13}C NMR δ 171.26, 156.36, 136.32, 132.10, 131.43, 129.32, 127.53, 123.88, 121.98, 120.87, 119.42, 113.79, 72.58, 56.57, 33.82. HRMS (TOF MS EI+) calcd for C16H14O3Na m/z 277.0841, found: 277.0829.

17. 5-(2-(benzyloxy)naphthalen-1-yl)-3-methylenedihydrofuran-2(3H)-one: ^{1}H NMR ($CDCl_3$) δ 8.19 (d, *J* = 9.0 Hz, 1H), 7.78 (d, *J* = 9.5 Hz, 1H), 7.51 – 7.33 (m, 8H), 6.32 (s, 1H), 5.84 – 5.81 (m, 1H), 5.60 (s, 1H), 5.30 – 5.25 (m, 1H), 3.05 – 3.00 (m, 1H), 2.90 – 2.86 (m, 1H); ^{13}C NMR δ 172.27, 154.03, 137.05, 136.47, 131.86, 130.21, 129.78, 129.68, 129.10, 128.78, 128.70, 127.97, 127.19, 124.40, 123.27, 114.45, 71.75, 68.98, 40.05. HRMS (TOF MS EI+) calcd for C22H18O3Na m/z 353.1154, found: 353.1143.

18. 3-methylene-5-(naphthalen-2-yl)dihydrofuran-2(3H)-one:[ii] ^{1}H NMR ($CDCl_3$) δ 7.88 – 7.79 (m, 4H), 7.52 – 7.50 (m, 2H), 7.38 (dd, *J* = 2.0 Hz, 9.0 Hz 1H), 6.34 (t, *J* = 3.0 Hz, 1H),

5.71 – 5.67 (m, 2H), 3.49 – 3.43 (m, 1H), 3.01 – 2.95 (m, 1H); ^{13}C NMR δ 170.22, 137.29, 134.34, 133.41, 133.25, 129.19, 128.28, 127.95, 126.85, 126.73, 124.75, 122.97, 122.80, 76.97, 36.45.

19. 4-methylene-3,4-dihydro-5H-spiro[furan-2,3'-indoline]-2',5-dione:[iv]

20. 1'-(but-3-yn-1-yl)-4-methylene-3,4-dihydro-5H-spiro[furan-2,3'-indoline]-2',5-dione: ^{1}H NMR ($CDCl_3$) δ 7.40 (t, *J* = 8.0 Hz, 1H), 7.33 (d, *J* = 7.5 Hz, 1H), 7.13 (t, *J* = 8.0 Hz, 1H), 6.97 (d, *J* = 8.0 Hz, 1H), 6.42 (t, *J* = 2.5 Hz, 1H), 5.80 (t, *J* = 2.5 Hz, 1H), 3.94 – 3.83 (m, 2H), 3.34 – 3.29 (m, 1H), 3.14 – 3.09 (m, 1H), 2.62 – 2.59 (m, 2H), 2.00 – 1.99 (m, 2H); ^{13}C NMR δ 173.74, 169.12, 143.11, 132.90, 131.42, 126.95, 124.63, 123.94, 123.44, 109.48, 80.22, 79.39, 71.03, 39.20, 36.69, 17.68. HRMS (TOF MS EI+) calcd for C16H13NO3Na m/z 290.0793, found: 290.0775.

21. 4-methylene-3,4-dihydro-5H-spiro[furan-2,3'-indoline]-2',5-dione:[iv]

22. Methyl 2-((3-hydroxy-2-oxoindolin-3-yl)methyl)acrylate:[iv]

23. 1'-methyl-4-methylene-3,4-dihydro-5H-spiro[furan-2,3'-indoline]-2',5-dione:[iv]

24. 1'-benzyl-4-methylene-3,4-dihydro-5H-spiro[furan-2,3'-indoline]-2',5-dione:[v]

25. 4-methylene-3-phenyl-3,4-dihydro-5H-spiro[furan-2,3'-indoline]-2',5-dione: ^{1}H NMR ($CDCl_3$) δ 8.37 (s, 1H), 7.22 – 7.20 (m, 3H), 7.18 – 7.13 (m, 1H), 7.03 – 7.00 (m, 2H), 6.80 – 6.78 (m, 2H), 6.68 (d, *J* = 3.0 Hz, 1H), 6.61 (d, *J* = 7.5 Hz, 1H), 5.81 (d, *J* = 3.0 Hz, 1H), 4.68 (t, *J* = 3.0 Hz, 1H); ^{13}C NMR δ 175.86, 169.49, 140.84, 136.55, 135.42, 131.05, 129.21, 128.82, 128.45, 126.43, 125.63, 124.21, 122.97, 110.79, 84.65, 53.16. HRMS (TOF MS EI+) calcd for C18H13NO3Na m/z 314.0793, found: 314.0789.

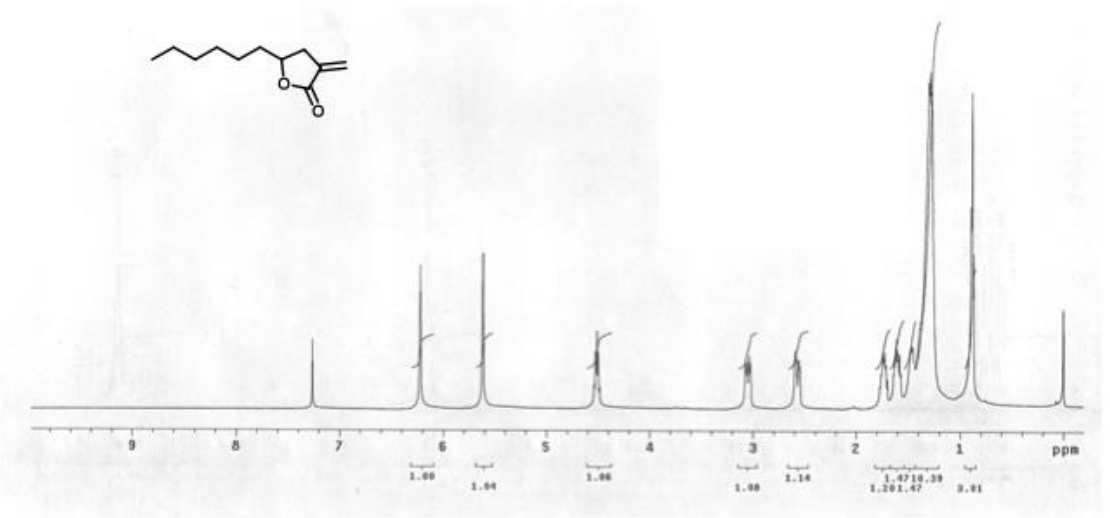
26. 5'-iodo-4-methylene-3,4-dihydro-5H-spiro[furan-2,3'-indoline]-2',5-dione:[v]

27. 5'-methoxy-4-methylene-3,4-dihydro-5H-spiro[furan-2,3'-indoline]-2',5-dione: ^{1}H NMR ($CDCl_3$) δ 8.28 (s, 1H), 6.89 – 6.85 (m, 3H), 6.42 (t, *J* = 2.5 Hz, 1H), 5.82 (t, *J* = 2.5 Hz, 1H), 3.78 (s, 3H), 3.36 – 3.32 (m, 1H), 3.13 – 3.09 (m, 1H); ^{13}C NMR δ 175.81, 169.20, 156.86, 134.20, 132.75, 128.26, 123.57, 116.41, 111.77, 111.33, 80.20, 56.12, 36.68. HRMS (TOF MS EI+) calcd for C13H11NO4Na m/z 268.0586, found: 268.0570.

28. 5'-bromo-4-methylene-7'-nitro-3,4-dihydro-5H-spiro[furan-2,3'-indoline]-2',5-dione: ^{1}H NMR ($CDCl_3$) δ 9.09 (s, 1H), 8.33 (d, *J* = 1.5 Hz, 1H), 7.71 (s, 1H), 6.48 (d, *J* = 2.5 Hz, 1H), 5.90 (d, *J* = 2.5 Hz, 1H), 3.41 – 3.37 (m, 1H), 3.15 – 3.11 (m, 1H); ^{13}C NMR δ 173.25, 167.88, 137.27, 133.91, 132.25, 130.98, 128.84, 125.21, 115.74, 36.43.

29. 4',7'-dimethyl-4-methylene-3,4-dihydro-5H-spiro[furan-2,3'-indoline]-2',5-dione:[iv]

NMR spectra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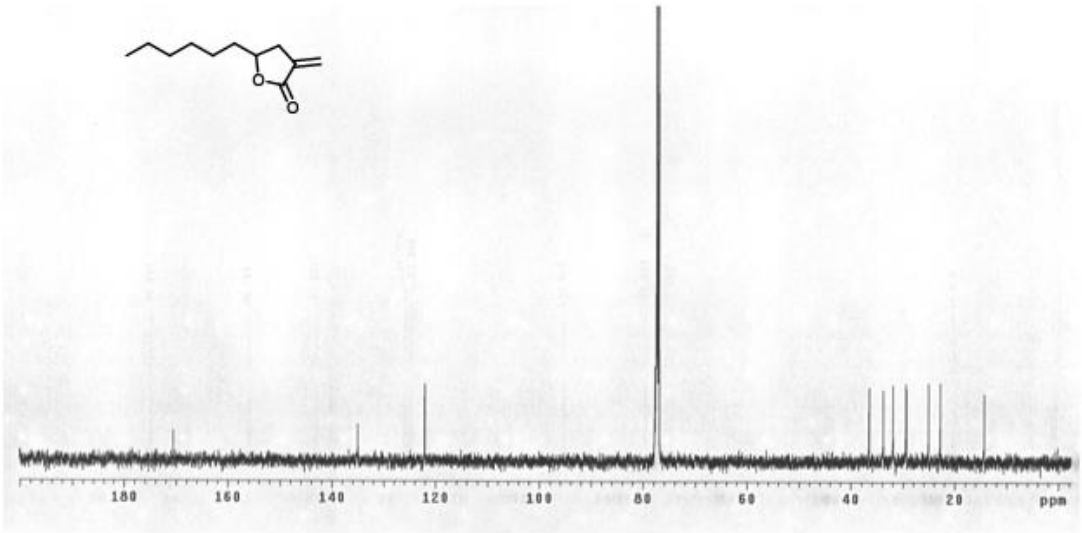
1.2011.47
3.01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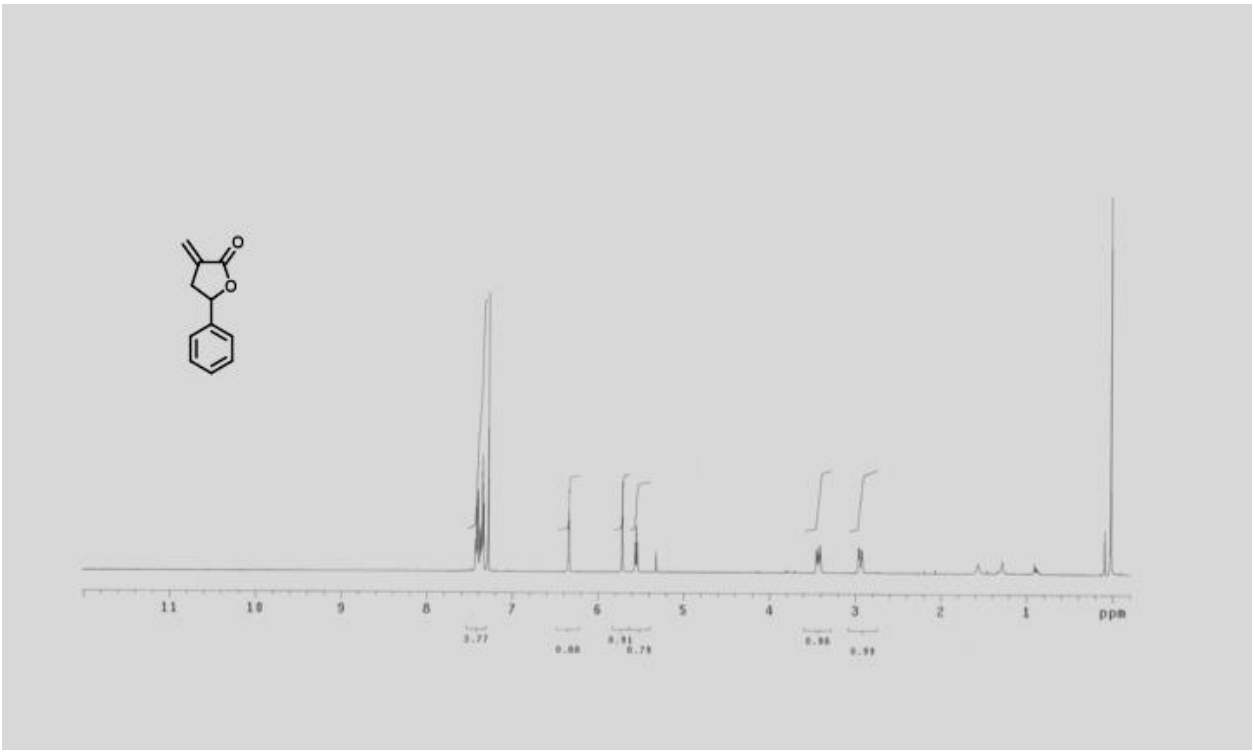
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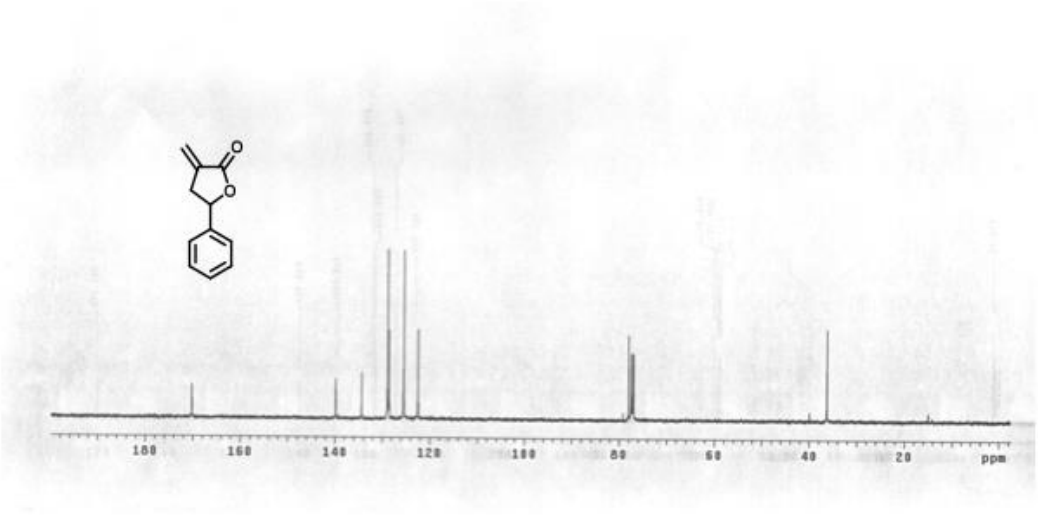
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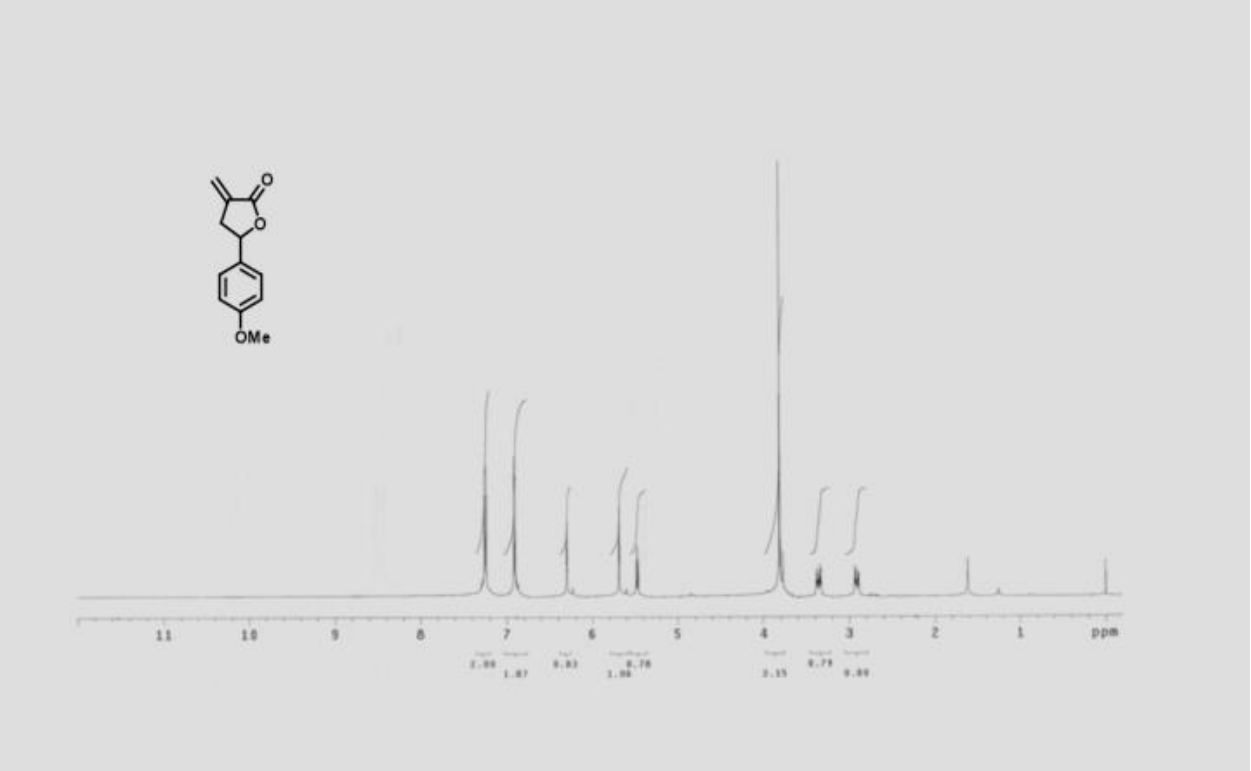
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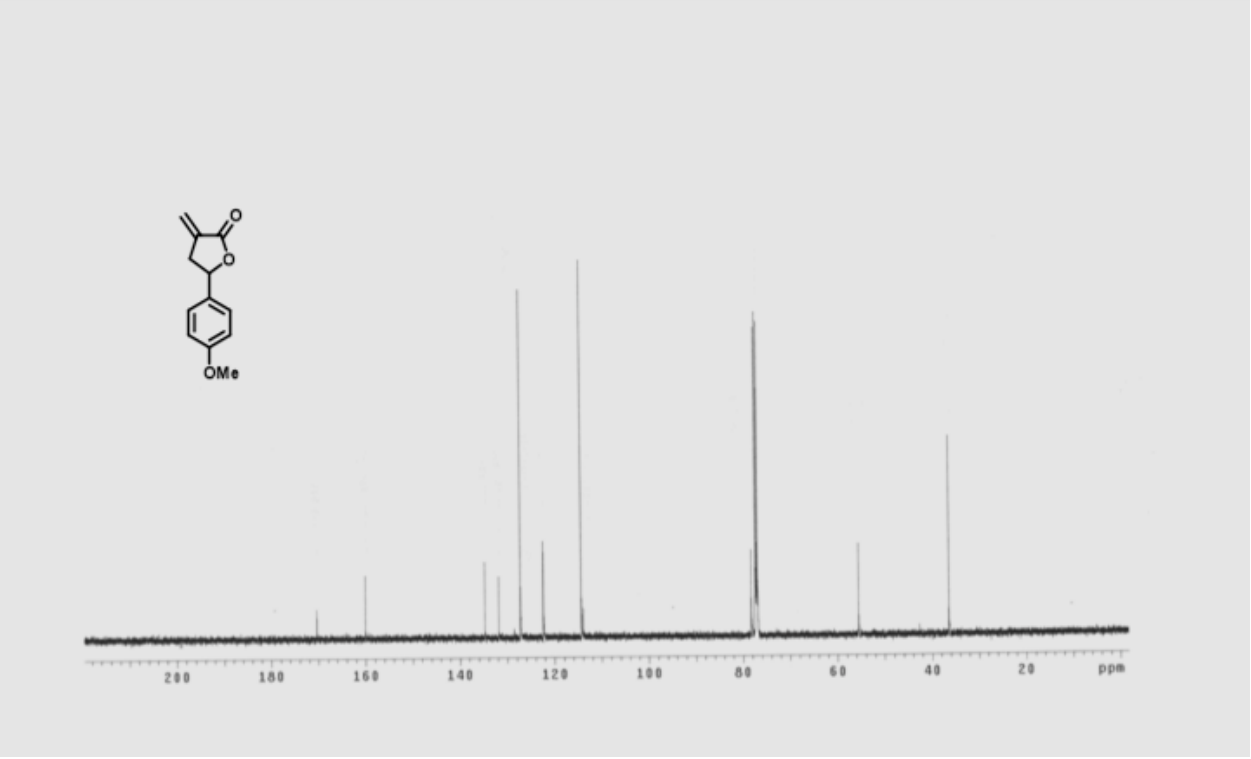
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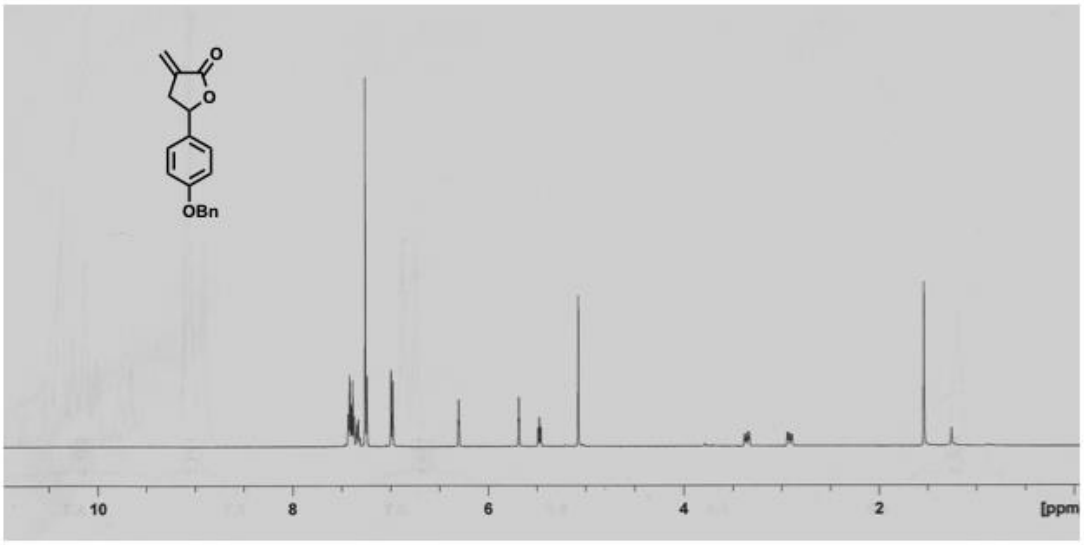
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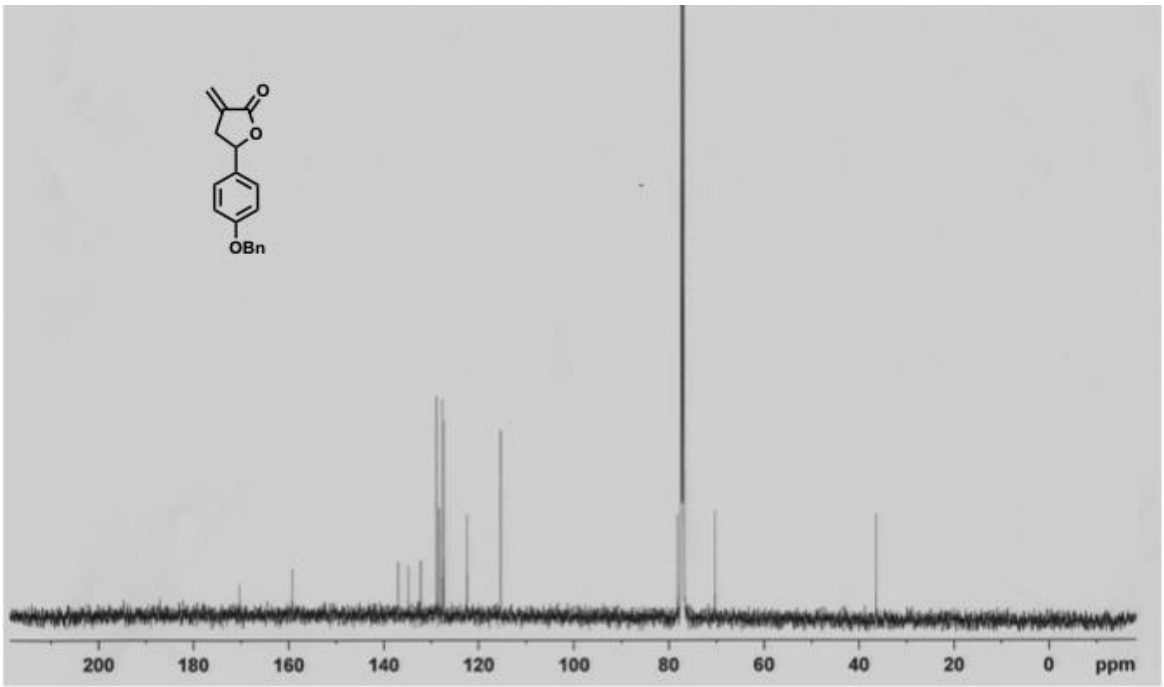
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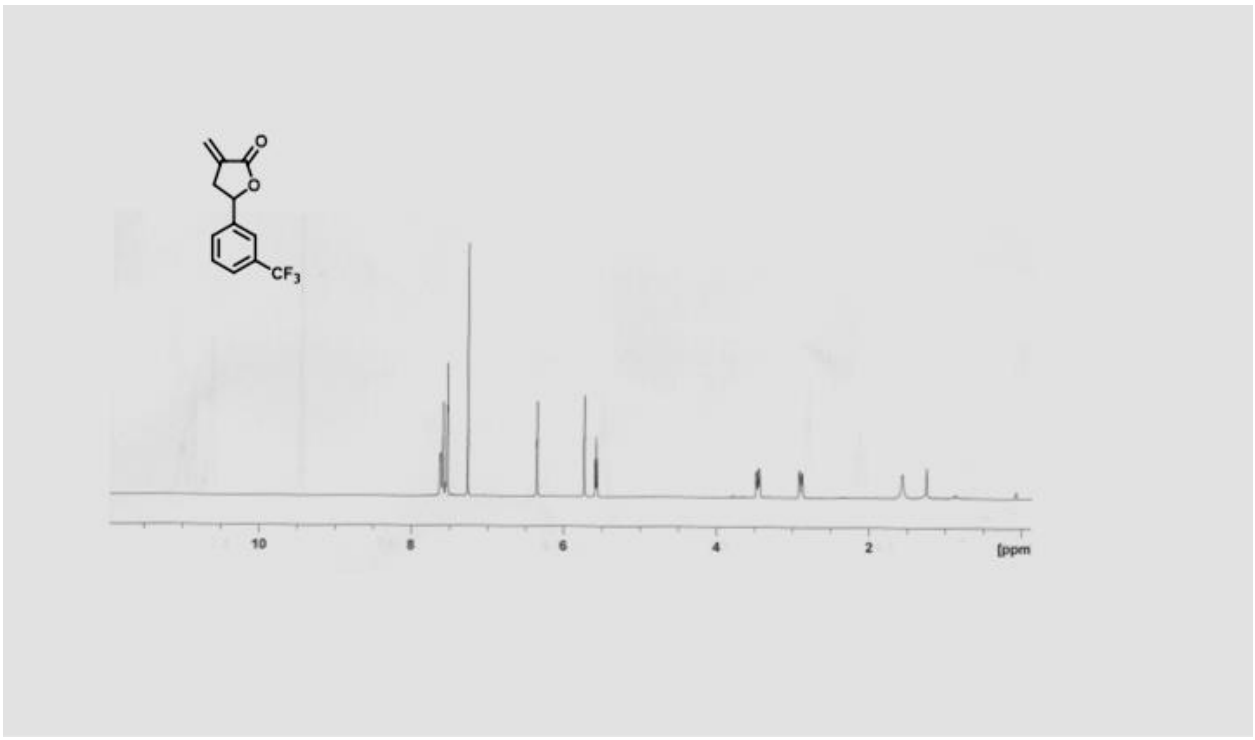
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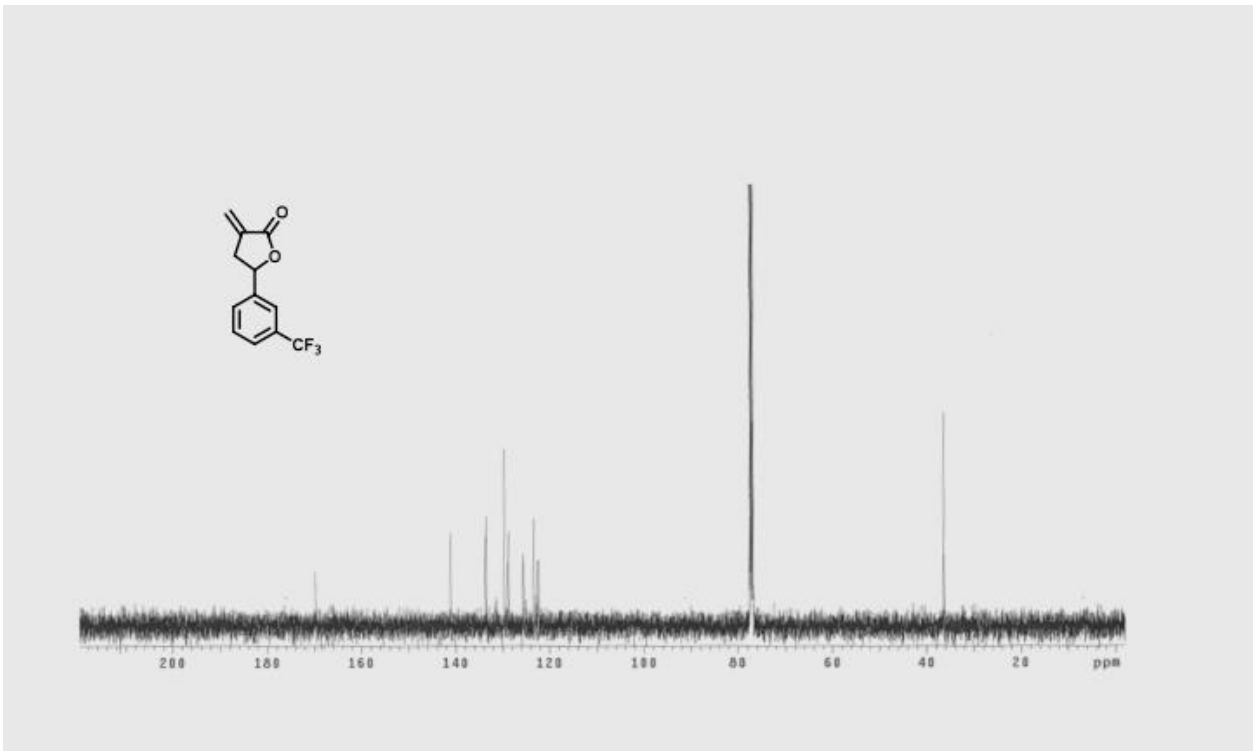
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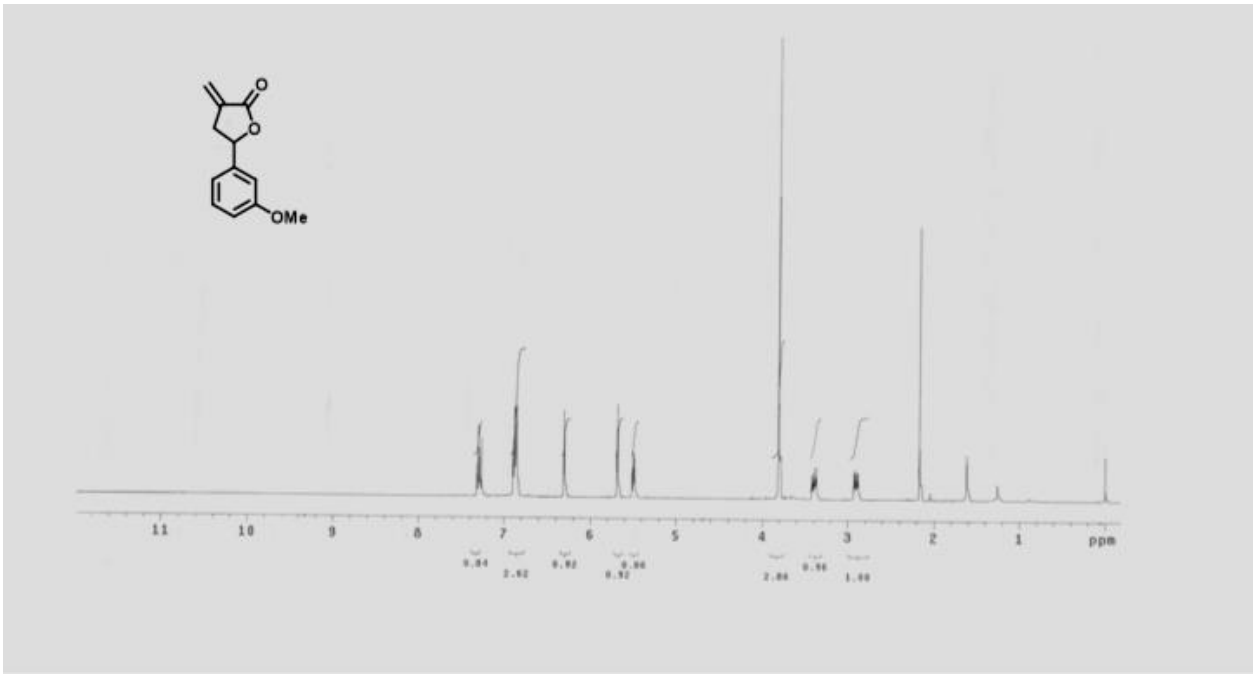
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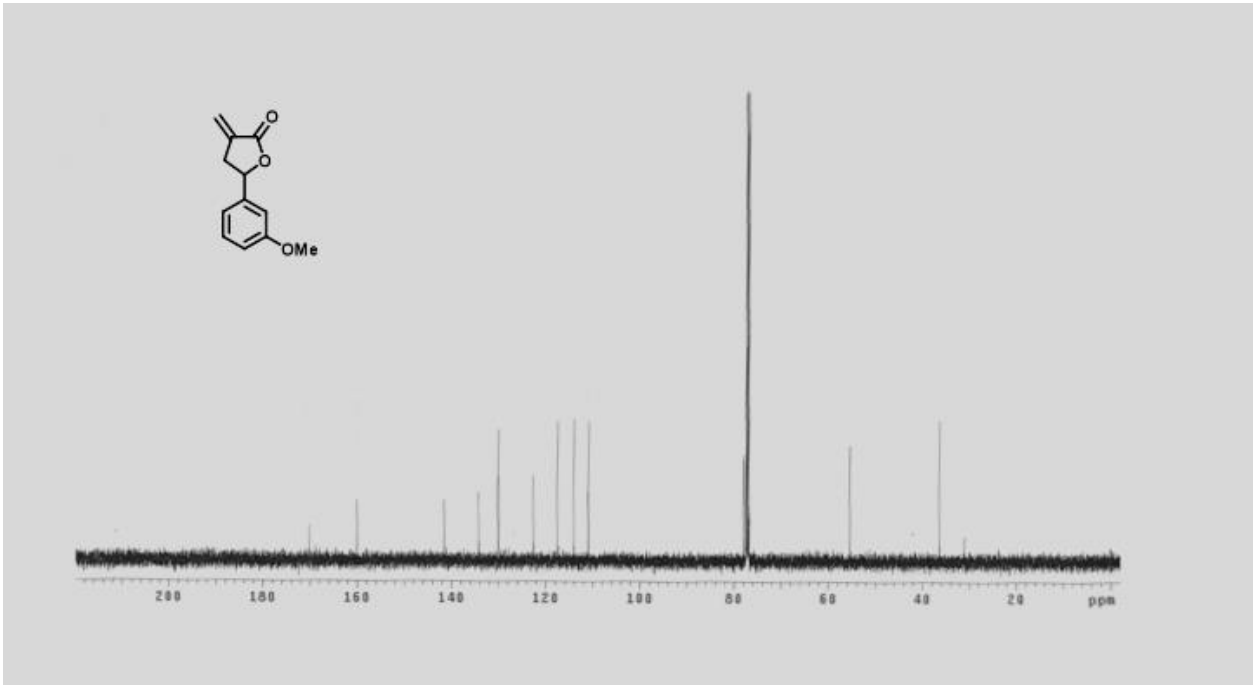
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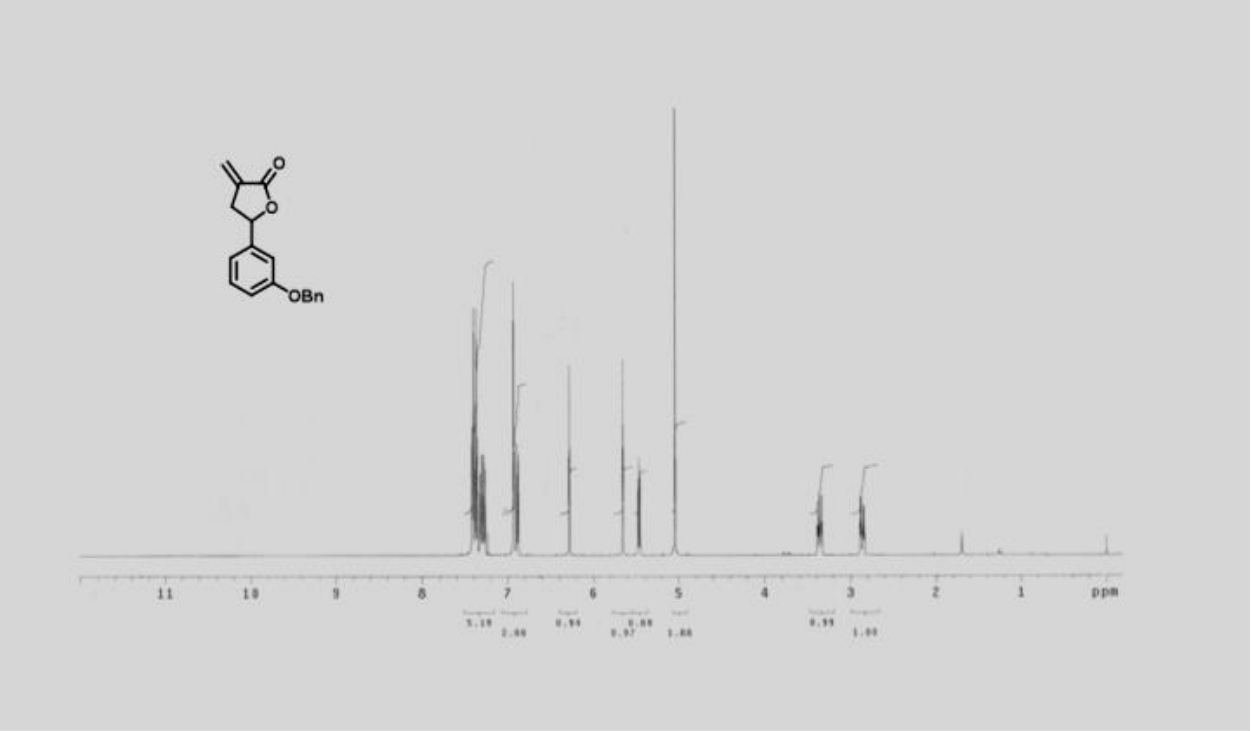
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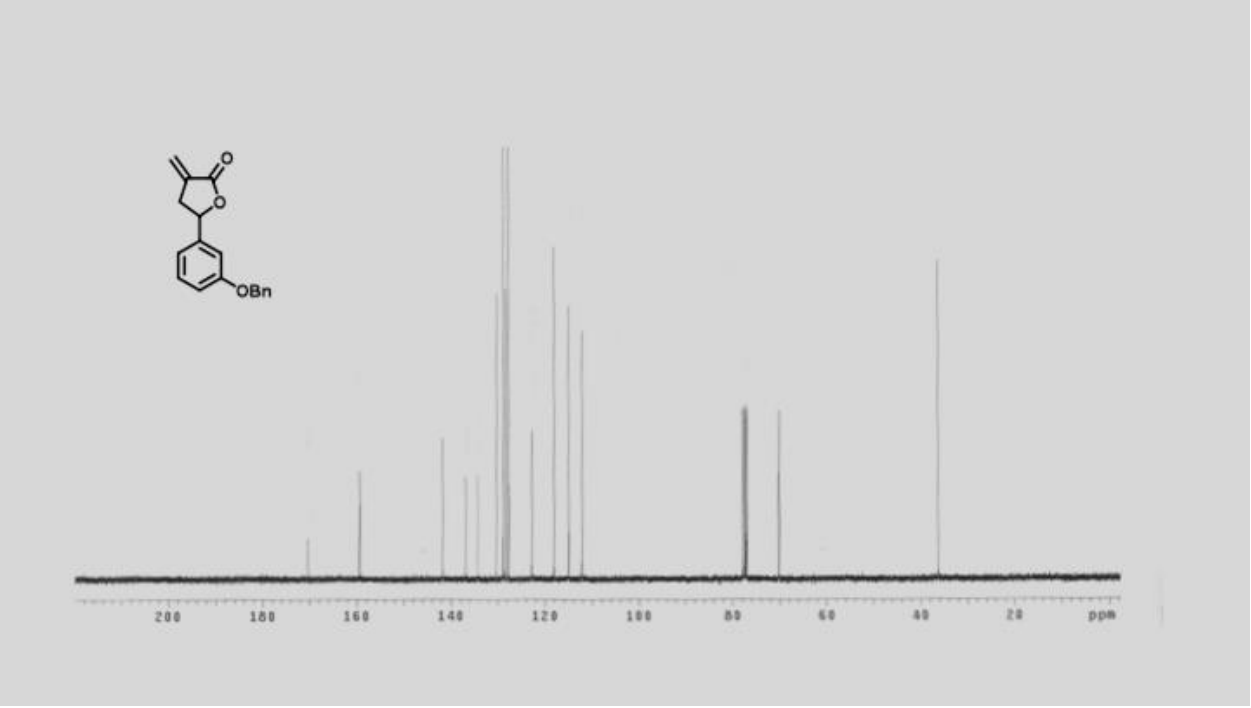
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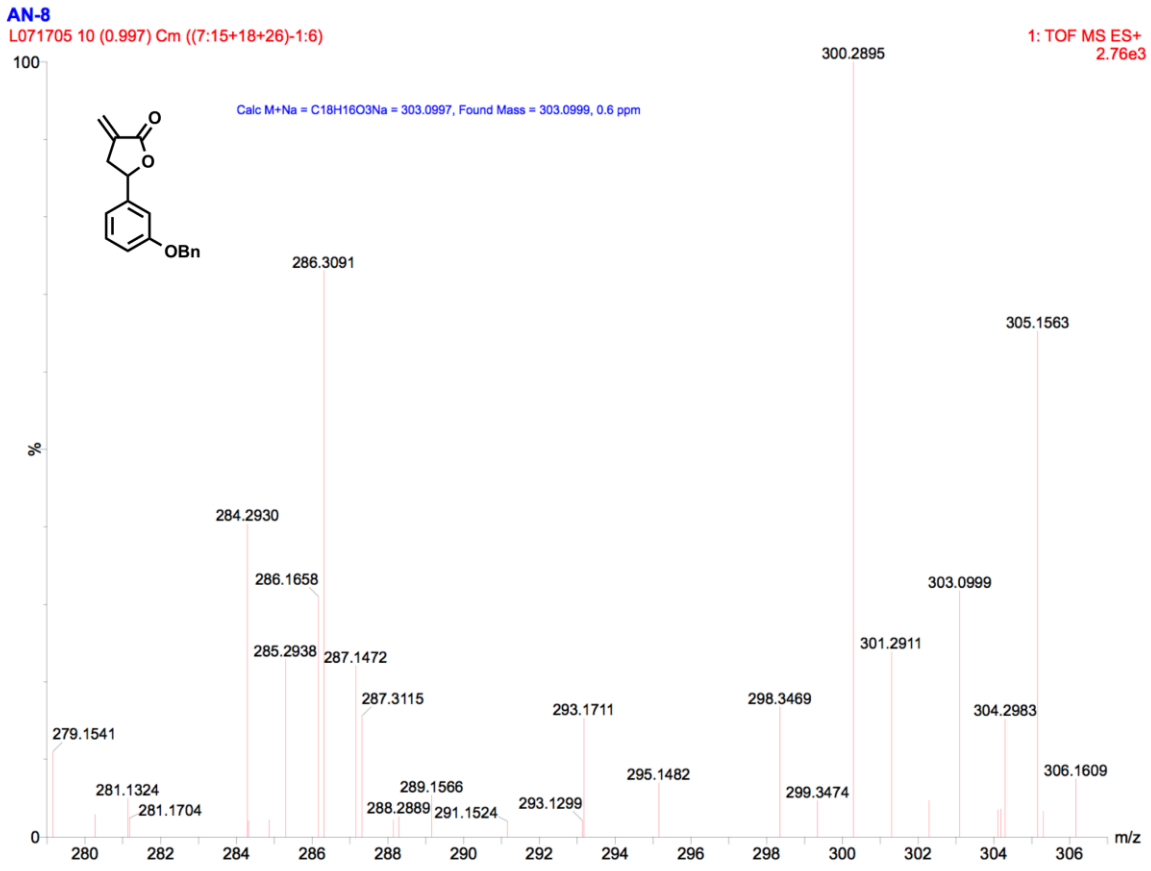
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OBn
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%
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m/z
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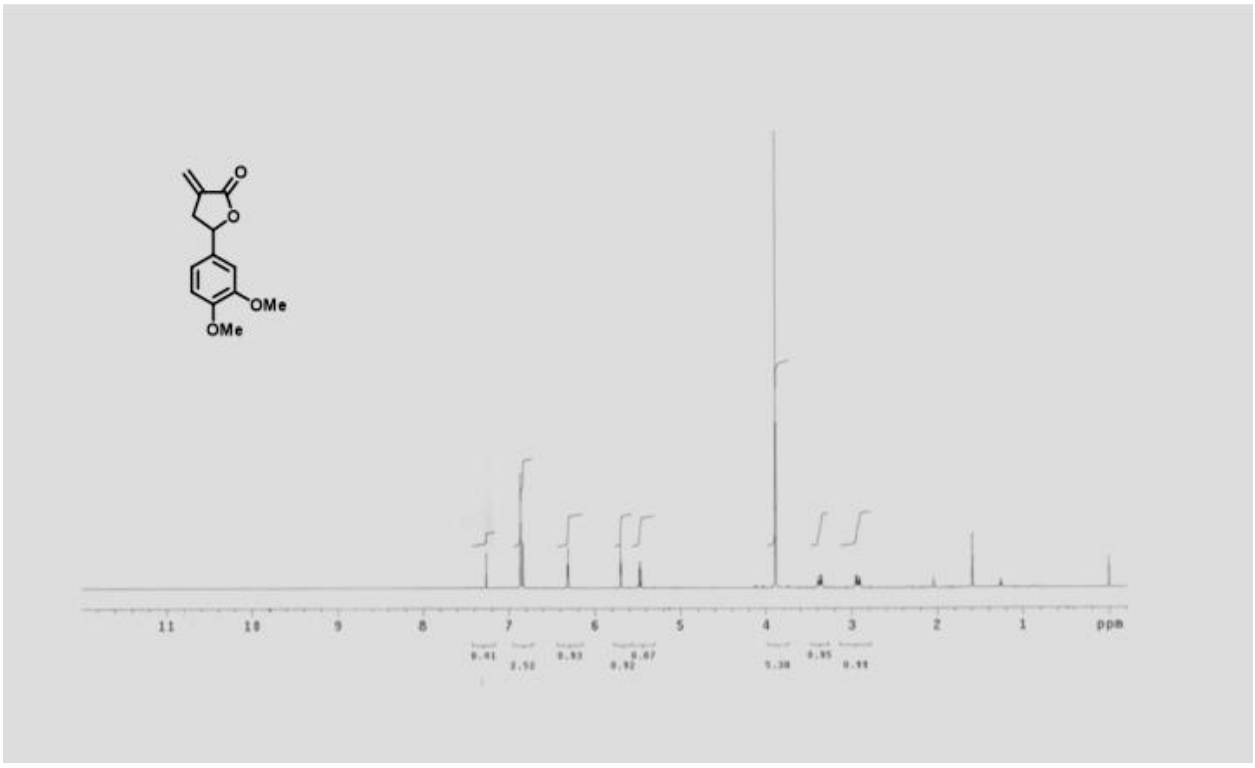
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OMe
OMe
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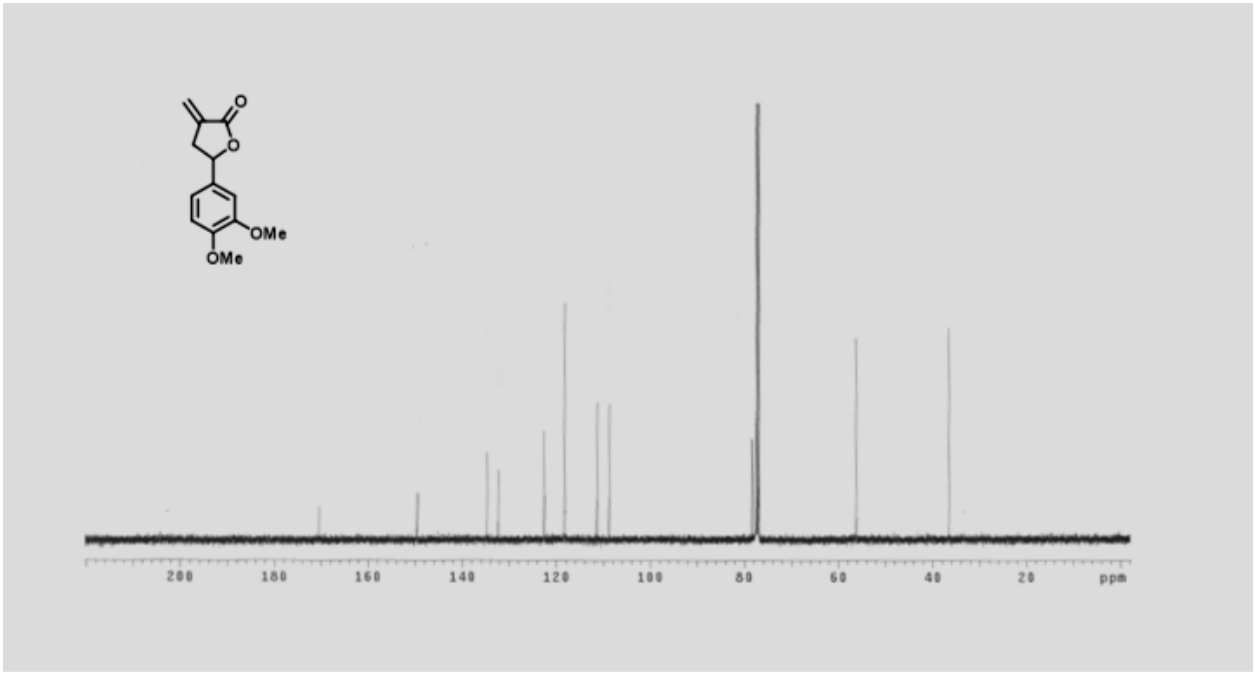
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OMe
OMe
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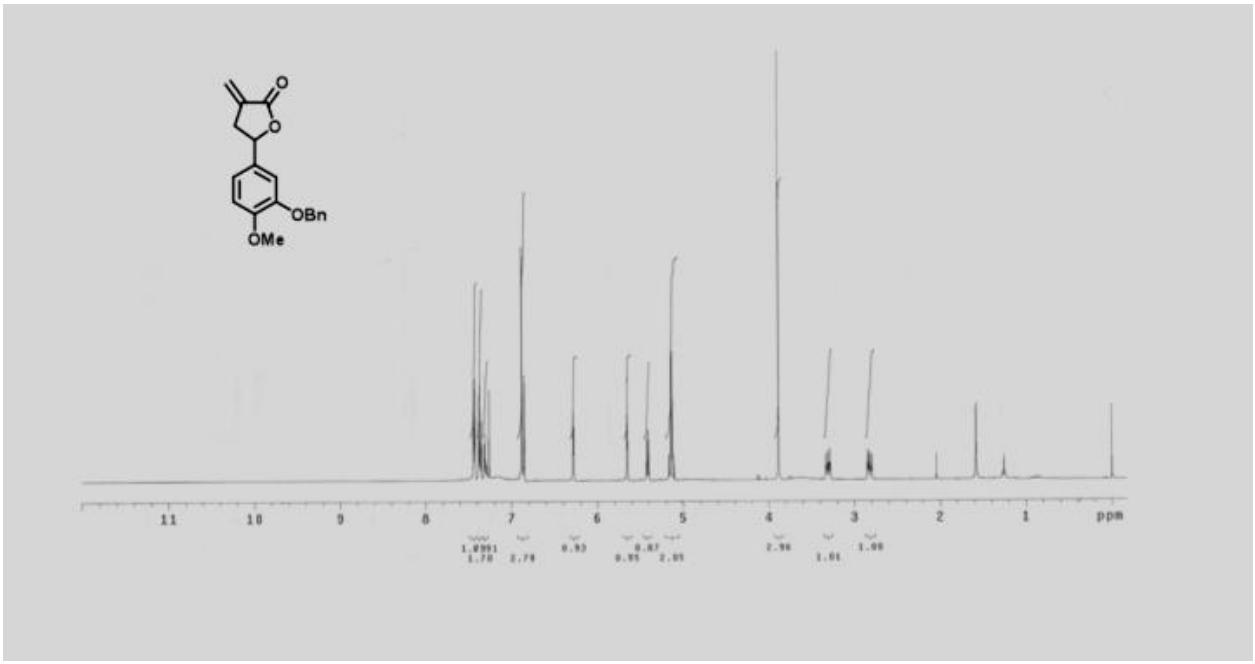
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OMe
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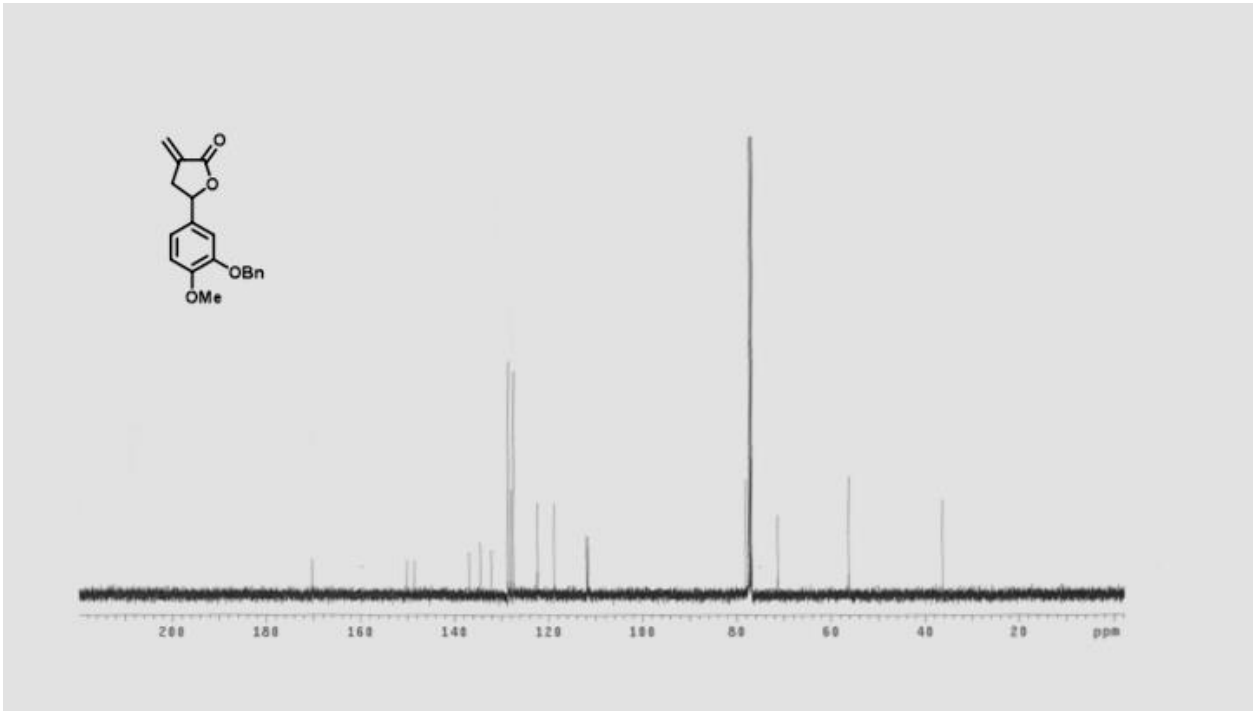
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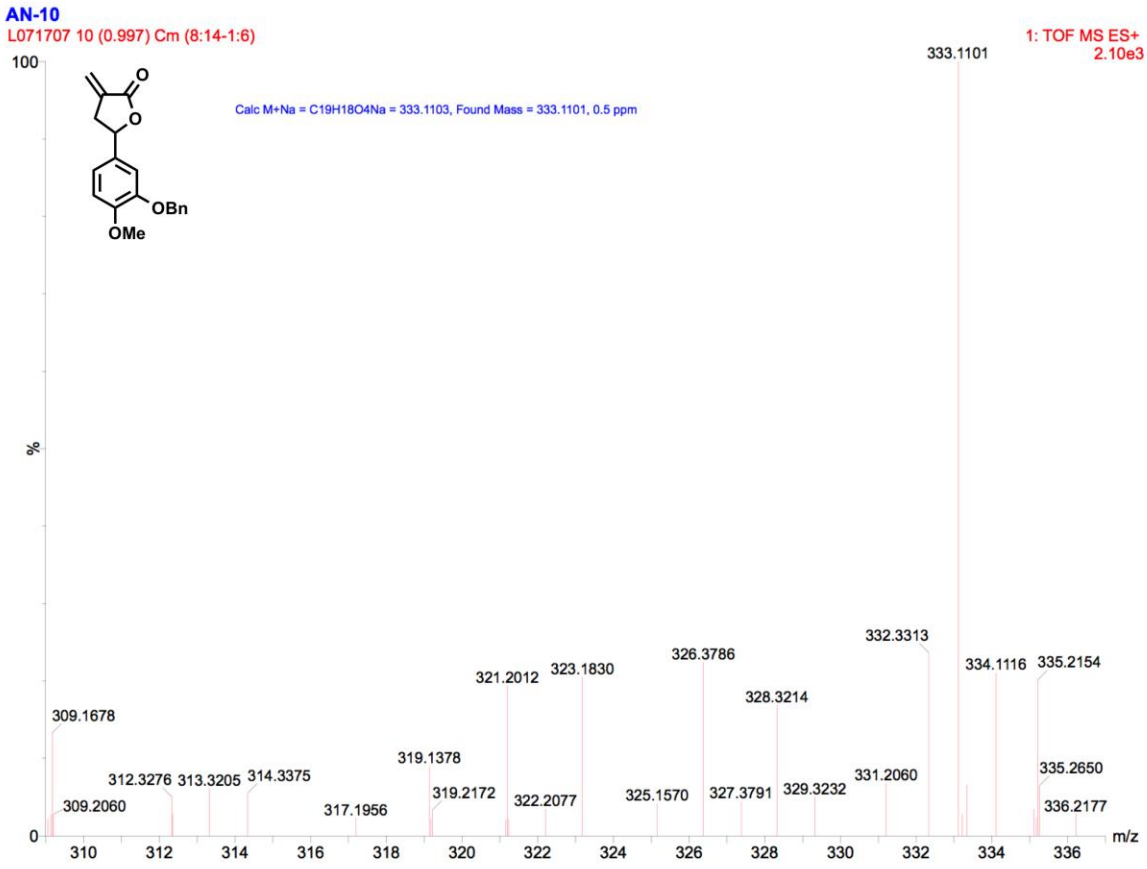
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OMe
%
m/z
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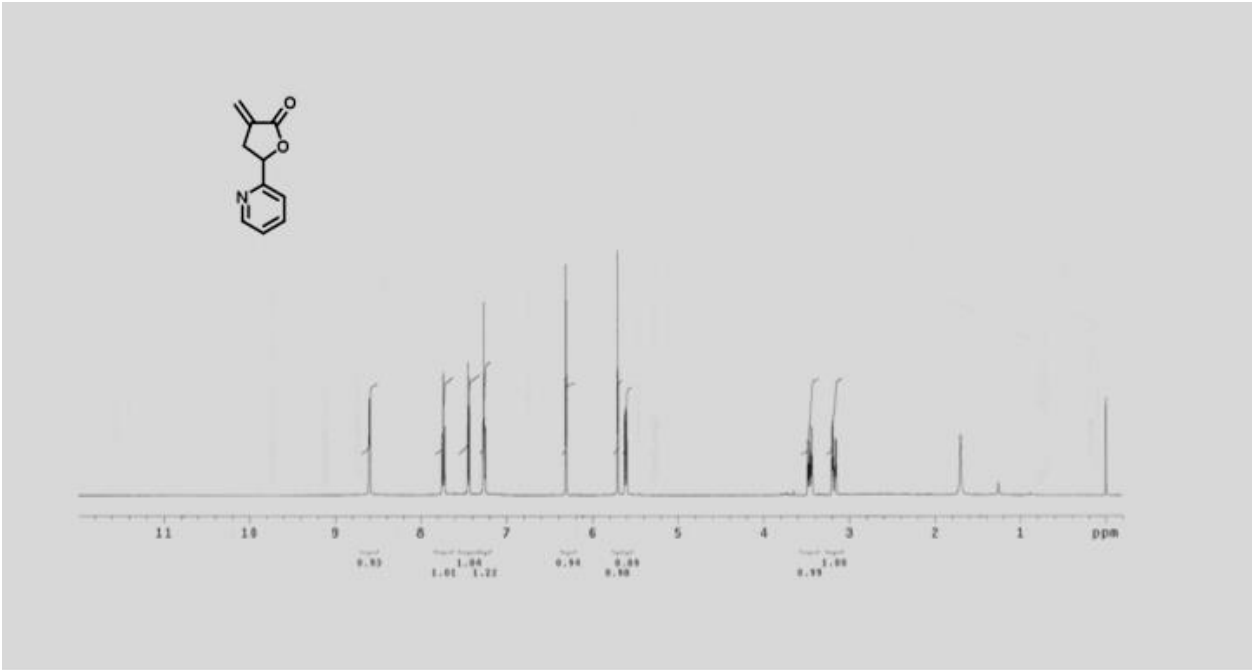
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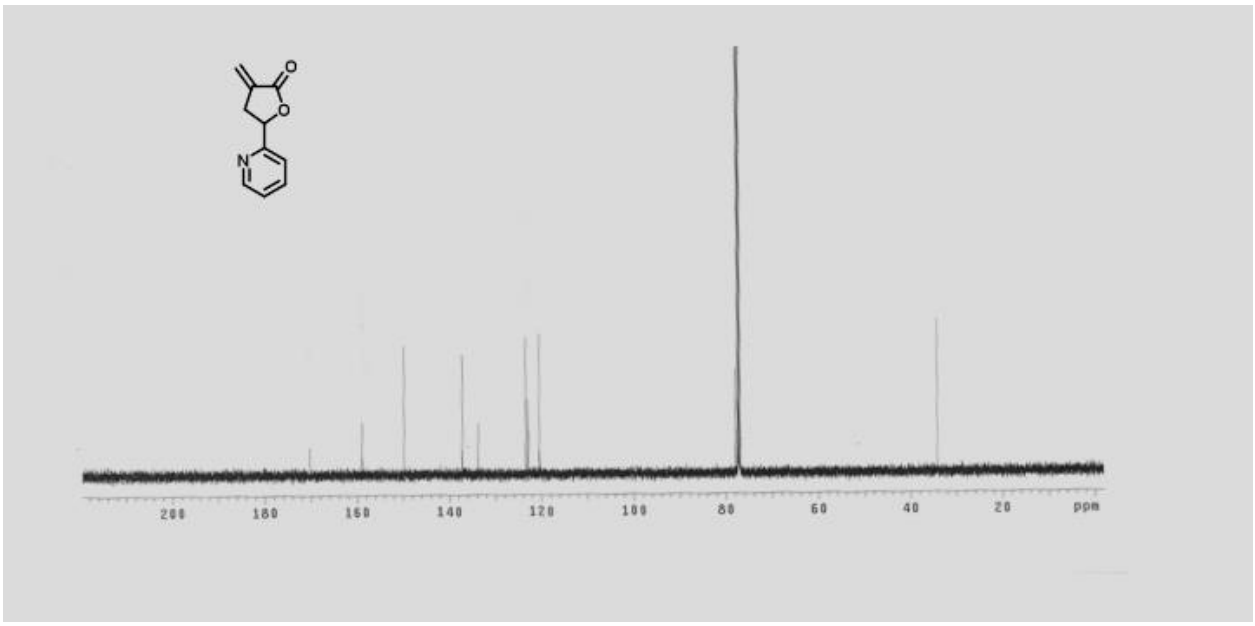
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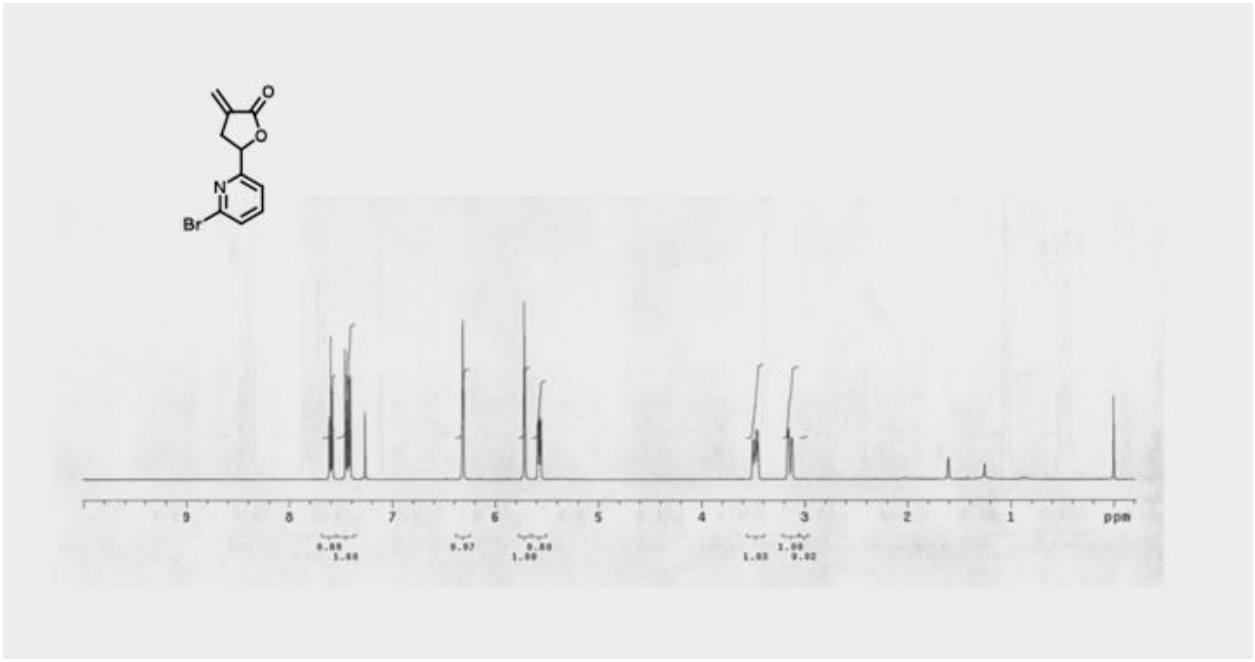
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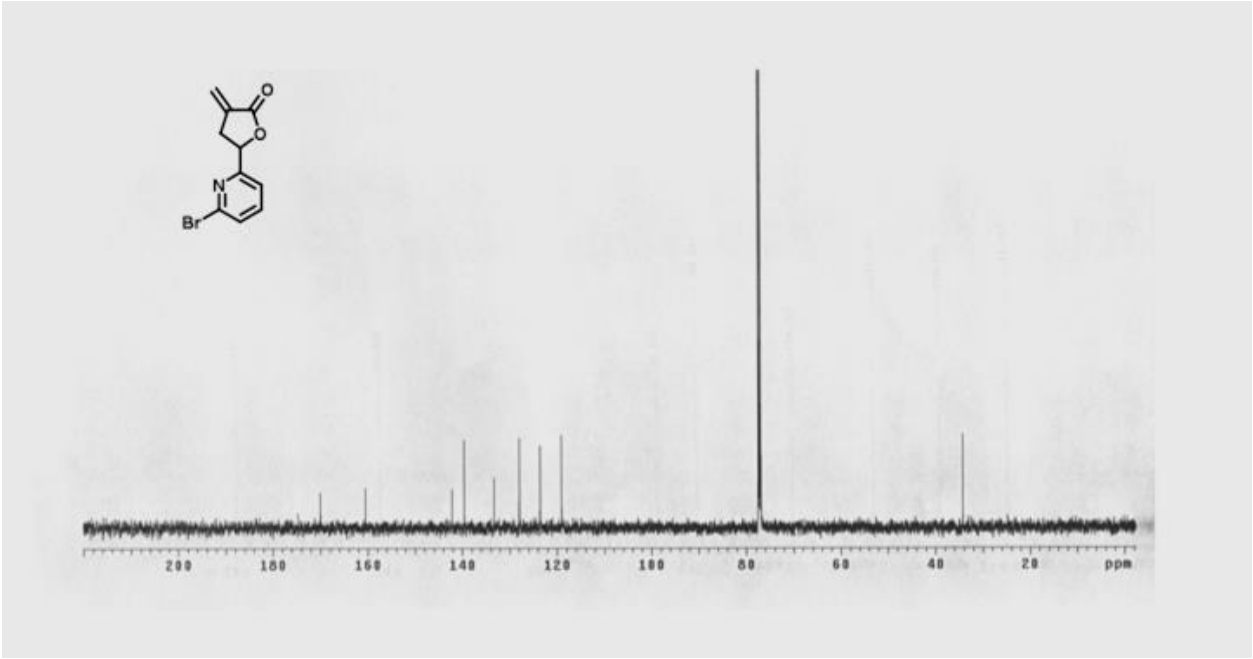
ppm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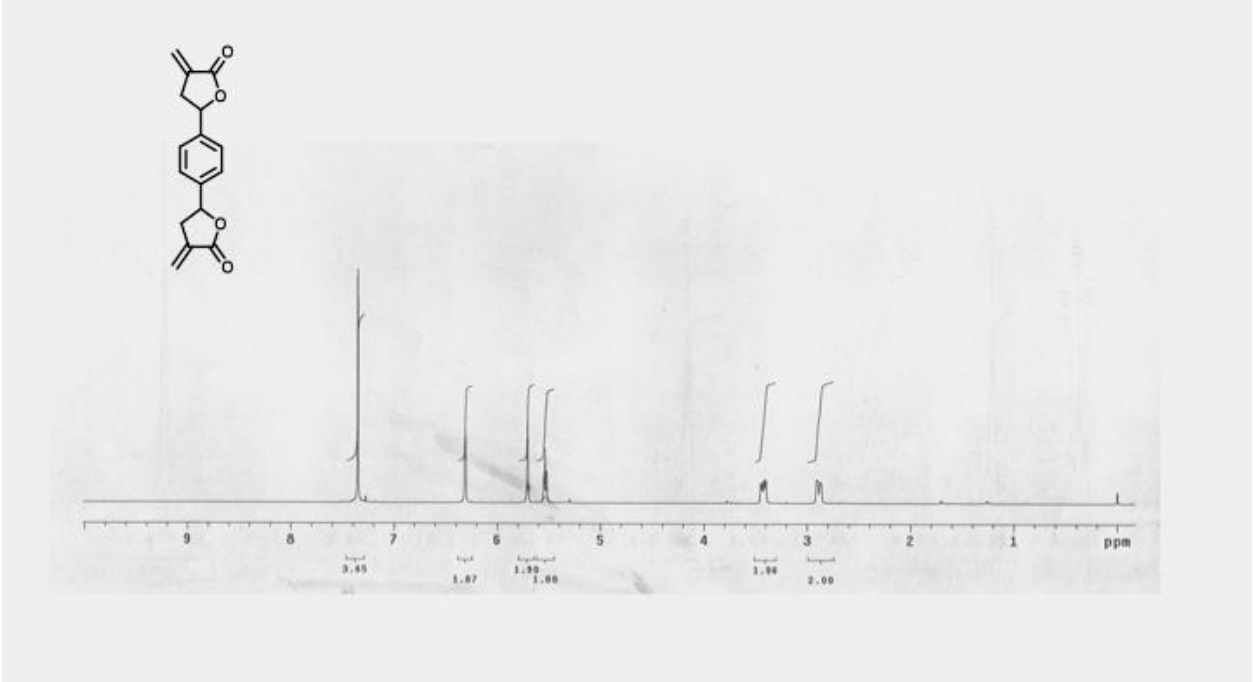
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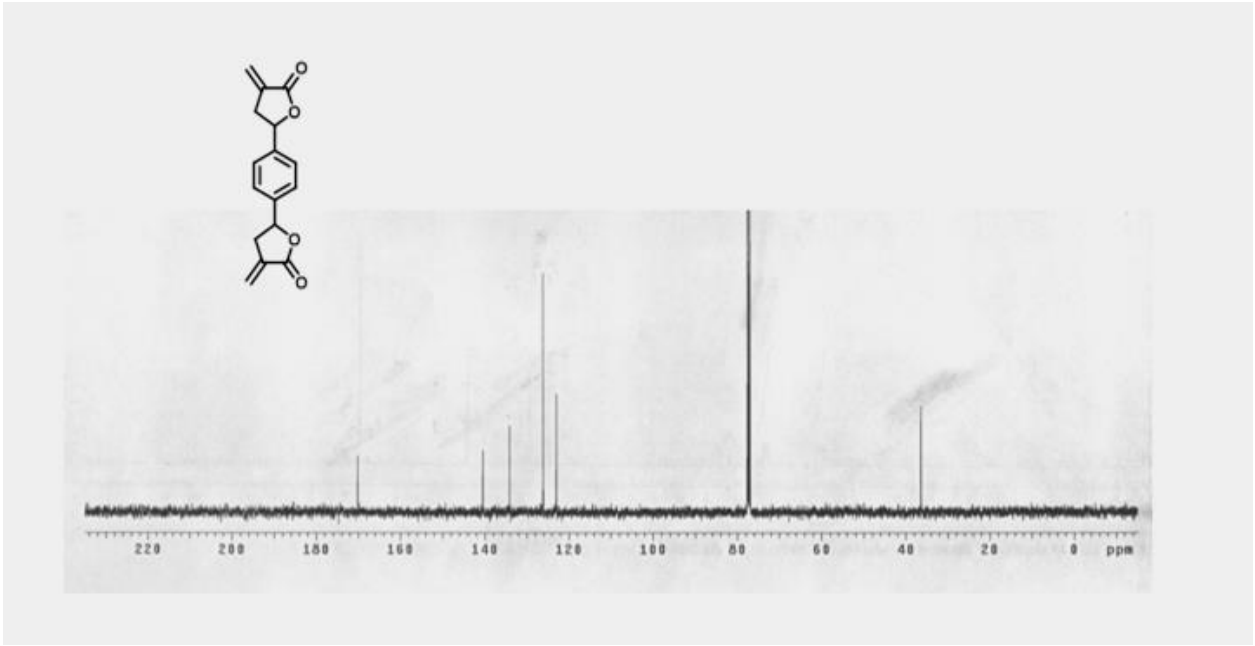
2.00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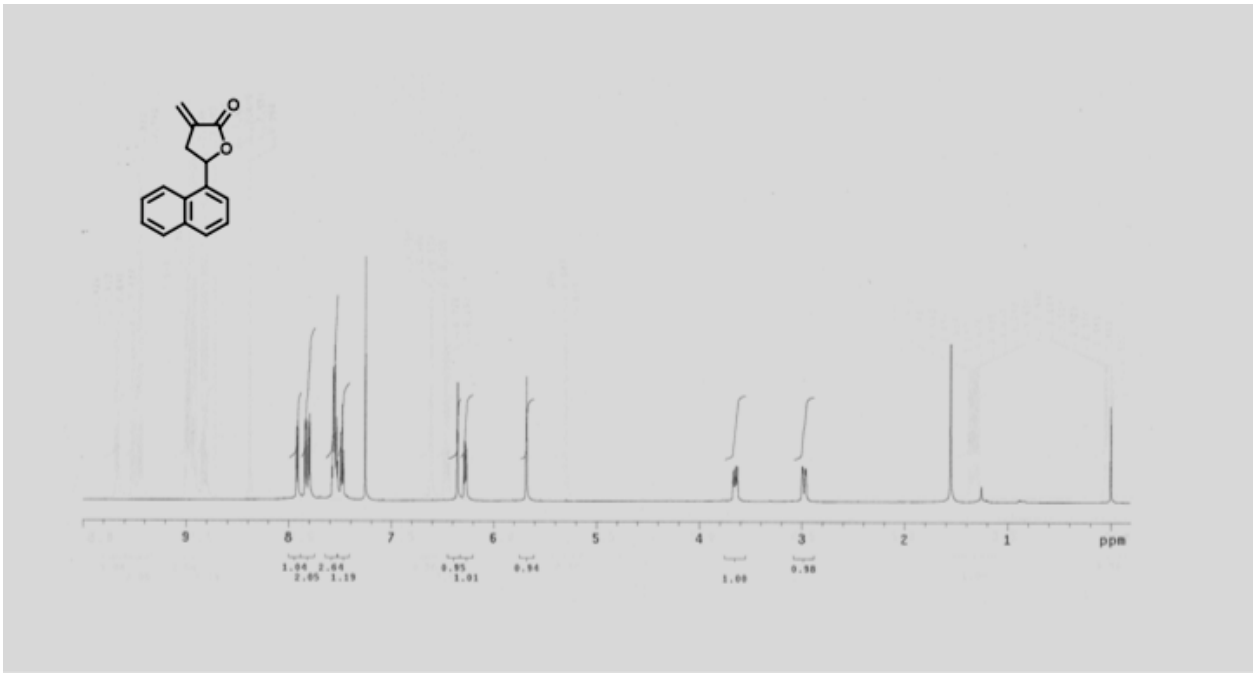
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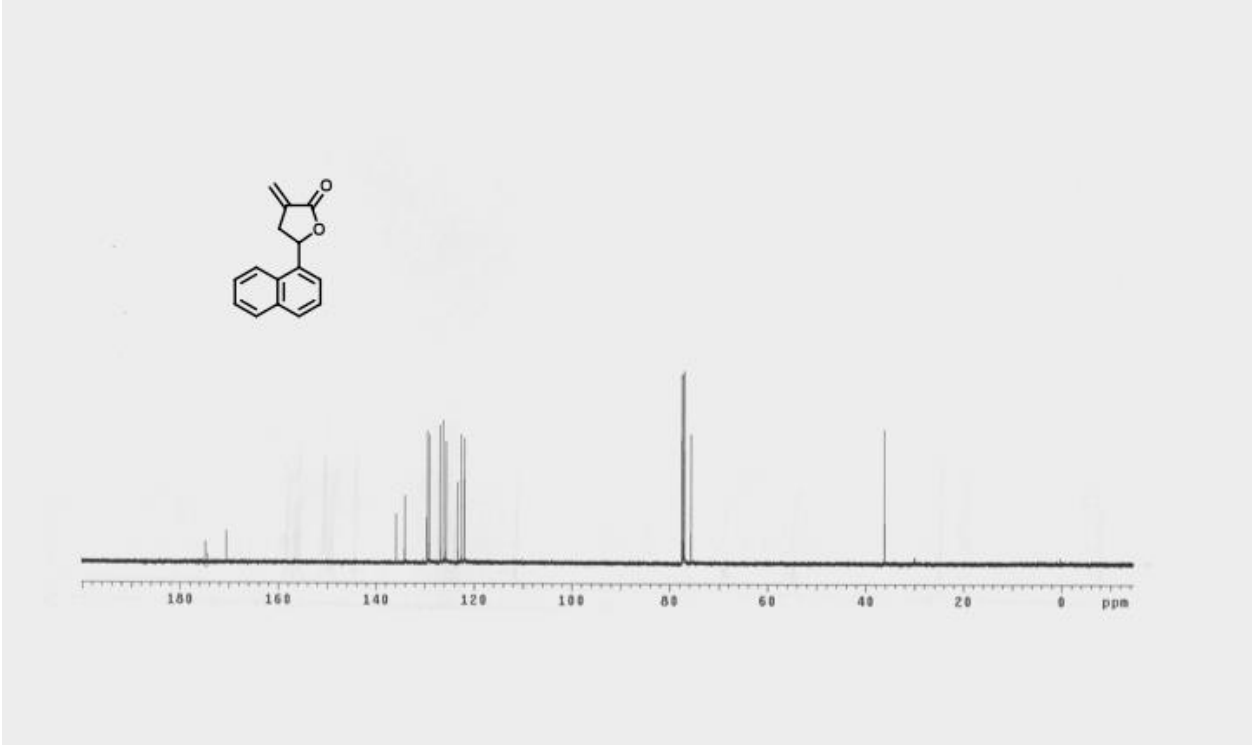
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40
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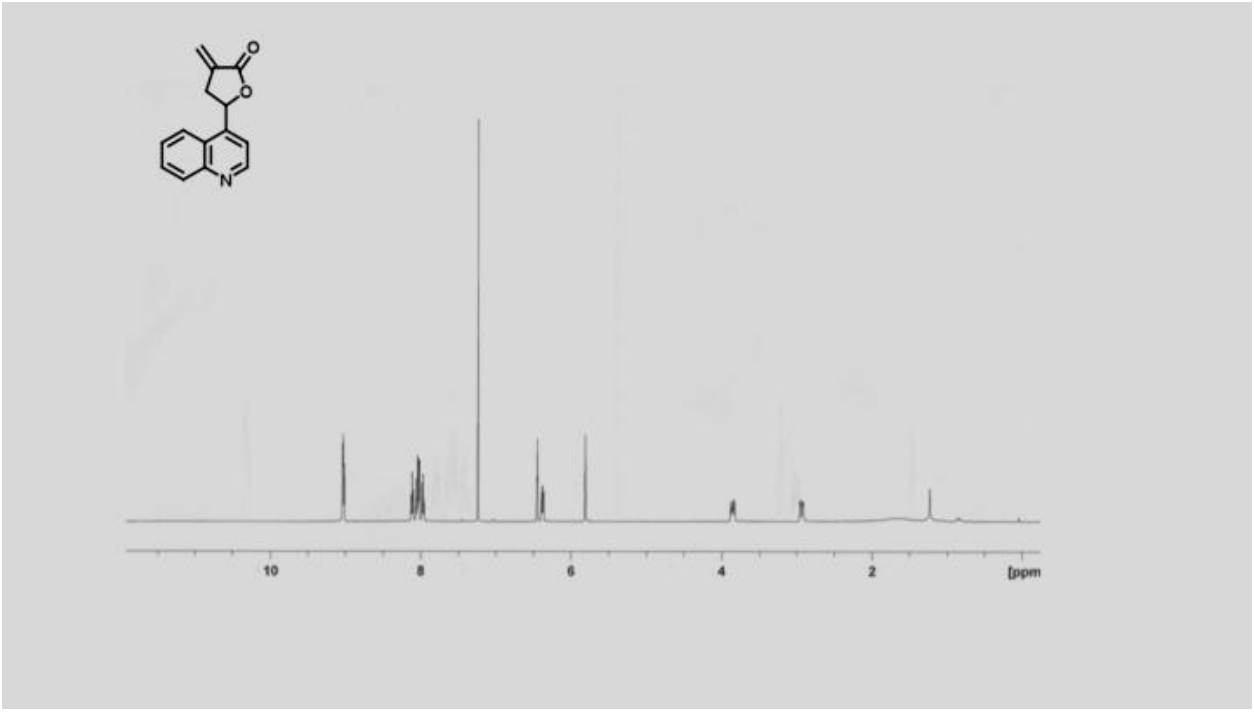
ppm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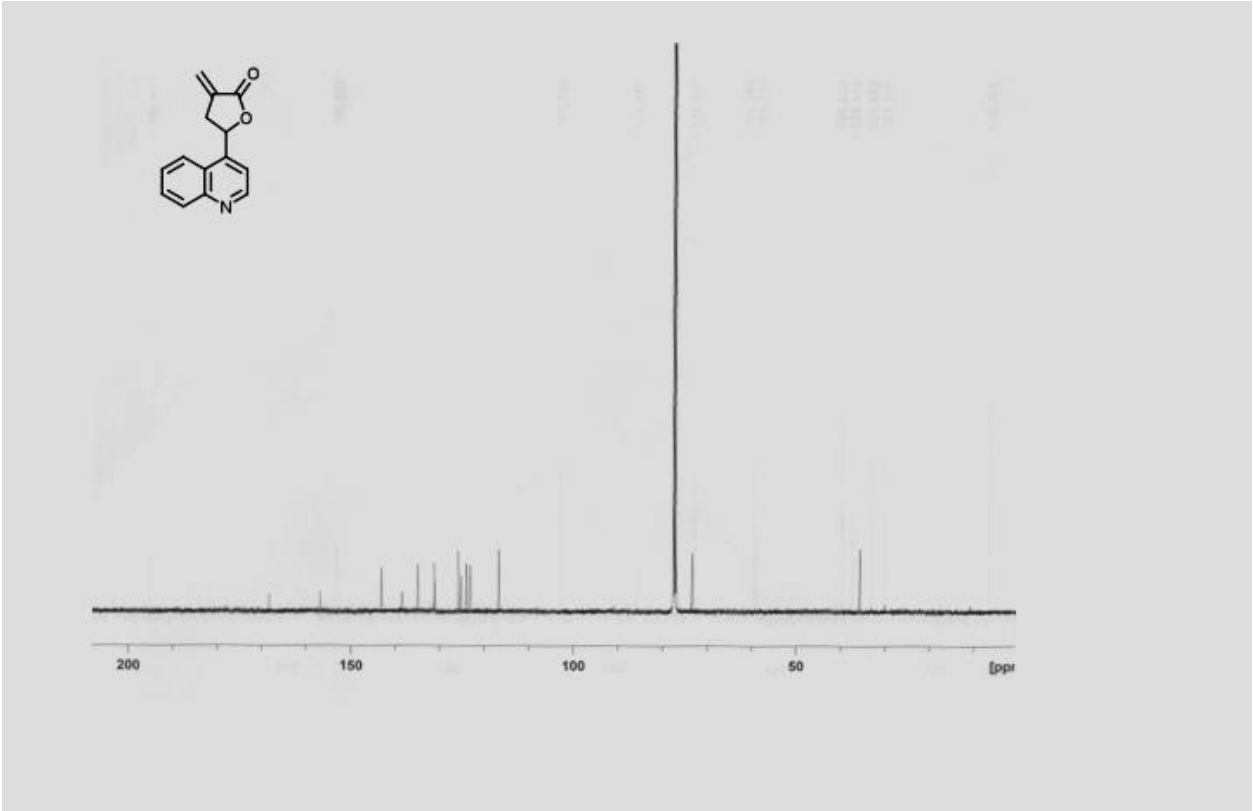
8
6
4
2
[ppm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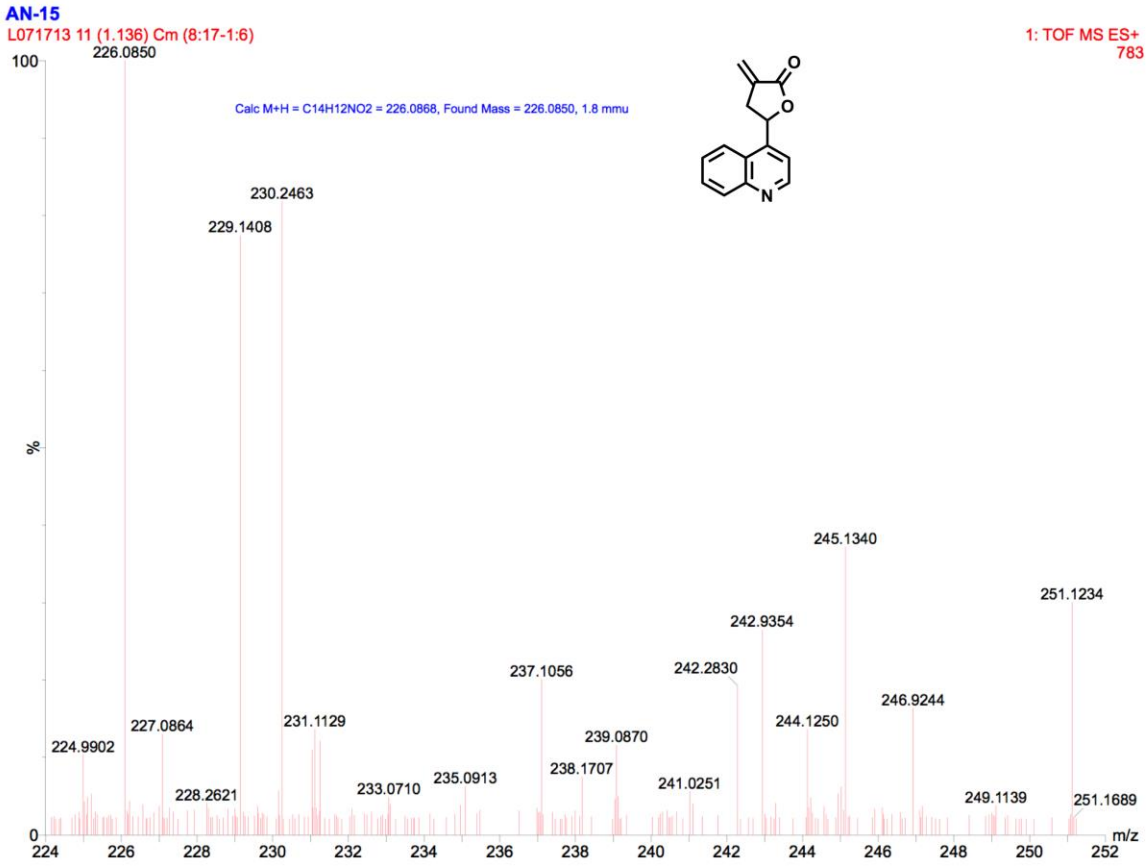
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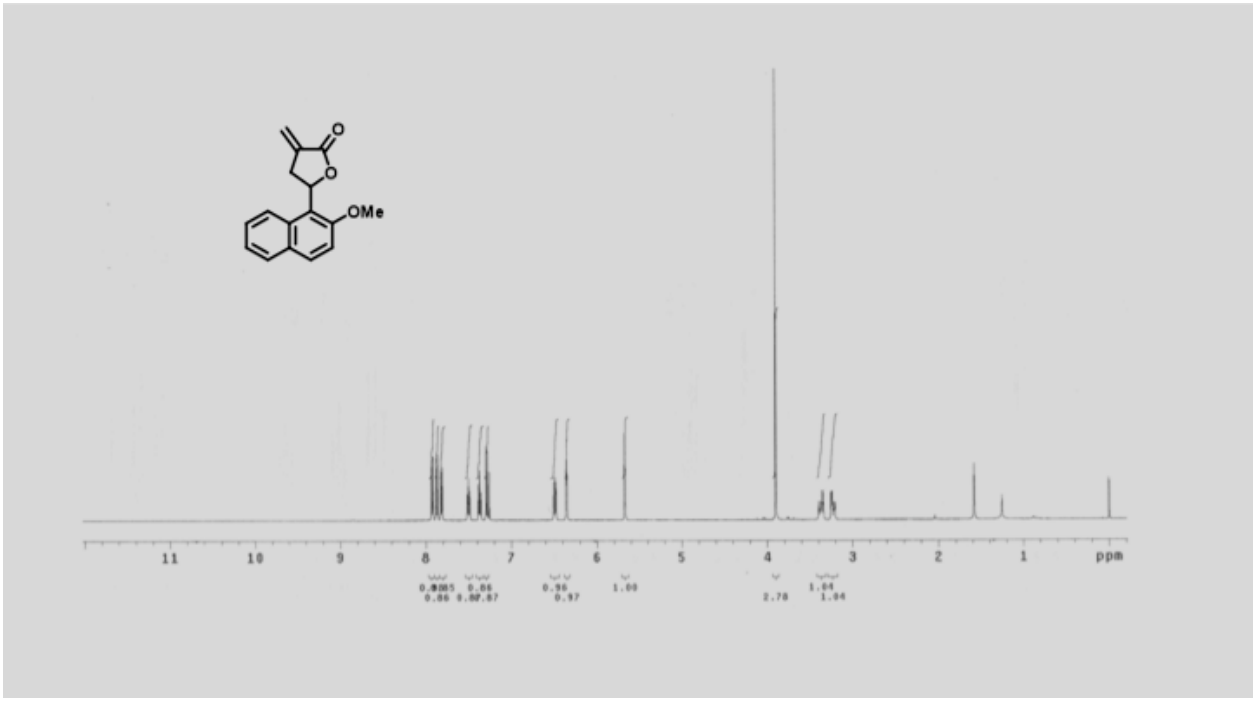
m/z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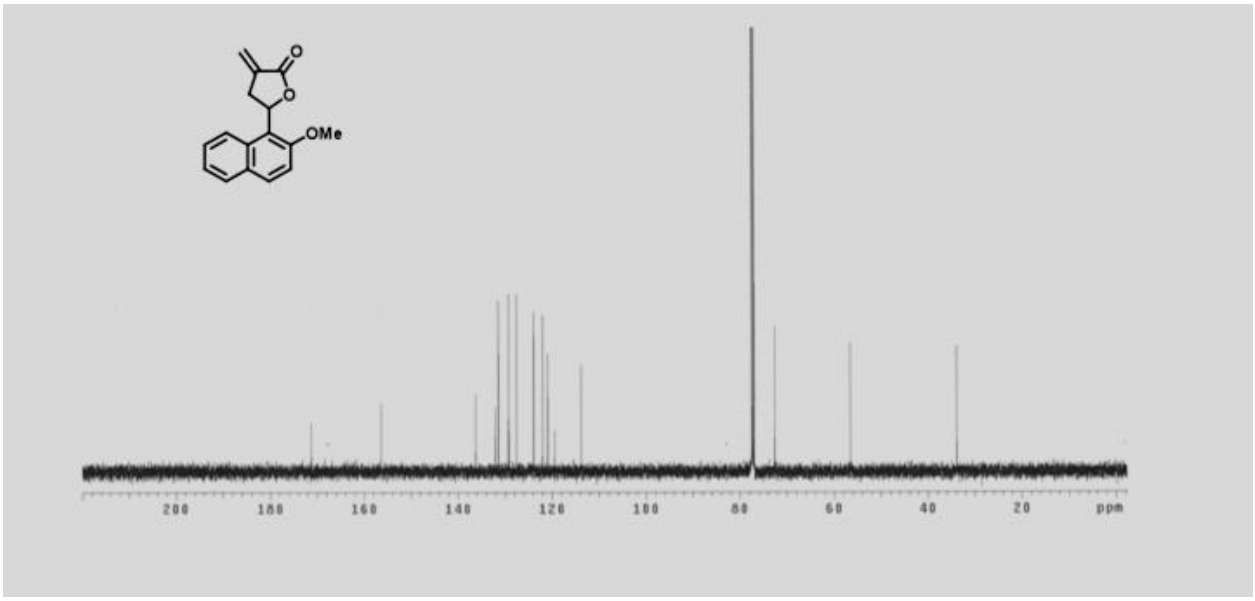
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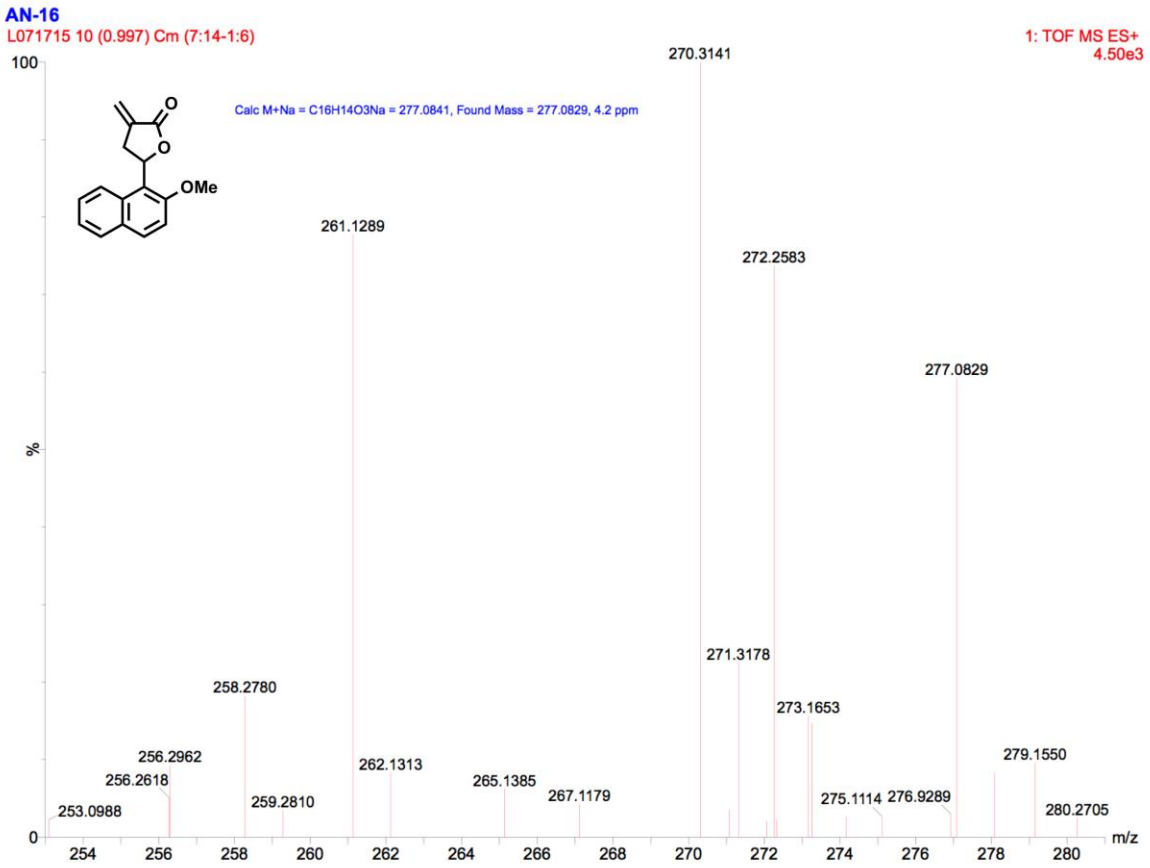
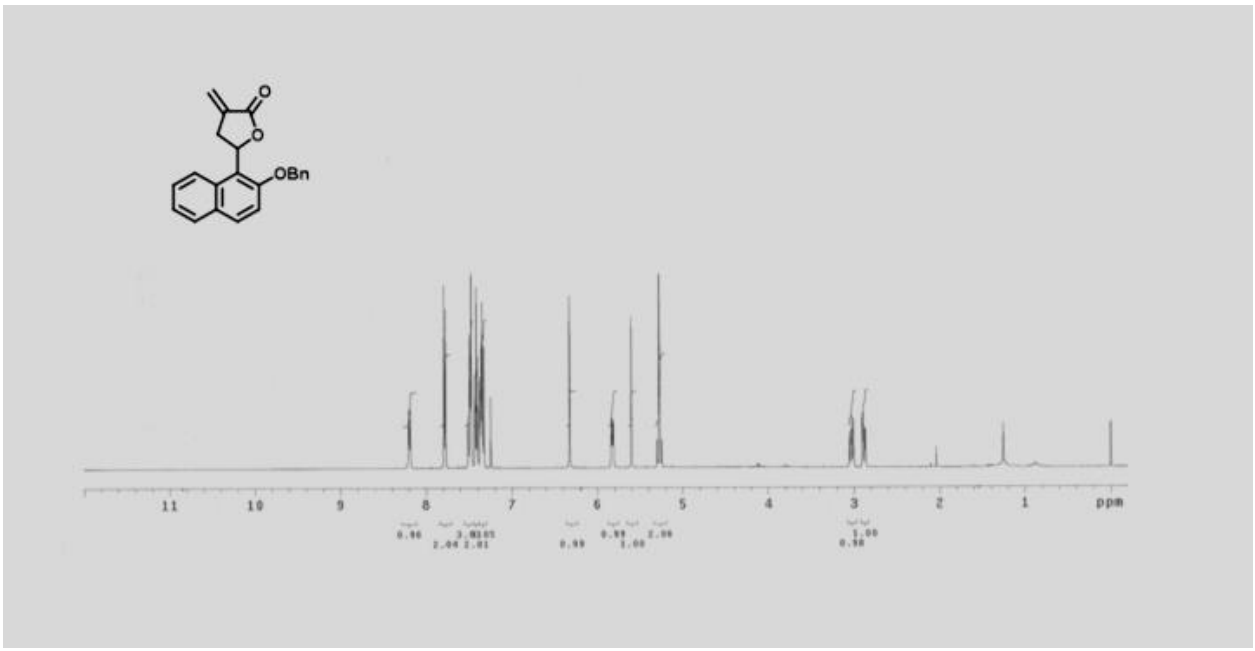
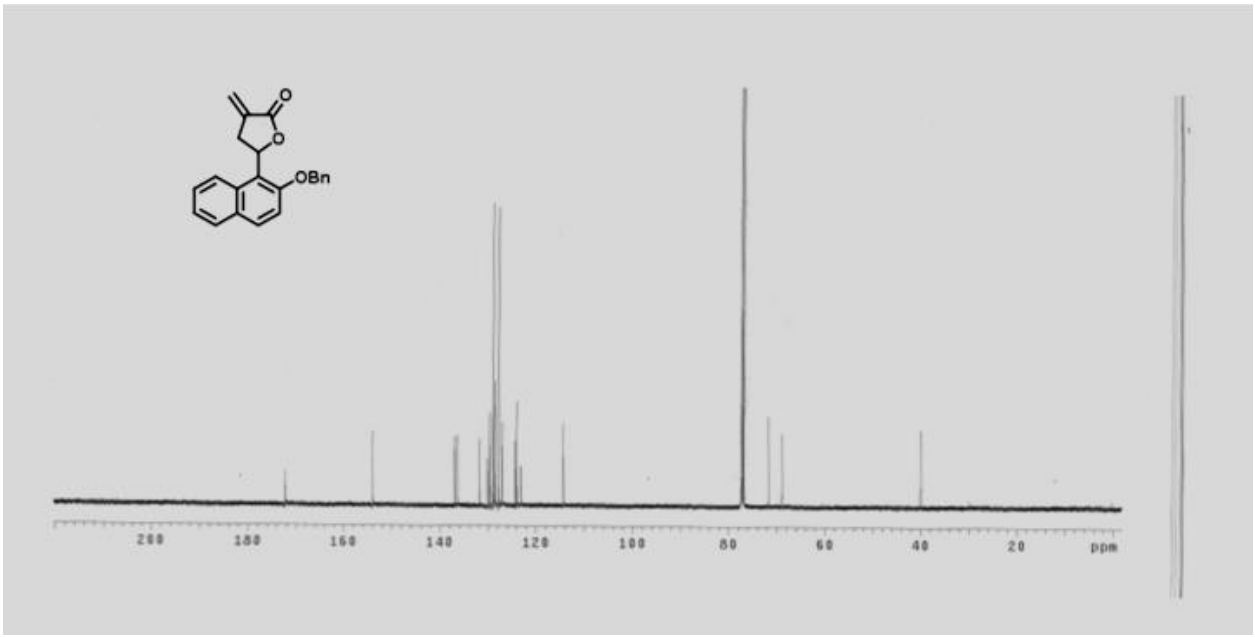
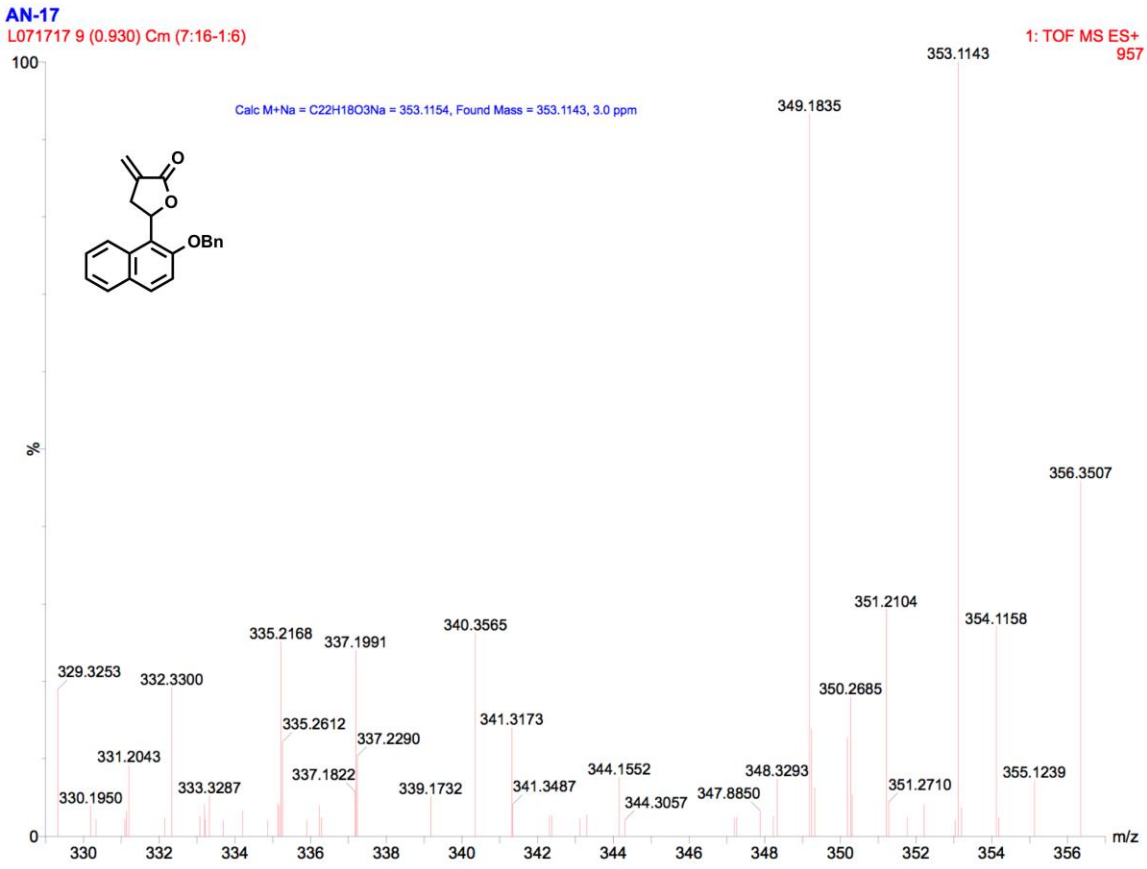
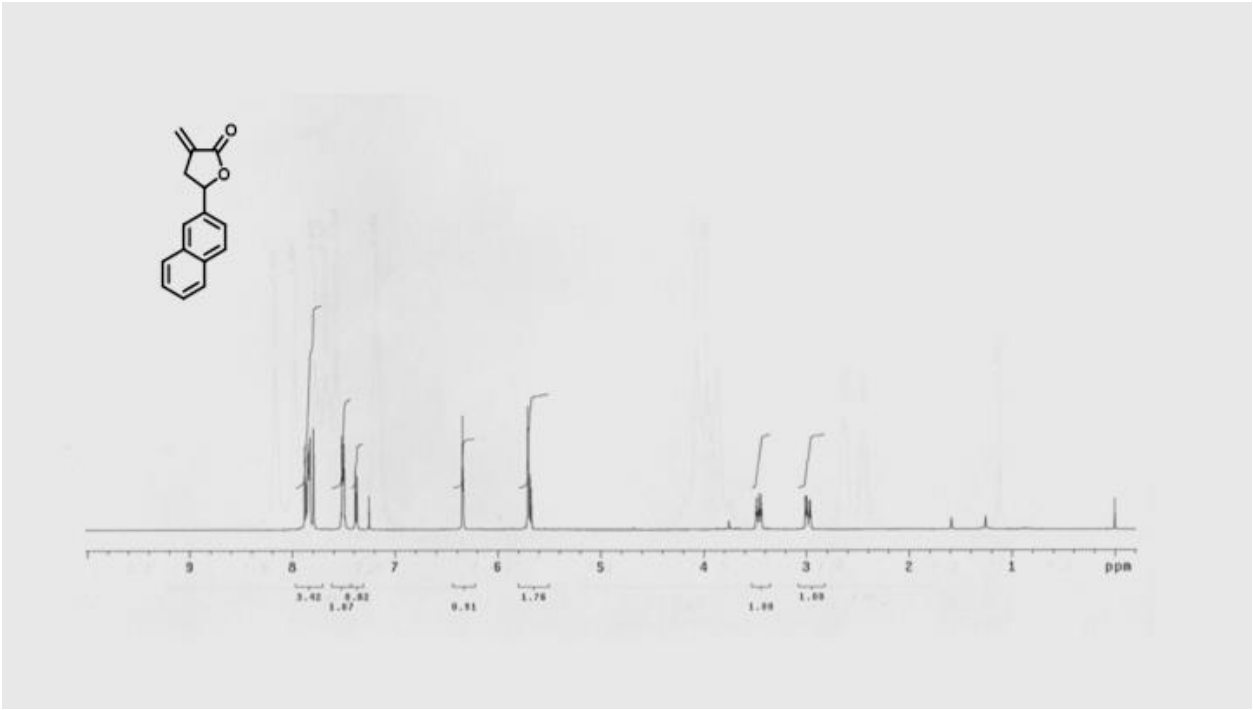
20
ppm

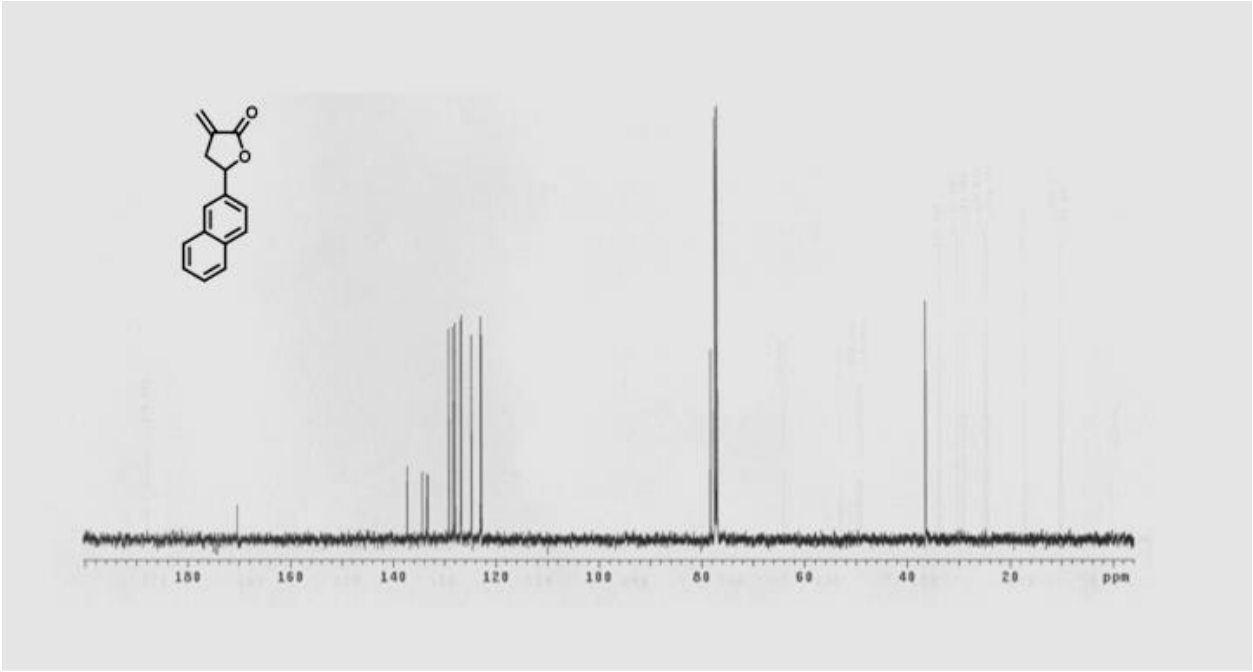

AN-16
L071715 10 (0.997) Cm (7:14-1:6)
1: TOF MS ES+
4.50e3
100
%
0
Calc M+Na = C16H14O3Na = 277.0841, Found Mass = 277.0829, 4.2 ppm
OMe
270.3141
261.1289
272.2583
277.0829
271.3178
258.2780
273.1653
256.2962
256.2618
262.1313
265.1385
279.1550
259.2810
267.1179
275.1114
276.9289
253.0988
280.2705
254
256
258
260
262
264
266
268
270
272
274
276
278
280
m/z

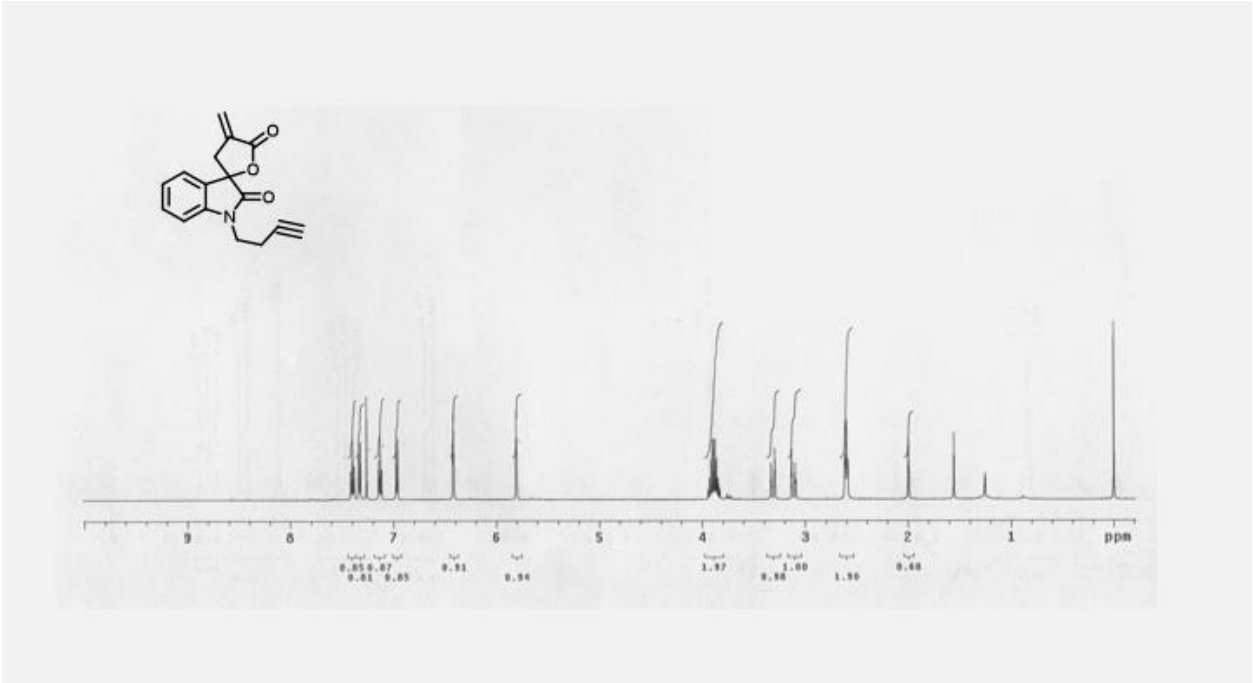

OBn
11
10
9
8
7
6
5
4
3
2
1
ppm

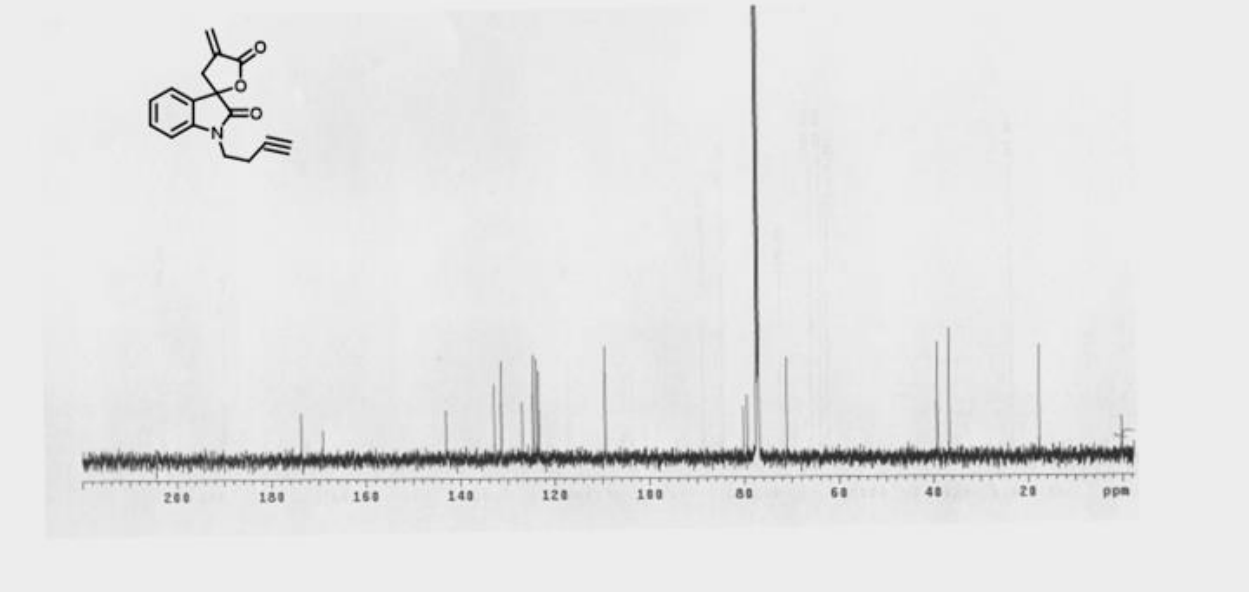

OBn
200
180
160
140
120
100
80
60
40
20
ppm

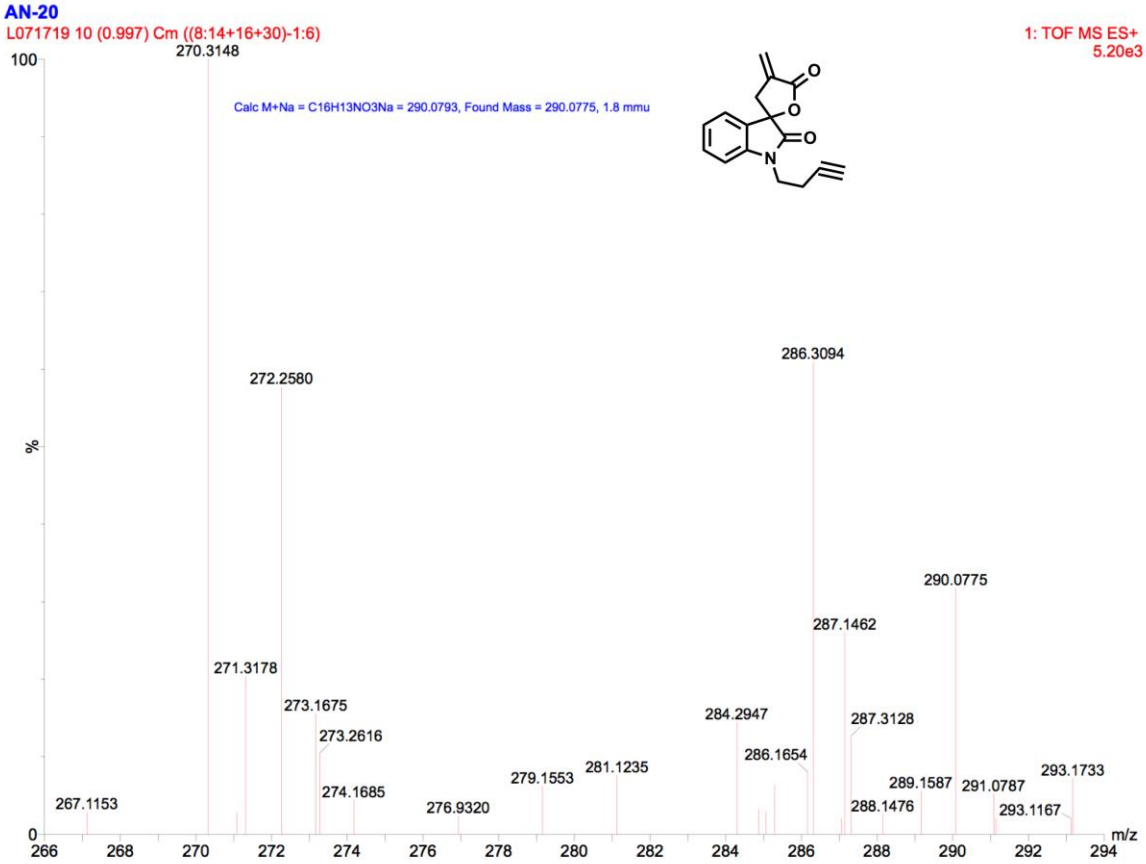

AN-17
L071717 9 (0.930) Cm (7:16-1:6)
1: TOF MS ES+
957
Calc M+Na = C22H18O3Na = 353.1154, Found Mass = 353.1143, 3.0 ppm
OBn
100
%
0
m/z
353.1143
349.1835
356.3507
351.2104
354.1158
340.3565
335.2168
337.1991
329.3253
332.3300
350.2685
335.2612
337.2290
341.3173
331.2043
344.1552
348.3293
337.1822
333.3287
339.1732
341.3487
351.2710
355.1239
330.1950
344.3057
347.8850
330
332
334
336
338
340
342
344
346
348
350
352
354
356

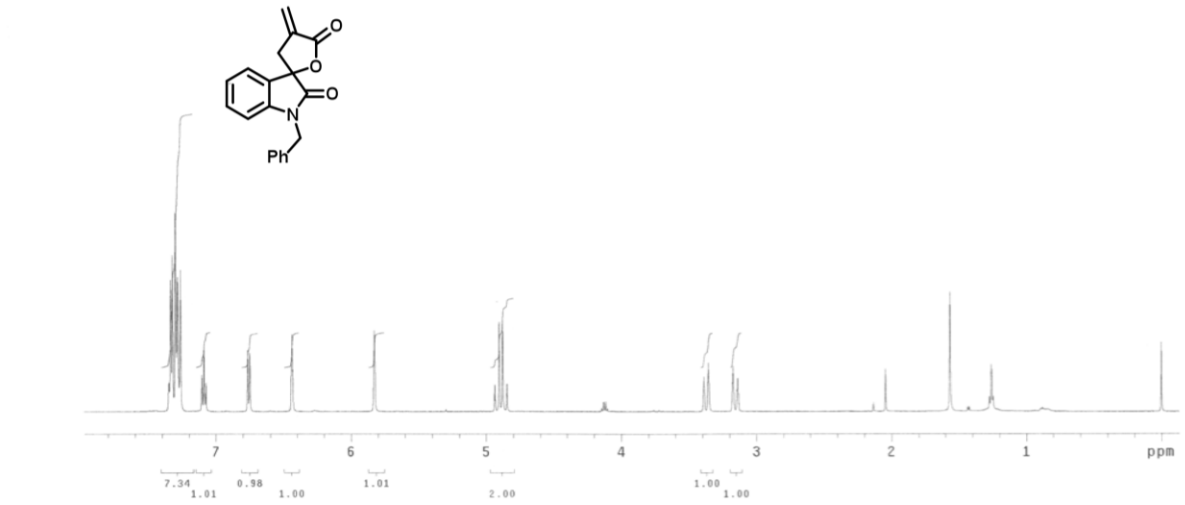

9
8
7
6
5
4
3
2
1
ppm

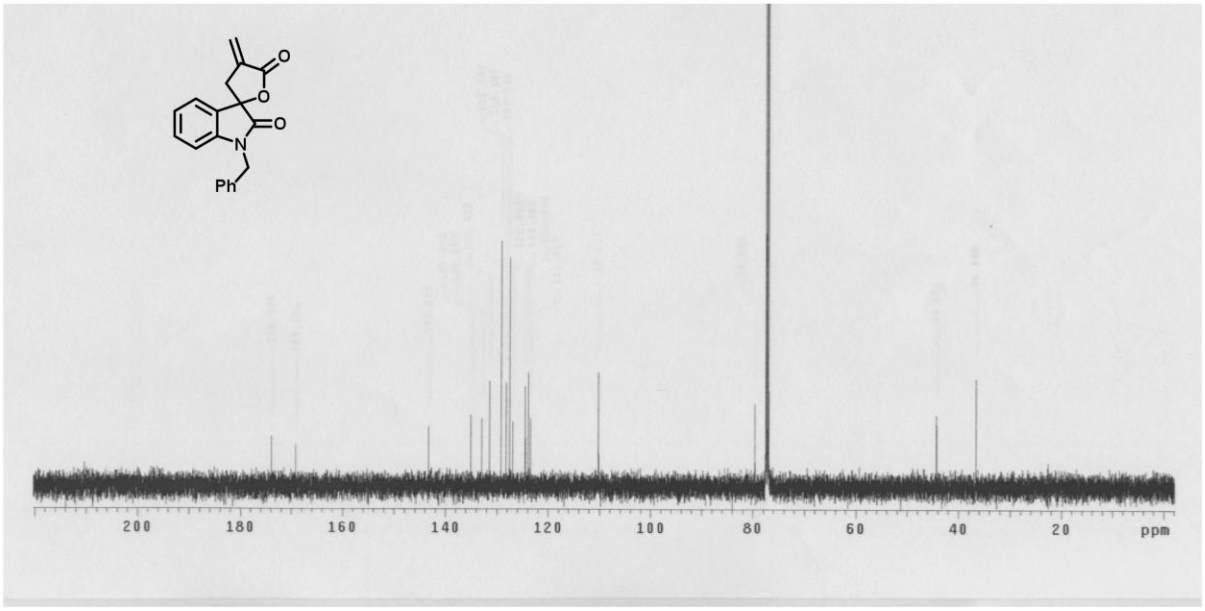

180
160
140
120
100
80
60
40
20
ppm

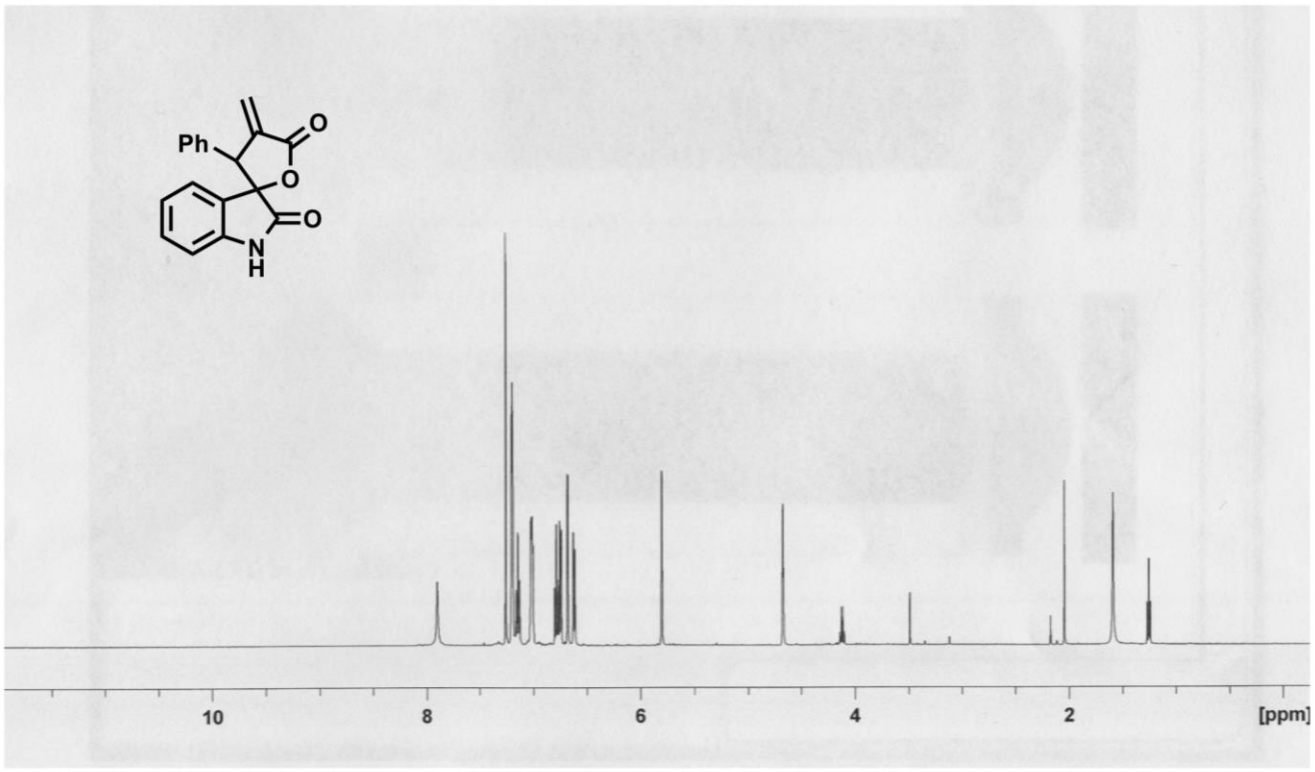

9
8
7
6
5
4
3
2
1
ppm

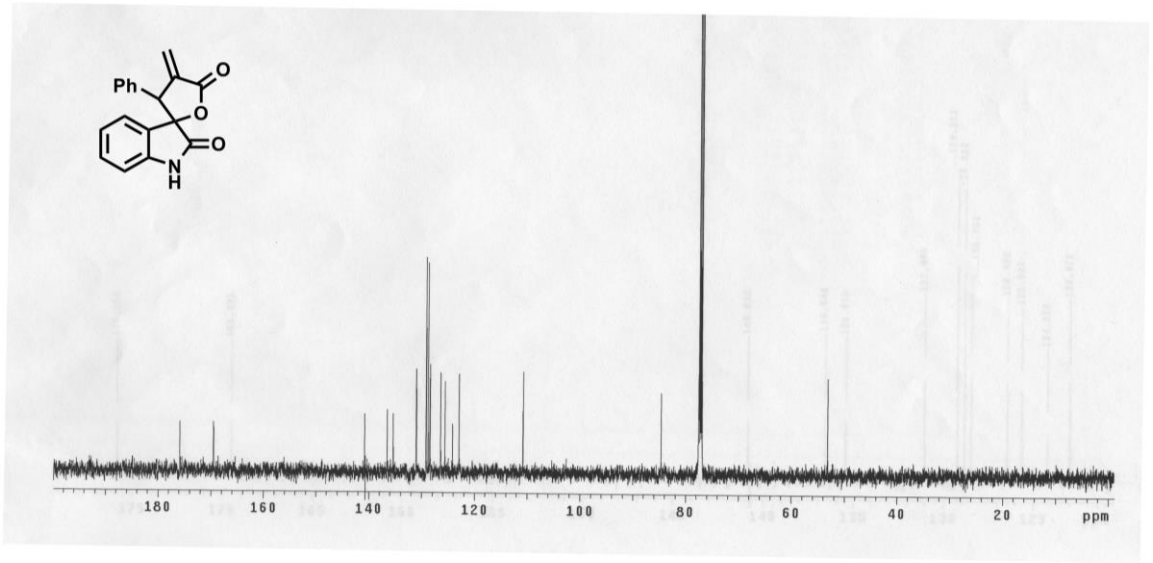

200
180
160
140
120
100
80
60
40
20
ppm

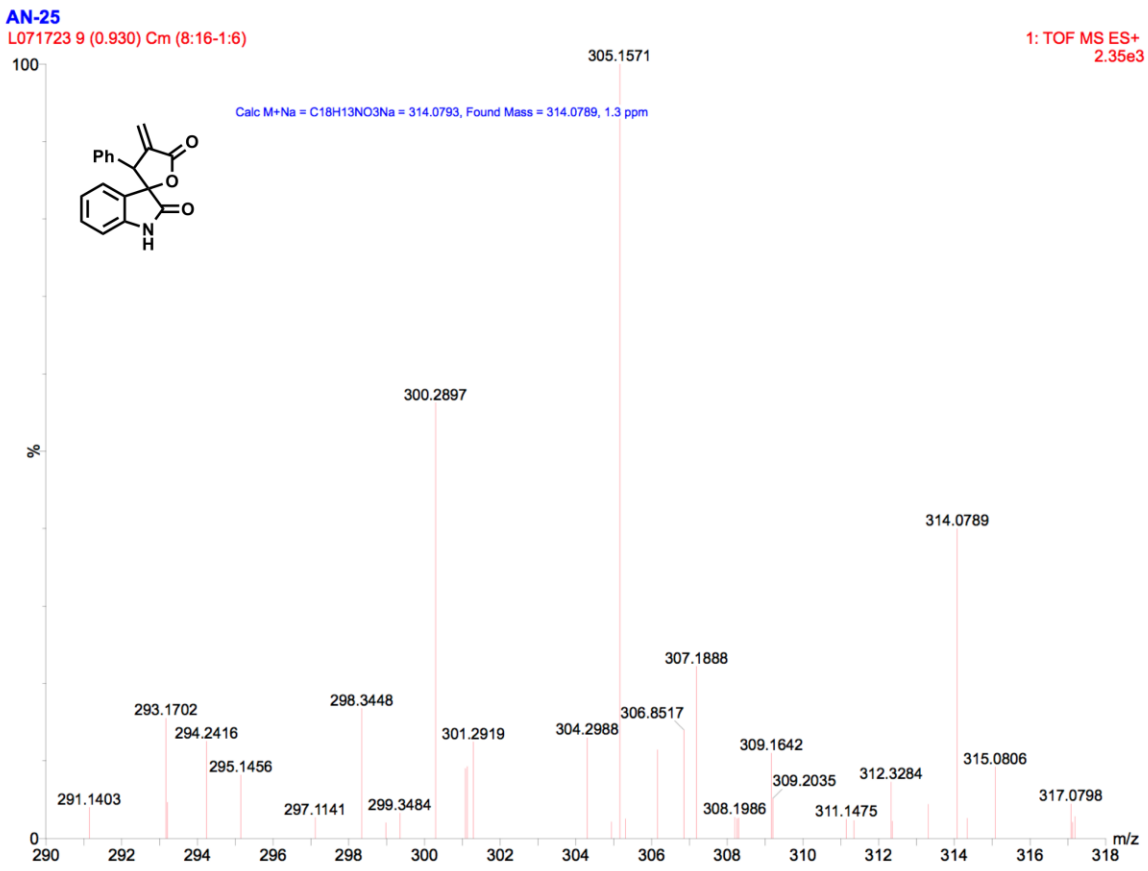

AN-20
L071719 10 (0.997) Cm ((8:14+16+30)-1:6)
1: TOF MS ES+
5.20e3
Calc M+Na = C16H13NO3Na = 290.0793, Found Mass = 290.0775, 1.8 mmu
100
%
0
270.3148
272.2580
286.3094
290.0775
287.1462
271.3178
273.1675
273.2616
284.2947
287.3128
286.1654
279.1553
281.1235
274.1685
267.1153
276.9320
289.1587
291.0787
293.1733
288.1476
293.1167
m/z
266
268
270
272
274
276
278
280
282
284
286
288
290
292
294

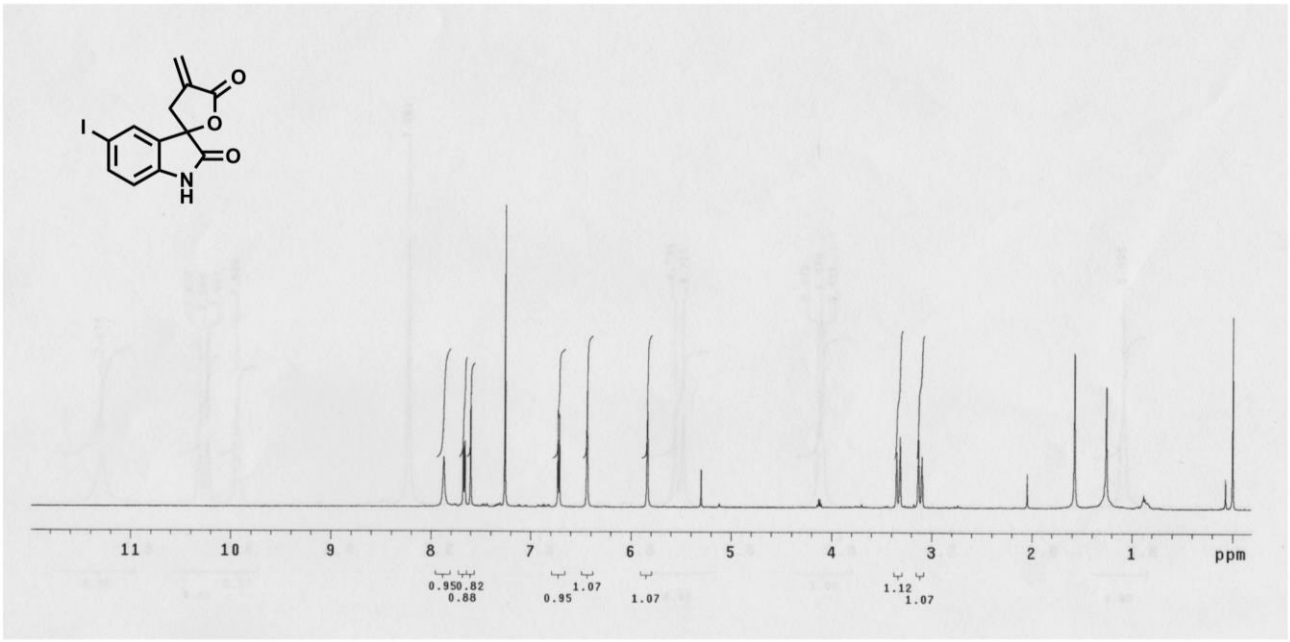

O
O
O
N
Ph
7
6
5
4
3
2
1
ppm
7.34
1.01
0.98
1.00
1.01
2.00
1.00
1.00

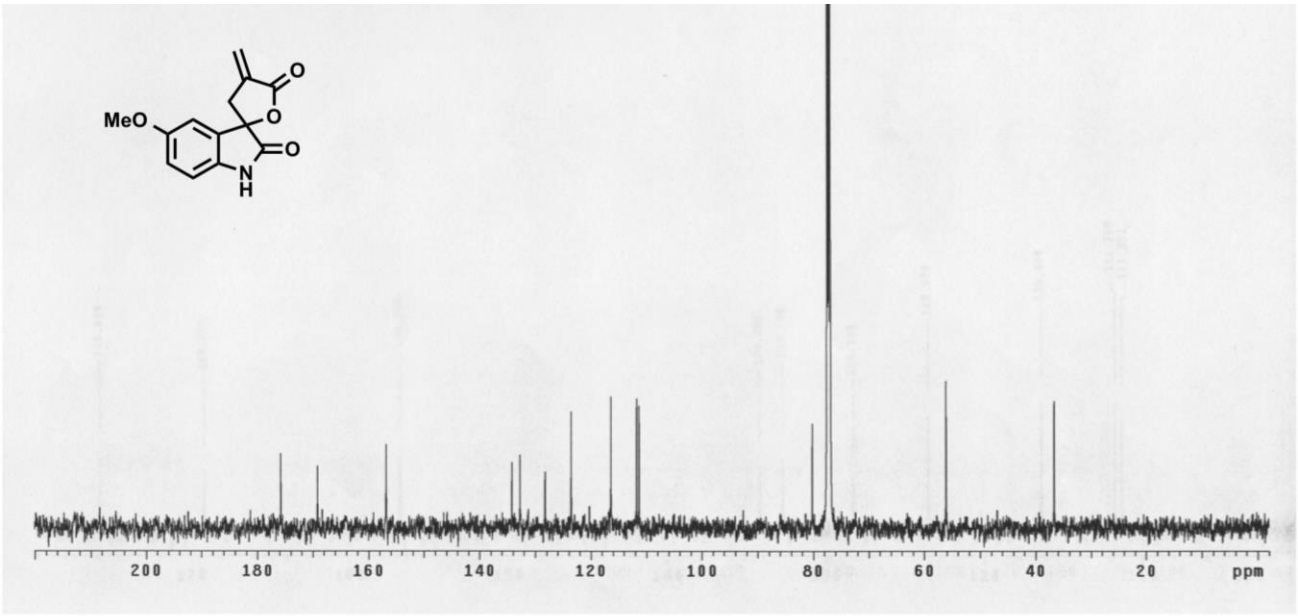

O
O
O
N
Ph
200
180
160
140
120
100
80
60
40
20
ppm

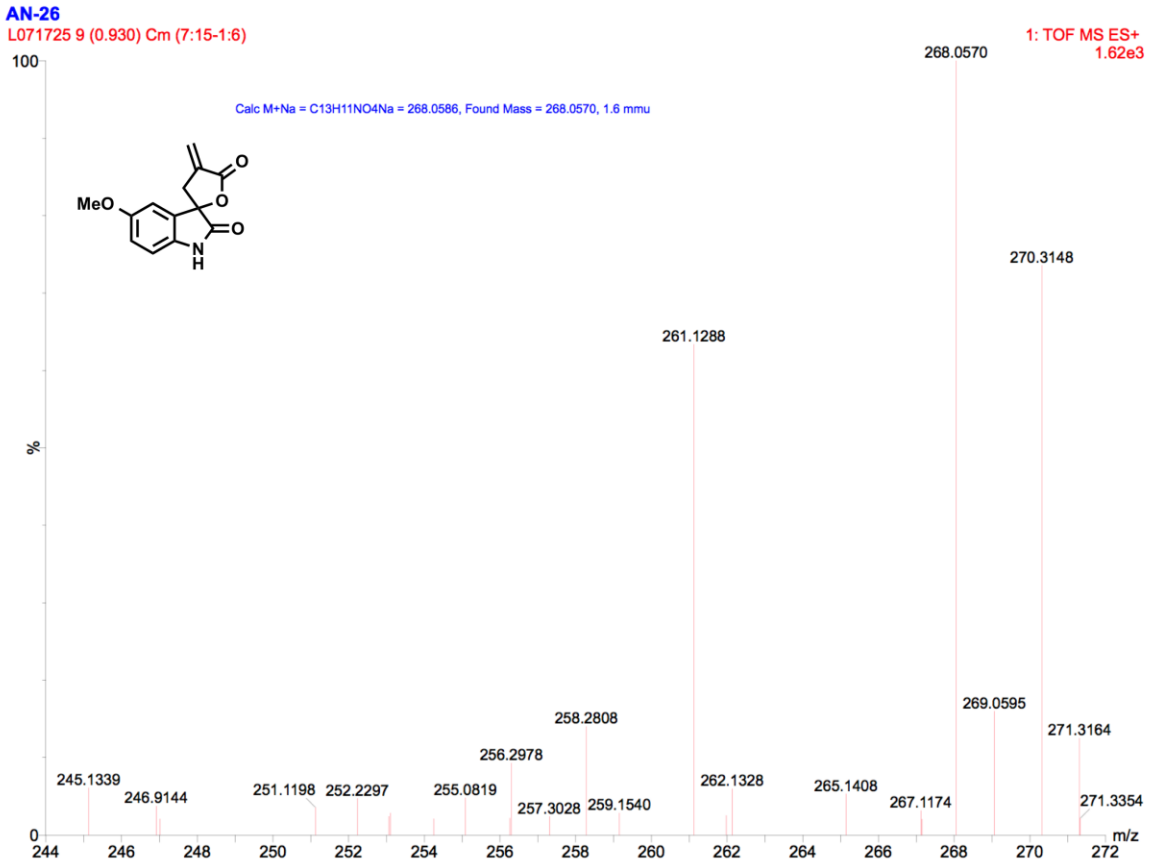

Ph
O
O
O
N
H
10
8
6
4
2
[ppm]

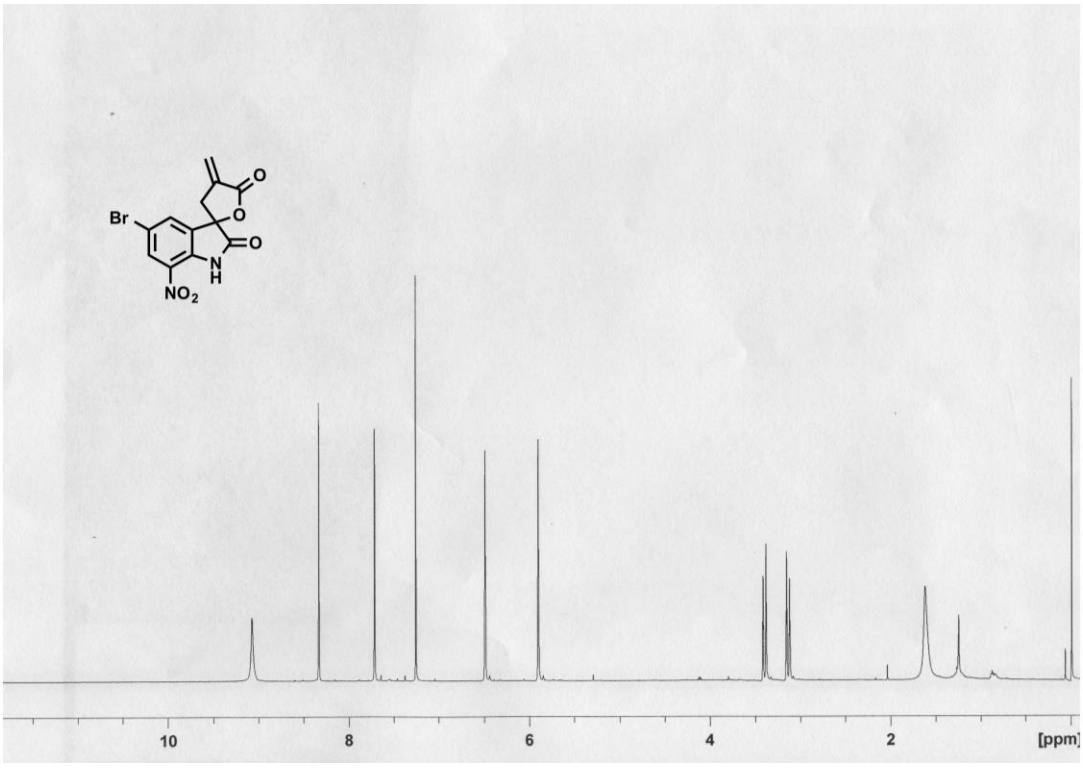

Ph
O
O
O
N
H
180
160
140
120
100
80
60
40
20
ppm

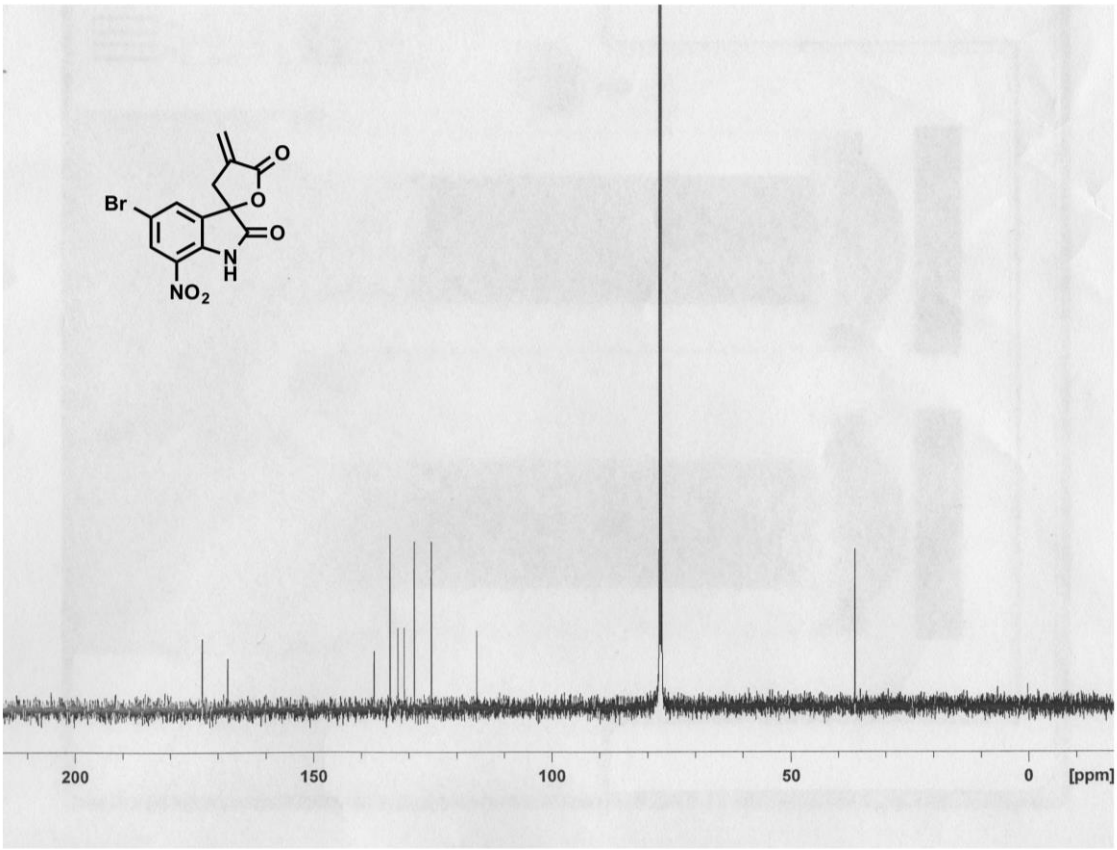

AN-25
L071723 9 (0.930) Cm (8:16-1:6)
1: TOF MS ES+
2.35e3
Calc M+Na = C18H13NO3Na = 314.0793, Found Mass = 314.0789, 1.3 ppm
Ph
O
O
O
N
H
100
%
0
305.1571
300.2897
314.0789
307.1888
293.1702
298.3448
306.8517
294.2416
301.2919
304.2988
309.1642
295.1456
315.0806
309.2035
312.3284
291.1403
317.0798
297.1141
299.3484
308.1986
311.1475
290
292
294
296
298
300
302
304
306
308
310
312
314
316
318
m/z


I
O
O
O
N
H
11
10
9
8
7
6
5
4
3
2
1
ppm
0.95
0.82
0.88
1.07
0.95
1.07
1.12
1.07


MeO
O
O
O
N
H
200
180
160
140
120
100
80
60
40
20
ppm


AN-26
L071725 9 (0.930) Cm (7:15-1:6)
1: TOF MS ES+
1.62e3
Calc M+Na = C13H11NO4Na = 268.0586, Found Mass = 268.0570, 1.6 mmu
MeO
O
O
O
N
H
100
%
0
268.0570
270.3148
261.1288
269.0595
271.3164
258.2808
256.2978
262.1328
265.1408
245.1339
246.9144
251.1198
252.2297
255.0819
257.3028
259.1540
267.1174
271.3354
m/z
244
246
248
250
252
254
256
258
260
262
264
266
268
270
272


Br
O
O
O
N
H
NO2
10
8
6
4
2
[ppm]

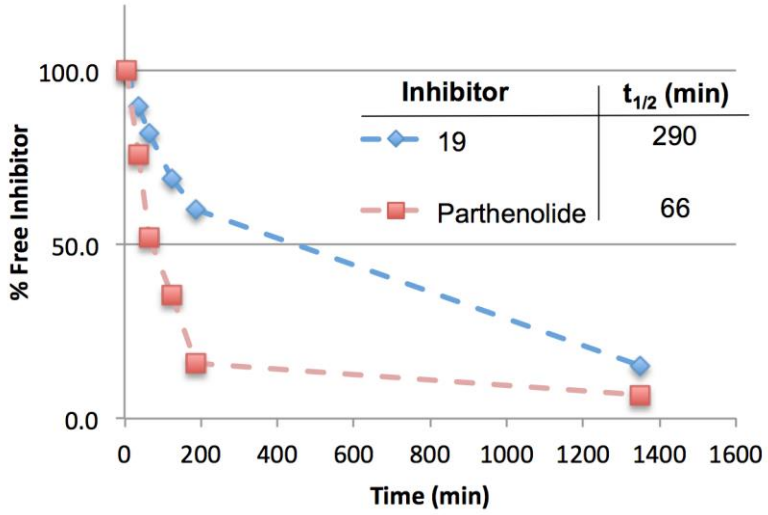

Br
O
O
O
N
H
NO2
200
150
100
50
0
[ppm]

Supplementary Table S1

Inhibition of cancer cell growth by **19** and known IKKβ inhibitors

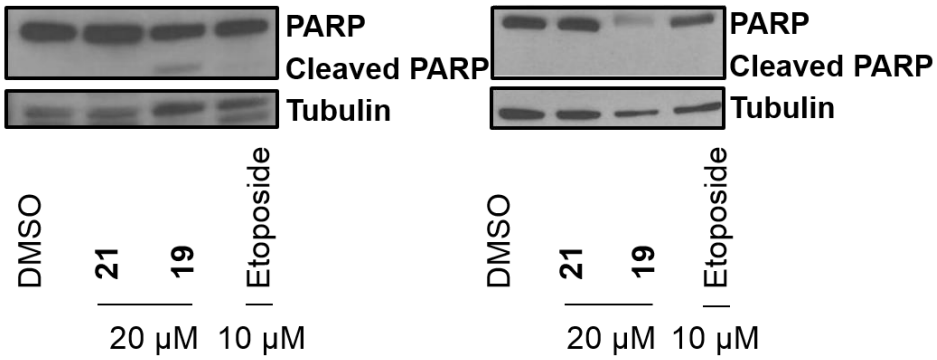
IKKβ inhibitor	Cancer Cell line	IC50 (μM)
19	MDA-MB-231 (Breast)	5.6 ± 0.9
19	PANC1 (Pancreas)	3.0 ± 0.6
19	MiaPaCa2 (Pancreas)	5.7 ± 3.2
19	A2780 (Ovarian)	2.6 ± 0.3
13-197	MDA-MB-231 (Breast)	2.1 ± 0.2
13-197	MiaPaCa-2 (Pancreas)	4.9 ± 0.7
Bayer VIII	MDA-MB-231 (Breast)	8.4 ± 1.2
Bayer VIII	MiaPaCa-2 (Pancreas)	7.3 ± 0.7
TPCA1	MDA-MB-231 (Breast)	6.6 ± 1.9
TPCA1	MiaPaCa-2 (Pancreas)	1.8 ± 0.7

Supplementary Figure S1



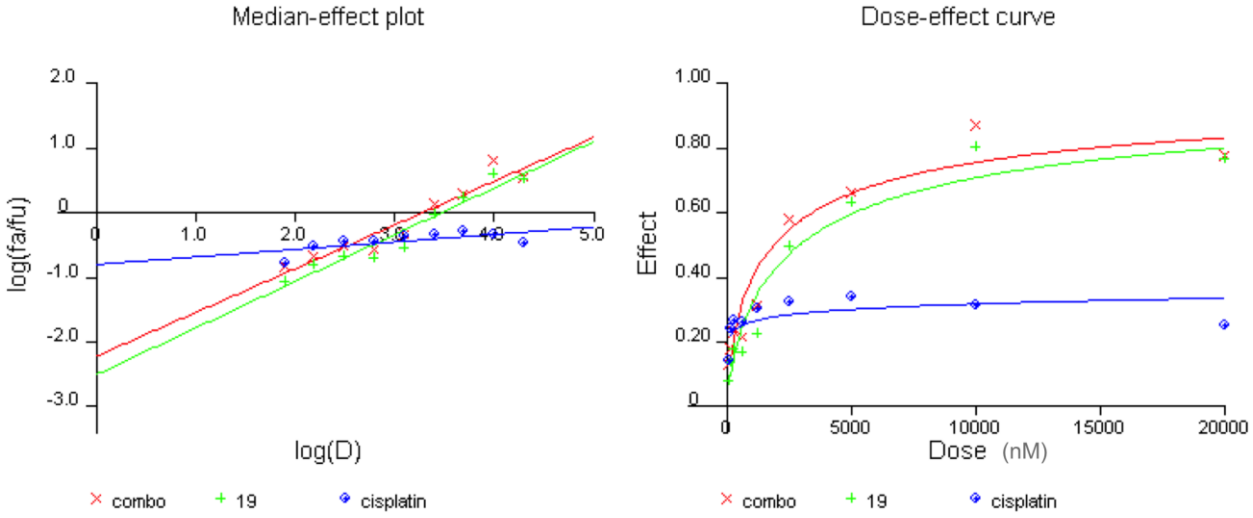
Covalent binding of **19** and parthenolide to serum albumin measured by HPLC

Supplementary Figure S2



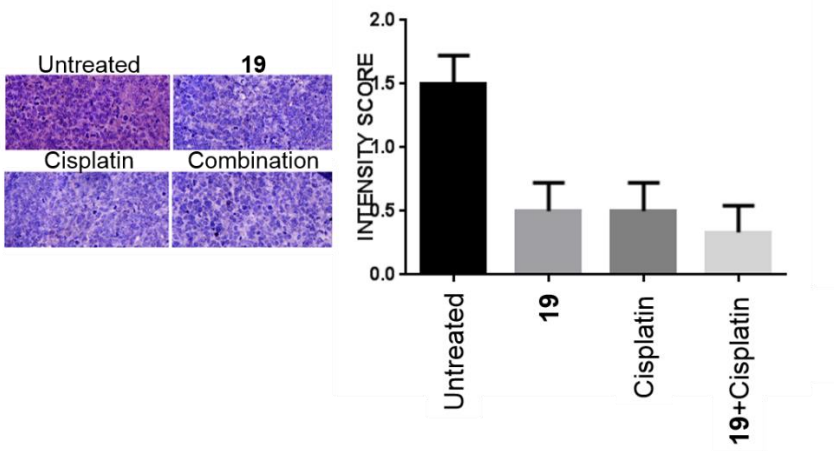
MiaPaCa2 (left) and SKOV3 cells were treated with the indicated concentrations of **21** and **19** and Etoposide for 24 h and lysed. Lysates were subjected to Western blot analysis for PARP cleavage and tubulin was used as a loading control. In 24 h, **19** induced PARP cleavage while **21** and Etoposide do not. This was the same Etoposide used in the main text, which produces PARP cleavage in HeLa (48 h) and A2780 cells (96 h), indicating the compound is indeed active at later time points.

Supplementary Figure S3



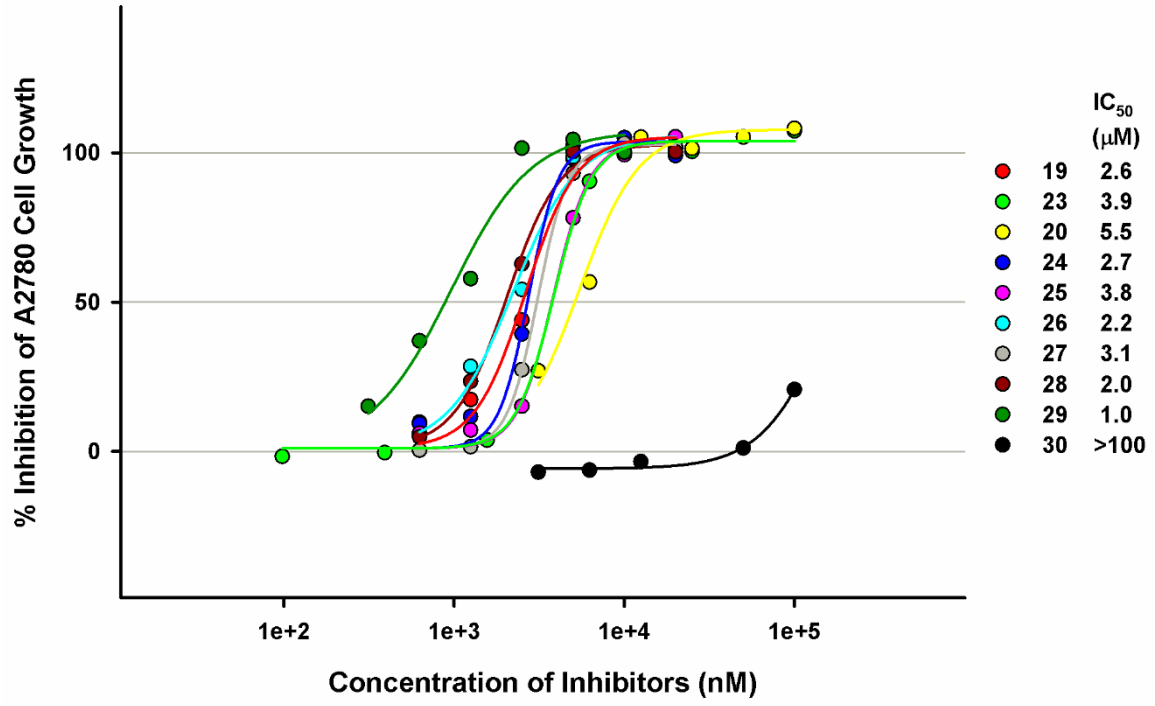
The data was analyzed and combination index (CI) values for the various combinations were derived from median effect plot and dose effect curves (Figure S2) using calcusyn.

Supplementary Figure S4

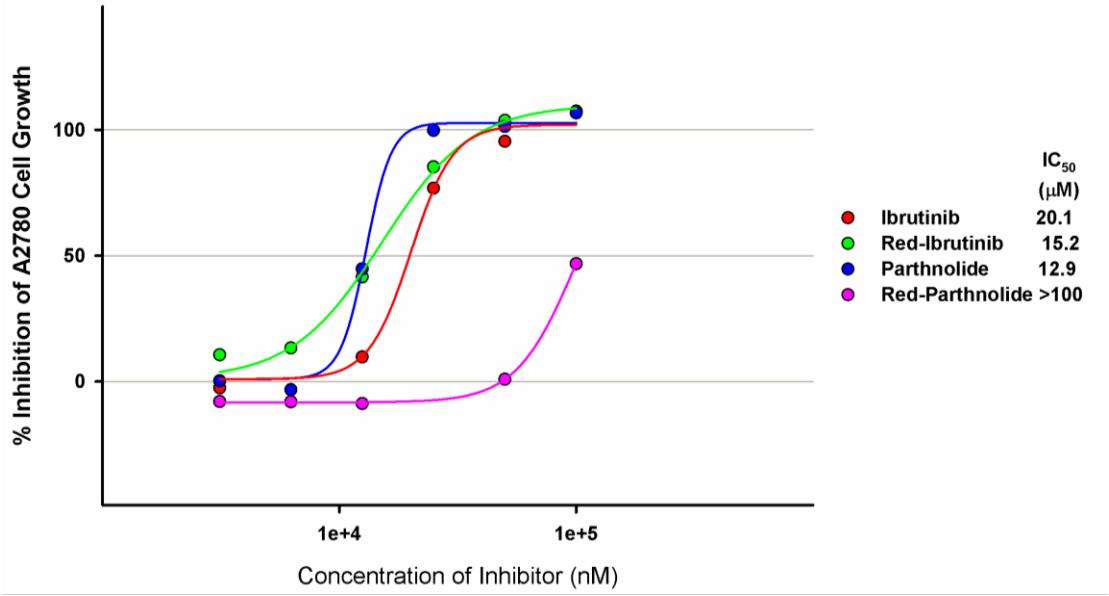


Mcl-1 staining of A2780 tumors from mice treated with vehicle control, **19**, cisplatin, or the combination with quantification.

Supplementary Figure S5

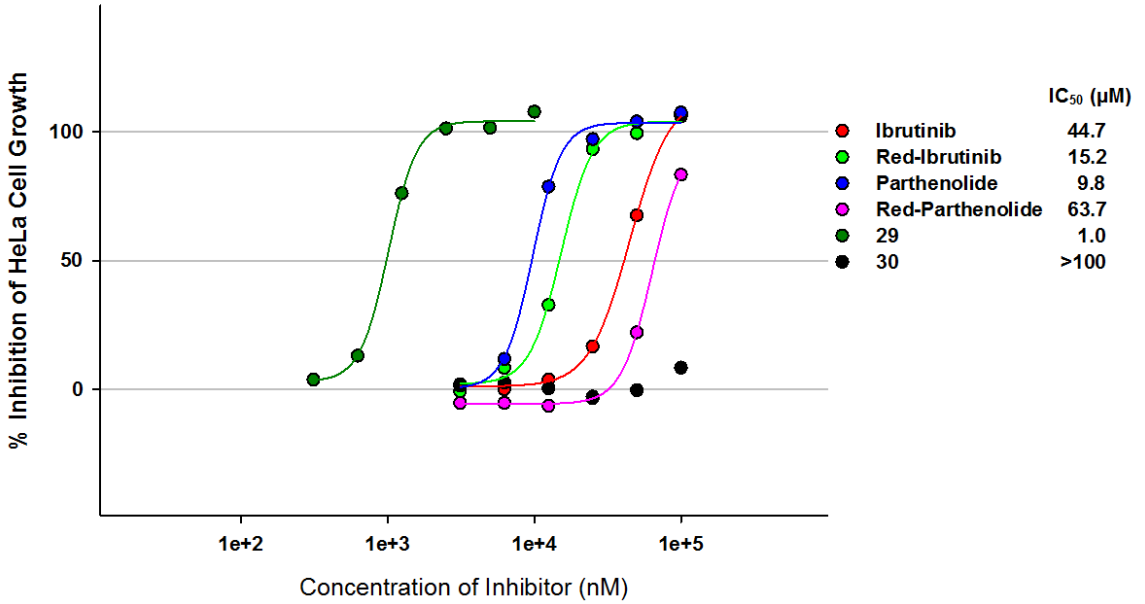


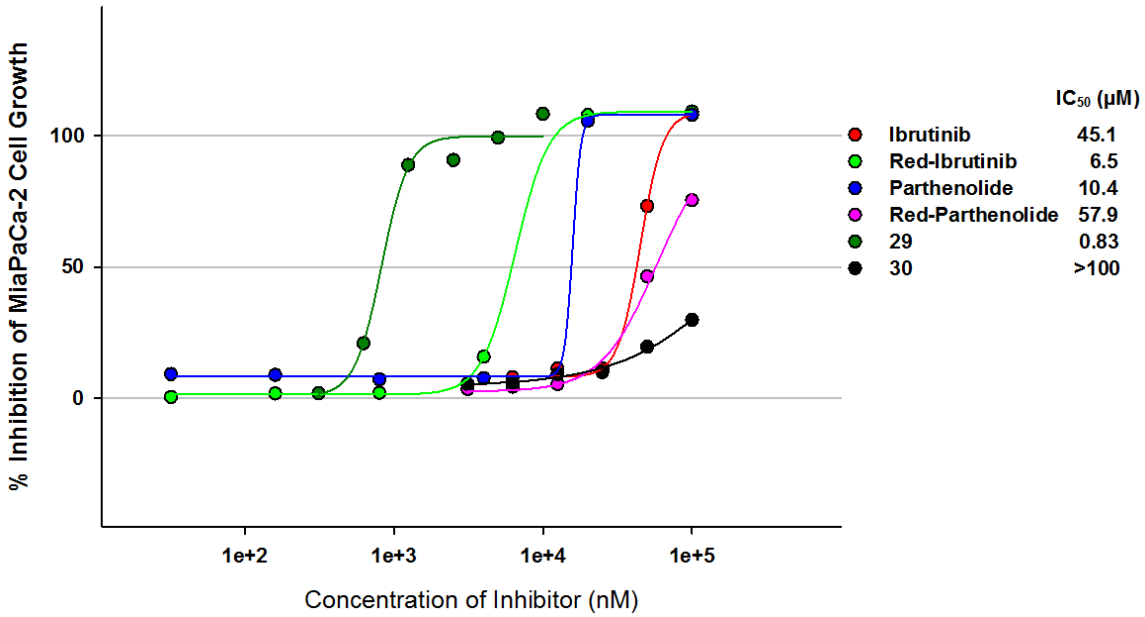
Inhibition of A2780 cancer cell growth by substituted α-methylene-γ-butyrolactone – oxindoles.

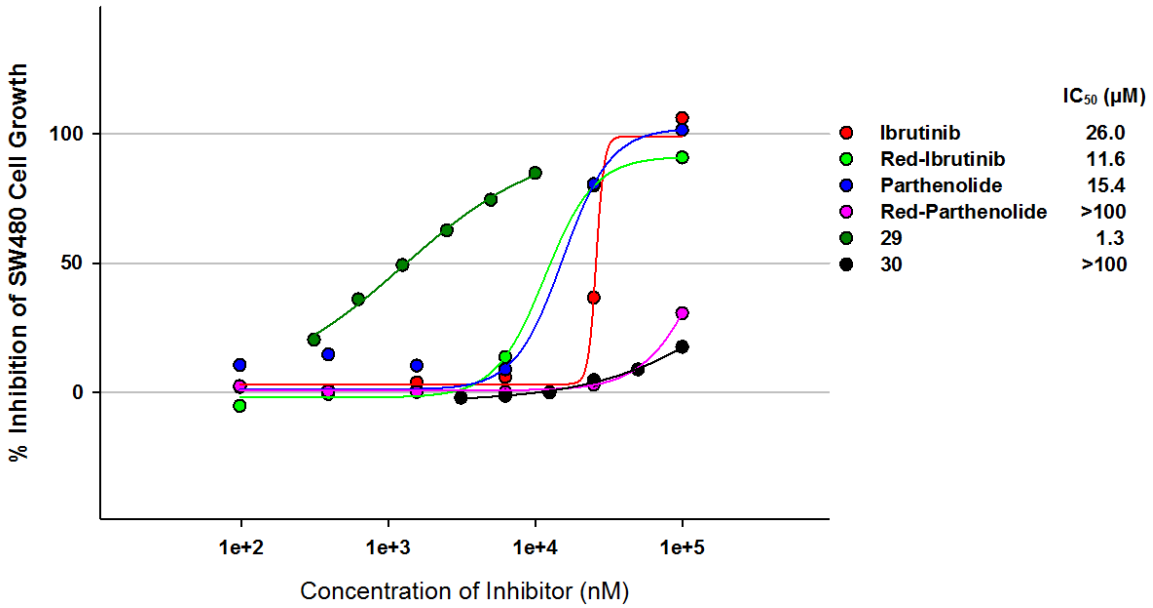


Inhibition of A2780 cancer cell growth by Ibrutinib, Reduced-Ibrurtinib, Parthenolide, and Reduced-parthenolide.

Supplementary Figure S6







Inhibition of HeLa, MiaPaCa-2 and SW480 cancer cell growth.

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[i] Jun-Tao, F; De-Long, W.; Yong-Ling; Yan, H.; Zhang, X. New antifungal scaffold derived from a natural pharmacophore: Synthesis of α-methylene-γ-butyrolactone derivatives and their antifungal activity against Colletotrichum lagenarium. *Bioorg. Med. Chem. Lett.*, **2013**, *23*, 4393-4397; 2013

[ii] Paira, M.; Banerjee, B.; Jana, S.; Mandal, S. K.; Roy, S. C. Titanocene(III) chloride-mediated radical-induced one-pot synthesis of α-methylene-γ-butyrolactones. *Tet. Lett.*, **2007**, *48*, 3205-3207.

[iii] Elford, T. G.; Ulaczyk-Lesanko, A.; Pascale, G. D.; Wright, G. D.; Hall, D. G. Diversity-Oriented Synthesis and Preliminary Biological Screening of Highly Substituted Five-Membered Lactones and Lactams Originating From an Allylboration of Aldehydes and Imines. *J. Combi. Chem.*, **2009**, *11*, 155-168.

[iv] Rana, S.; Natarajan, A. Face Selective Reduction of the Exocyclic Double Bond in Isatin Derived Spirocyclic Lactones. *Org. Biomol. Chem.*, **2013**, *11*, 244-247.

[v] Takahashi, M.; Murata, Y.; Yagishita, F.; Sakamoto, M.; Sengoku, T.; Yoda, H. Catalytic Enantioselective Amide Allylation of Isatins and Its Application in the Synthesis of 2-Oxindole Derivatives Spiro- Fused to the a-Methylene-g-Butyrolactone Functionality. *Chem. Eur. J.*, **2014**, *20*, 11091-11100.