SUPPORTING INFORMATION

The Oxetanyl Sulfoxide MMS-350 Ameliorates Pulmonary Fibrosis *in Vitro, in Vivo,* and *ex Vivo*

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I. General Information

All glassware was flame dried or oven-dried and cooled under dry N_2 or Ar prior to use. All moisture sensitive reactions were performed under dry N_2 or Ar. Reactions carried out below 0 °C employed an acetone/dry ice bath or a cyrocool and an isopropanol/ethanol bath. Reagents obtained from commercial sources were used as received unless otherwise specified. THF, Et_2O, and 1,4-dioxane were distilled from sodium/benzophenone ketyl; DIPEA and TEA were distilled from CaH_2 and stored over KOH; *t*-BuOH was distilled over CaH_2; and CH_2Cl_2 and toluene were purified by passage through an activated alumina filtration system. HFIP was distilled from 4Å MS and stored over 4Å MS. Benzaldehyde was distilled under vacuum (~30 mmHg) immediately prior to use. Concentrating under reduced pressure refers to the use of a rotary evaporator connected to a membrane vacuum pump to remove solvent.

Melting points were determined using a Laboratory Devices Mel-Temp II in open capillary

tubes and are uncorrected. Infrared spectra were determined as neat solids or oils (unless otherwise specified) on a Smiths Detection IdentifyIR FT-IR spectrometer or Perkin Elmer Spectrum 100; or as KBr pellets or thin films on a Nicolet Avatar 360 FT-IR. Low-resolution mass spectra were obtained on a Shimadzu 2020-LCMS or Agilent Technologies 1260 Infinity II LCMS. High-resolution mass spectra were obtained on a Micromass UK Limited, Q-TOF Ultima API or a Thermo Scientific Exactive Orbitrap LCMS. Purity of compounds tested in biological assays was assessed using an Agilent Technologies 1260 Infinity II LC at 220 nm UV absorption Waters XBridge BEH C_{18} 2.1 x 50 mm, 2.5 µm) or an Agilent Technologies 385-ELSD (Microsolv Cogent 2.0 Bidentate C_{18} 2.1 x 50 mm, 2.2 µm; ELSD conditions: evaporator and nebulizer set at 45 °C; gas flow set at 1.80 standard liter/min). All assay samples were >95% purity by ELSD and/or UVD.

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III 300MHz, 400 MHz, 500 MHz, and a cryoprobe equipped 600MHz instruments. $CDCl_3$ was filtered through basic Al_2O_3 immediately prior to sample preparation. Chemical shifts (δ) were reported in parts per million with the residual solvent peak used as an internal standard δ ^{1}H / ^{13}C (Solvent); 7.26 / 77.16 ($CDCl_3$); 2.50 / 39.52 (DMSO-d_6); 2.05 / 29.84 (acetone-d_6) and are tabulated as follows: chemical shift, multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet), number of protons, and coupling constant(s). ^{13}C NMR spectra were obtained at 75 MHz, 100 MHz, and 125 MHz using a proton-decoupled pulse sequence and are tabulated by observed peak. Thin-layer chromatography was performed using pre-coated silica gel 60 F_{254} plates (EMD, 250 µm thickness) and visualization was accomplished with a 254 nm UV light and by staining with a phosphomolybdic acid solution (5 g of phosphomolybdic acid in 100 mL of 95% EtOH), a *p*-anisaldehyde solution (2.5 mL of *p*-anisaldehyde, 2 mL of AcOH, and 3.5 mL of conc. H_2SO_4

in 100 mL of 95% EtOH), a $KMnO_4$ solution (1.5 g of $KMnO_4$ and 1.5 g of K_2CO_3 in 100 mL of a 0.1% NaOH solution), or Vaughn's reagent (4.8 g of $(NH_4)_6Mo_7O_{24}$•4 H_2O and 0.2 g of $Ce(SO_4)_2$ in 100 mL of a 3.5 N H_2SO_4 solution). Flash chromatography on SiO_2 (Silicycle, Silia-P Flash Silica Gel or SiliaFlash® P60, 40-63 μm) was used to purify crude reaction mixtures.

II. Experimental Procedures

MMS-350 was synthesized as previously reported.[1]

KRL507-031 (3-methyl-3-((phenylsulfinyl)methyl)oxetane) was prepared as follows: To a solution of (3-methyloxetan-3-yl)methyl 4-methylbenzenesulfonate (**1**, 1.50 g, 5.85 mmol) and NaOH (0.255 g, 6.25 mmol, 1.1 equiv) in EtOH (7.5 mL, 0.78 M) was added thiophenol (0.597 mL, 5.85 mmol, 1 equiv). The reaction mixture was heated at 80 °C for 3 h, and a white precipitate formed. The suspension was cooled to room temperature, and the EtOH was evaporated. The residue was diluted with EtOAc (100 mL) and washed with 1 M NaOH (2 x 50 mL) and brine (50 mL). The combined aqueous layers were back-extracted with EtOAc, and the combined organic layers were dried ($MgSO_4$), and evaporated. Removal of residual solvent and thiophenol overnight on high vacuum at room temperature gave 3-methyl-3-((phenylthio)methyl)oxetane (0.968 g, 4.98 mmol, 85%) as a yellow oil. A solution of 3-methyl-3-((phenylthio)methyl)oxetane (0.968 g, 4.98 mmol) in MeOH (25.0 mL) was cooled to 0 °C and treated via addition funnel over 30 min with a solution of $NaIO_4$ (1.07 g, 4.98 mmol, 1 equiv) in H_2O (12.5 mL). After addition was complete, the ice bath was removed and the resulting solution allowed to warm to room temperature. The

[1] Sprachman, M. M.; Wipf, P. A Bifunctional Dimethylsulfoxide Substitute Enhances the Aqueous Solubility of Small Organic Molecules. *Assay Drug Dev. Technol.* **2012**, *10*, 269–277.

mixture was stirred at room temperature for 15 h followed by filtration. The precipitate was washed with MeOH and the filtrate and subsequent washings were collected and concentrated. Toluene was added to help remove any additional H_2O azeotropically. The residue was diluted with CH_2Cl_2 and dried ($MgSO_4$), filtered and concentrated to afford **KRL507-031** (1.01 g, 4.81 mmol, 96%) as a pale yellow oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65 (d, *J* = 6.4 Hz, 2 H), 7.56-7.52 (m, 3 H), 4.81 (d, *J* = 6.4 Hz, 1 H), 4.51 (d, *J* = 6.0 Hz, 1 H), 4.47 (d, *J* = 6.0 Hz, 1 H), 4.44 (d, *J* = 6.0 Hz, 1 H), 3.20 (d, *J* = 13.2 Hz, 1 H), 2.84 (d, *J* = 13.2 Hz, 1 H), 1.69 (s, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.5, 131.3, 129.5, 123.8, 82.4, 82.2, 66.9, 38.7, 23.5; HRMS (ESI$^+$) *m/z* calcd for $C_{11}H_{15}O_2S$ 211.0787 (M+H), found 211.0786; purity was 97% by HPLC-ELSD analysis.

CL-613-091 (3-fluoro-3-(((3-fluorophenyl)sulfinyl)methyl)oxetane) was prepared as follows: A solution of (3-fluorooxetan-3-yl)methyl 4-methylbenzenesulfonate (44.8 mg, 0.17 mmol; prepared by tosylation of (3-fluorooxetan-3-yl)methanol[2]), 3-fluorothiophenol (0.019 mL, 0.18 mmol, 1 equiv) and K_2CO_3 (48 mg, 0.34 mmol) in DMF (0.5 mL) was stirred at room temperature for 27 h. The reaction mixture was poured into H_2O (10 mL) and extracted with EtOAc (3 x 10 mL). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated. The crude mixture was purified by chromatography on SiO_2 (hexanes/EtOAc, 15:1 to 10:1) to afford 3-fluoro-3-(((3-fluorophenyl)thio)methyl)oxetane (34 mg, 0.16 mmol, 92%) as a colorless oil: IR (CH_2Cl_2) 3081, 2945, 2876, 1597, 1577, 1472, 1424, 1351, 1286, 1262, 1213, 1105, 971, 910, 878, 844, 774, 677 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29-7.24 (m, 1 H), 7.20-7.18 (m, 1 H), 7.13 (dt, *J* = 9.2, 2.2 Hz, 1 H), 6.93 (tdd, *J* = 8.5, 2.5, 0.9 Hz, 1 H), 4.78 (dd, *J* = 19.2, 8.4 Hz, 2 H), 4.57 (dd, *J* = 18.4,

[2] Boyd, S.; Davies, C. D. A New and Versatile Synthesis of 3-Substituted Oxetan-3-yl Methyl Alcohols. *Tetrahedron Lett.* **2014**, *55*, 4117–4119.

8.4 Hz, 2 H), 3.47 (d, J = 21.6 Hz, 2 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.7 (d, J_{CF} = 247.0 Hz), 137.6 (d, J_{CF} = 8.0 Hz), 130.4 (d, J_{CF} = 8.0 Hz), 125.6 (d, J_{CF} = 3.0 Hz), 116.9 (d, J_{CF} = 23.0 Hz), 113.9 (d, J_{CF} = 21.0 Hz), 94.2 (d, J_{CF} = 210.0 Hz), 79.9 (d, J_{CF} = 24.0 Hz), 39.2 (d, J_{CF} = 25.0 Hz); HRMS (ESI^+) *m/z* calcd for $C_{10}H_{11}OF_2S$ 217.0493 (M+H), found 217.0493. To a solution of 3-fluoro-3-(((3-fluorophenyl)thio)methyl)oxetane (30 mg, 0.14 mmol) in MeOH (0.5 mL) at 0 °C was added slowly a solution of $NaIO_4$ (30 mg, 0.14 mmol, 1 equiv) in water (0.2 mL). The resulting heterogeneous mixture was allowed to warm to room temperature and stirred for 31 h. The reaction mixture was filtered through a plug of Celite (MeOH), and the solvent was evaporated. The residue was dissolved in CH_2Cl_2, dried ($MgSO_4$), filtered and concentrated. The crude mixture was purified by chromatography on SiO_2 (hexanes/EtOAc, 1:1) to afford **CL-613-091** (30 mg, 0.13 mmol, 94%) as a colorless solid: Mp 105–108 °C; IR (CH_2Cl_2) 3070, 2962, 2925, 2878, 1594, 1473, 1421, 1352, 1270, 1251, 1217, 1105, 1079, 1035, 975, 930, 913, 899, 735, 705 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54 (td, J = 8.0, 5.2 Hz, 1 H), 7.47-7.44 (m, 1 H), 7.41 (dt, J = 7.7, 1.2 Hz, 1 H), 7.25-7.21 (m, 1 H), 5.07-4.84 (m, 3 H), 4.57 (bdd, J = 20.2, 7.8 Hz, 1 H), 3.50 (dd, J = 29.6, 14.0 Hz, 1 H), 3.23 (ddd, J = 13.7, 12.7, 1.2 Hz, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.2 (d, J_{CF} = 252.0 Hz), 146.3 (d, J_{CF} = 6.0 Hz), 131.3 (d, J_{CF} = 8.0 Hz), 119.4 (d, J_{CF} = 3.0 Hz), 118.7 (d, J_{CF} = 21.0 Hz), 111.2 (d, J_{CF} = 24.0 Hz), 92.4 (d, J_{CF} = 209.0 Hz), 80.7 (d, J_{CF} = 24.0 Hz), 80.1 (d, J_{CF} = 24.0 Hz), 62.2 (d, J_{CF} = 23.0 Hz); HRMS (ESI) $[M+H]^+$ *m/z* calcd for $C_{10}H_{11}O_2F_2S$ 233.0442, found 233.0441; purity was 100% by HPLC-ELSD analysis.

III. Biological Assays

Cell Culture and Treatment. All tissues were obtained under a protocol approved by the Institutional Review Board of the University of Pittsburgh and with written consent. Primary

human fibroblasts were cultured from the lung tissues of normal donors and patients undergoing lung transplantation. Fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) (Corning-Cellgro Inc., Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), 100 U/mL penicillin, 10 mg/mL streptomycin and 2.5 mg/mL amphotericin B (Invitrogen Life Technologies, Carlsbad, CA), and maintained at 37 °C in 5% CO_2 humidified incubator. Three strains of normal primary human fibroblasts were plated on 6-well plates at a seeding density of 2.5 x 10^5 cells per well. Cells were serum starved for 6-16 h and treated with vehicle (5mM HCl/0.1% BSA/1x PBS), TGFβ (10 ng/mL) (R&D Systems, Minneapolis, MN), MMS-350 (0.5 mg/mL), or TGFβ + MMS-350. Experiments using KRL507-031 and CL-613-091were performed similarly, except that vehicle was an equal volume of DMSO. The cell lysates and culture supernatants were harvested 72 h after treatment and analyzed by immunoblotting.

Immunoblotting. Fibroblasts were rinsed with 1x PBS, scraped into 100 μL of 2X- sodium dodecyl sulfate (SDS) sample buffer, and boiled for 5 min. Equal protein amounts of cell lysates or supernatants were resolved on 10-12% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels. Proteins were transferred to 0.2 μm nitrocellulose membranes (Whatman, Germany) and blocked with 5% nonfat dry milk in TBS-Tween 20 for 1 h at room temperature. Membranes were incubated with primary antibodies overnight at 4 °C followed by horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Signals were detected with enhanced chemiluminescence (Perkin Elmer Life Sciences, Inc., Boston, MA) and imaged with the FluorChem E digital imaging system from Protein Simple (Santa Clara, California). Results were analyzed using the accompanying software Alphaview. The following antibodies were used for immunoblotting: anti-human collagen 1α1(Col1), anti-fibronectin (FN), anti-IGFBP-3(IGFBP3),

anti-CTGF, and anti-human GAPDH were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and α-SMA from Abcam (Cambridge, MA). Horseradish peroxidase-conjugated secondary antibodies were purchased from GE Healthcare (Little Chalfont, UK) and Santa Cruz, Inc.

In vivo Experiment. Pulmonary fibrosis was induced in male 6- to 8-week-old C57BL/6J mice by intratracheal administration of 1 mg/kg of Bleomycin (Enzo) in a total volume of 50 uL. Mice were given either 200 uL of PBS or MMS-350 (2%) via oral gavage on a daily basis and lungs were harvested after 5 and 14 days. Right lungs were fixed in 10% neutralized formalin and paraffin embedded. Left lungs were frozen for hydroxyproline assay and total RNA extraction.

Histological examination. Six-micrometer sections of paraffin-embedded mouse lung tissues were stained with H&E and images were taken on an Olympus Provis III microscope (Olympus America Inc.) with identical settings.

Hydroxyproline Assay. The hydroxyproline content of mouse lung tissues obtained on day 14 was measured as described[3] and adjusted by lung weight.

Quantitative Real-Time Polymerase Chain Reaction. Frozen mouse lung tissues obtained on day 5 were placed in 1.5 mL of RNase free Red Bead Lysis Kit tubes (Next Advance) containing 500 uL of TRIzol® Reagent (Life Technologies) and homogenized using BBX24 Bullet Blender® (Next Advance). Total RNA was extracted and purified with RNeasy Mini Kit (Qiagen). Fibroblasts were directly lysed in RLT buffer and RNA extracted using the RNeasy Mini Kit. Reverse transcription was performed with SuperScript II and IV (Invitrogen) followed by quantitative

[3] Santos, A. M.; Jung, J.; Aziz, N.; Kissil, J. L.; Pure, E. (2009). Targeting fibroblast activation protein inhibits tumor stromagenesis and growth in mice. *J. Clin. Invest.* **2009**, *119*, 3613-3625.

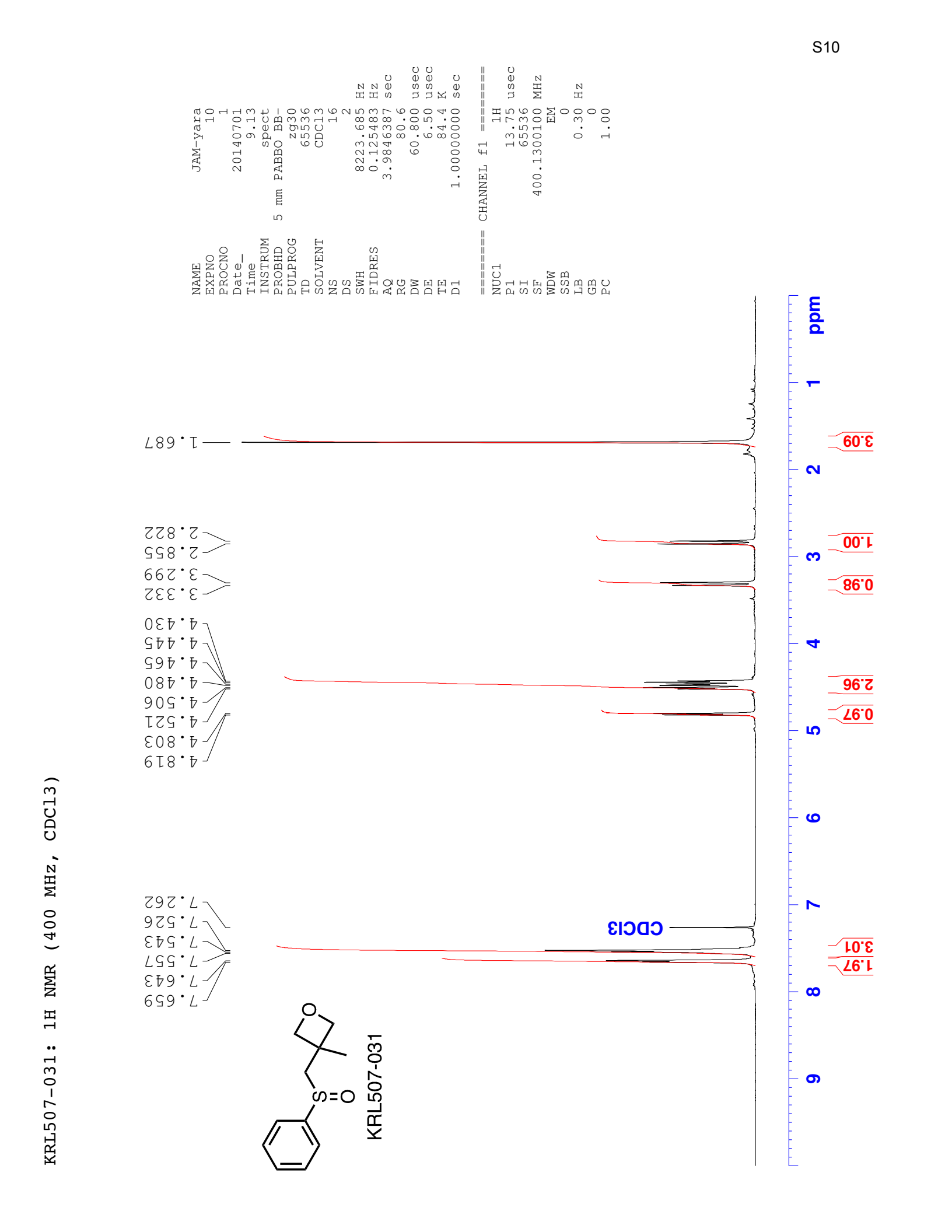
polymerase chain reaction (PCR) amplification with the TaqMan method (ABI Prism 7300 and StepOne Plus; Applied Biosystems). Premixed PCR primers and TaqMan probes for mouse collagen 1A2 (*COL1A2*) (Mm00483888_m1), mouse *GAPDH* (Mm99999915_g1), human collagen 1A1 (*COL1A1*) (Hs00164004_m1), human fibronectin (*FN*) (Hs00365052_m1), human *CTGF* (Hs01026927_g1), human *B2M* (Hs00187842_m1), and human *GAPDH* (Hs02758991_g1) were obtained from Applied Biosystems. Gene expression levels were normalized to GAPDH and compared with the $2^{-\Delta\Delta Ct}$ method.

Ex vivo human skin assay: Skin was obtained from discarded tissue of healthy donors undergoing cosmetic surgery and deemed as non-human subject research by the Institutional Review Committee of the Medical University of South Carolina. Human skin tissue was cut into 3 mm^2 cores using a biopsy punch (Integra LifeSciences, Plainsboro Township, NJ, USA) and four punches per well were placed into a 6-well plate in serum-free DMEM (Corning, Corning, NY, USA) supplemented with 1% penicillin, streptomycin, and amphotericin B (Invitrogen, Carlsbad, CA, USA). Skin punches were treated with vehicle or 10 ng/mL TGF-β1 (R&D Systems, Minneapolis, MN, USA) for 24 h, after which vehicle or 500 µg/mL MMS-350 was added. RNA was extracted from the skin tissues after 24, 48, and 72 h of incubation with MMS-350.

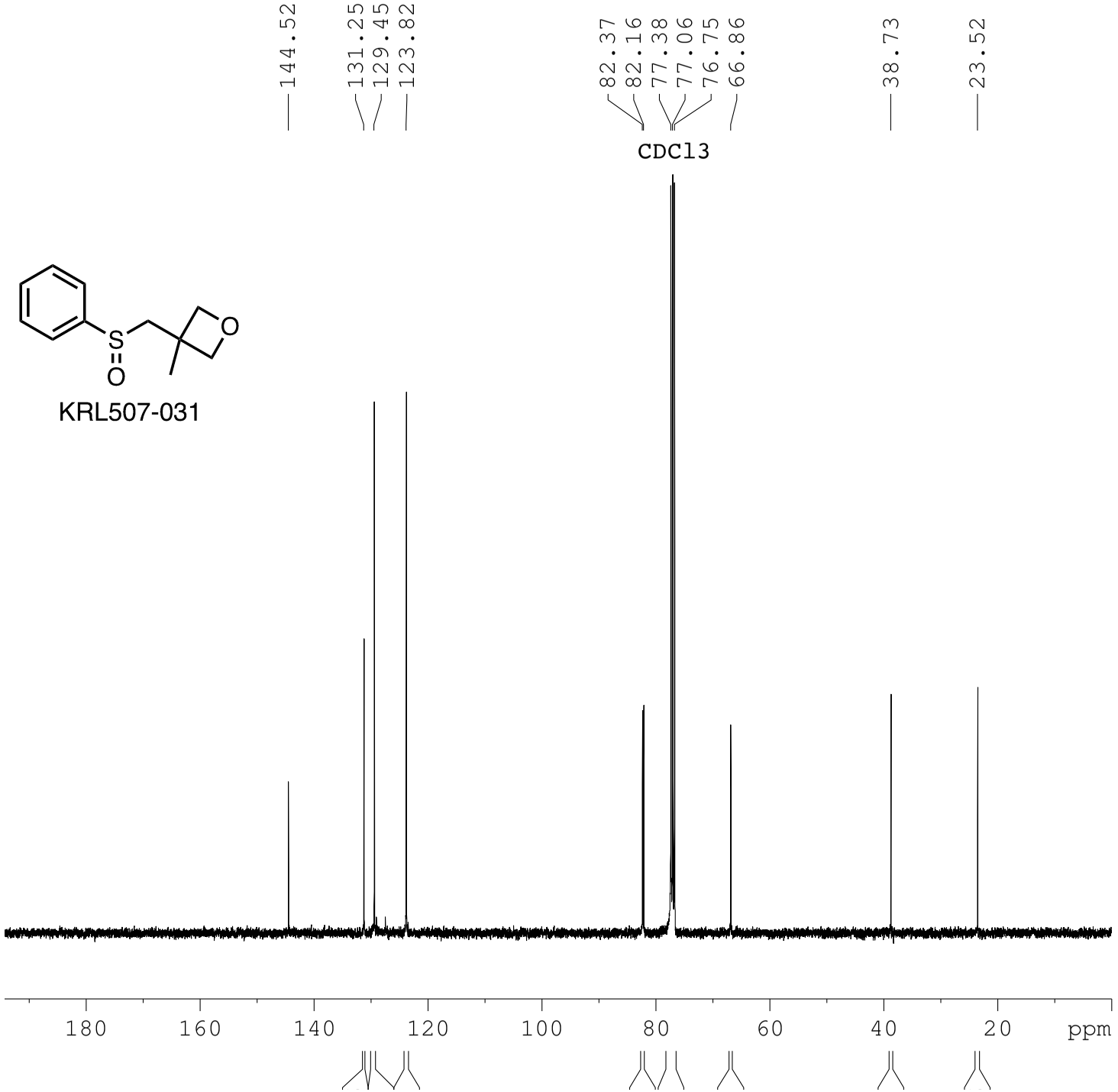
Statistical analysis: Data were analyzed with the unpaired student's t-test or one-way ANOVA with Tukey's multiple comparisons test as appropriate using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Data is represented as the standard error of the mean (SEM).

IV. ^{1}H NMR and ^{13}C NMR Spectra

KRL507-031: 1H NMR (400 MHz, CDCl3)



KRL507-031: 13C NMR (100 MHz, CDCl3)



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PROCNO	1
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PULPROG	zgpg30
TD	65536
SOLVENT	CDCl3
NS	630
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SWH	24038.461 Hz
FIDRES	0.366798 Hz
AQ	1.3631988 sec
RG	50.8
DW	20.800 usec
DE	6.50 usec
TE	298.0 K
D1	3.00000000 sec
D11	0.03000000 sec
TD0	1
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P1	10.00 usec
PL1	-0.44 dB
PL1W	39.19395828 W
SFO1	100.6479773 MHz
======== CHANNEL f2 ========	
CPDPRG2	waltz16
NUC2	1H
PCPD2	90.00 usec
PL2	-3.90 dB
PL12	15.81 dB
PL13	120.00 dB
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PL12W	0.23137002 W
PL13W	0.00000000 W
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SI	32768
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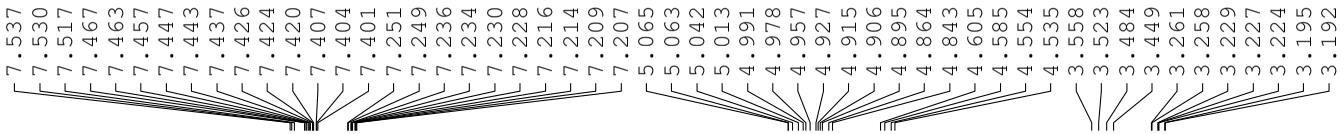
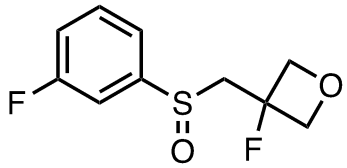
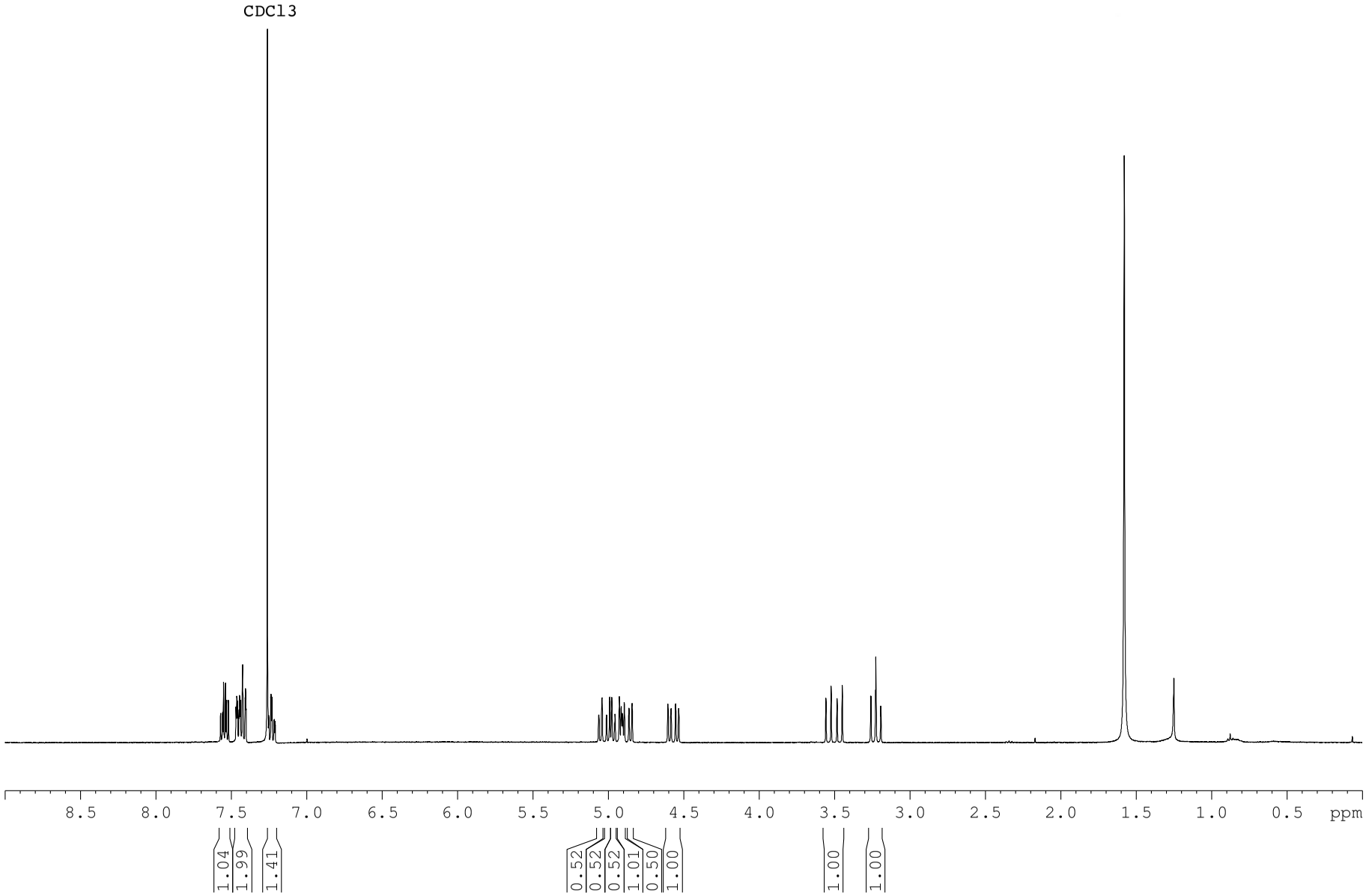
CL-613-091: 1H NMR (400 MHz, CDCl3)





CL-613-091



CL-613-091: 13C NMR (100 MHz, CDCl3)