Diverging Novobiocin Anti-cancer Activity from Neuroprotective Activity through Modification of the Amide Tail

Suman Ghosh[1#], Yang Liu[3#], Gaurav Garg[1], Mercy Anyika[1], Nolan T. McPherson[1], Jiacheng Ma[2], Rick T. Dobrowsky[2], and Brian S. J. Blagg[1]*

[1]Department of Medicinal Chemistry; [2]Department of Pharmacology and Toxicology, The University of Kansas, Lawrence, Kansas 66045; [3]Department of Medical Chemistry, Fujian Medical University, Fuzhou China 350004. [#]These authors contributed equally. *To whom correspondence should be addressed: Brian S. J. Blagg. The University of Kansas, Department of Medicinal Chemistry, 1251 Wescoe Hall Drive, 4070 Malott Hall, Lawrence, KS 66045-7563. Tel: 785-864-4495. Fax: 785-864-5326. E-mail: bblagg@ku.edu.

Supporting Information

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1. General Experimental

^{1}HNMR were recorded at 400 or 500 MHz (Bruker DRX-400 Bruker with a H/C/P/F QNP gradient probe) spectrometer and ^{13}C NMR spectra were recorded at 100 or 125 MHz (Bruker DRX 500 with broadband, inverse triple resonance, and high resolution magic angle spinning HR-MA probe spectrometer); chemical shifts are reported in δ (ppm) relative to the internal reference chloroform-*d* ($CDCl_3$, 7.27 ppm) or dimethyl sulfoxide- d_6 (DMSO-d_6, 2.50 ppm). High resolution mass spectra (FAB) were recorded with a LCT Premier (Waters Corp., Milford, MA) spectrometer. The purity of all compounds was determined to be >95% as determined by ^{1}HNMR and ^{13}CNMR spectra, unless otherwise noted. TLC was performed on glassbacked silica gel plates (Uniplate) with spots visualized by UV light. All solvents were reagent grade and, when necessary, were purified and dried by standard methods. Concentration of solutions occurred via the use of a rotary evaporator operating at reduced pressure.

General Method for the Synthesis of Compounds 5A−5I. N-(7-(((2R,3R,4S)-3,4-dihydroxy-5-methoxy-6,6-dimethyl tetrahydro-2H-pyran-2-yl)oxy)-8-methyl-2-oxo-2H-chromen-3-yl)propionamide (5A) Pyridine (0.24 mL, 3.0 mmol) was added to a solution of amino coumarin **4** (36.5 mg, 1.0 mmol), alkyl acid (111.12 mg, 1.5 mmol), and 4-DMAP (0.1 mmol) in CH_2Cl_2 at rt. The reaction mixture was cooled to 0 ^{O}C and N-(3-(dimethylamino)propyl)-N-ethylcarbo diimide hydrochloride (310.5 mg, 2.0 mmol) was added to the reaction mixture, and stirred at rt for 12 h. The reaction mixture was then concentrated and the residue purified by column chromatography (SiO_2, 40:1 CH_2Cl_2: $CH_3C(O)CH_3$) to afford the corresponding benzamide as a white solid (30 mg, 67%). Triethylamine (2% total volume) was added dropwise to a solution of benzamides (cyclic carbonate) in CH_3OH (5 mL) and the resulting mixture stirred at rt for 12 h. The reaction mixture was concentrated and the residue purified by column chromatography/prep TLC (SiO_2, 19:1 CH_2Cl_2:$CH_3C(O)CH_3$) to afford an amorphous white solid (20 mg, 71.4%). Yields and characterization of all compounds are provided in the supporting information.

Antibodies

The following antibodies were used for Western blotting and/or co-immunoprecipitation: rabbit anti-Hsp90α (Neomarkers, RB-119-P), goat anti-Hsp90β (SantaCruz), rat anti-Grp94 (SantaCruz), mouse anti-Trap-1(BD Biosciences), rabbit anti-Aha1 (Abcam), rabbit anti-Actin (SantaCruz), rabbit anti-Her2 (SantaCruz), rabbit anti-Akt (SantaCruz), rabbit anti-Raf-1 (SantaCruz), mouse anti-Hsp90α/β (SantaCruz).

Cell Lines

The PC3-MM2 cell lines were maintained in DMEM supplemented with 10% FBS, streptomycin, and penicillin at 37 °C, 5% CO_2. 50B11 cells were maintained in DMEM supplemented with 10% FBS, blasticidin (5 μg/ml), streptomycin, and penicillin at 37 °C, 5% CO_2. Breast cancer SkBr3 cells were maintained in ATCC-formulated F-12K media supplemented with 10% FBS, streptomycin and penicillin.

Western Blot Analysis

The various cell lines were harvested in cold PBS and lysed in mammalian protein extraction reagent (MPER, Pierce) lysis buffer containing protease and phosphatase inhibitor cocktails (Roche) on ice for 1 h. Cancer patients' tissue samples were

homogenized and lysed in Native Blue buffer containing protease and phosphatase inhibitor cocktails (Roche) on ice for 1h. Lysates were clarified at 14,000g for 10 min at 4 °C. Protein concentrations were determined using the Pierce BCA protein assay kit per the manufacturer's instructions. Equal amounts of protein (2.5–20 μg) were electrophoresed under reducing conditions (8% polyacrylamide gel), transferred to a polyvinylidene fluoride membrane (PVDF), and immunoblotted with the corresponding specific antibodies. Membranes were incubated with an appropriate horseradish peroxidase-labeled secondary antibody, developed with a chemiluminescent substrate, and visualized.

Co-immunoprecipitation

PC3-MM2 cell lines were plated in 10 cm cell culture dishes or T25 flasks and allowed to grow to ~80% confluency. PC3-MM2 cell lines were untreated, or received DMSO (0.1%), or the indicated drugs dissolved in DMSO. After drug treatments PC3-MM2 cell lines were harvested in lysis buffer containing 0.1% NP40, 50 mM Tris (pH 7.5), 150 mM NaCl, with or without 20 mM MoO_4, protease and phosphatase inhibitor cocktails (Roche). Lysates were clarified and protein concentration was determined using BCA assay. For co-immunoprecipitation, 500 μg of total protein was diluted to 500 μL total volume in lysis buffer and incubated with 10 μl of primary antibody overnight at 4 °C with rocking. Immune complexes were captured with 30 μL of DynaBeads Protein G (Invitrogen) for 3 h with rocking at 4 °C. Protein G Bead complexes were washed three times with lysis buffer and eluted with sample buffer. Samples were then boiled and subjected to SDS–PAGE and Western blot analysis.

Drug-Protein Interaction Assay

PC3-MM2 cell lines were harvested in lysis buffer containing 0.1% NP40, 50 mM Tris (pH 7.5), 150 mM NaCl, protease and phosphatase inhibitor cocktails (Roche). The cell lysates (200 μg) were incubated with biotinylated KU-32 (10 μM) overnight at 4 °C with rocking. Drug-protein complexes were captured with 30 μL of Streptavidin DynaBeads (Invitrogen) for 3 h with rocking at 4 °C. Streptavidin bead complexes were washed three times with lysis buffer and eluted with sample buffer. Samples were then boiled and subjected to SDS–PAGE and western blot analysis.

Luciferase Refolding Assay

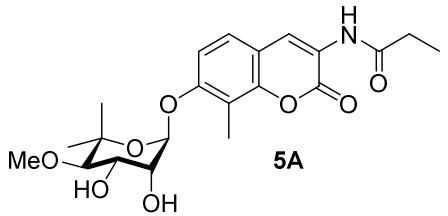
Compound at varying concentrations in DMSO (1% DMSO final concentration) was added to wells of a white, round-bottom 96-well plate containing 50 μL of MEME media. Luciferase-expressing PC3-MM2 cells were grown to confluence, collected, and incubated for 8–12 min at 50 °C in pre-warmed MEME media until bioluminescence of luciferase was reduced to 1% of the initial counts. Cells were added (60,000 cells/50 μL) to wells (final concentration of 60,000 cells/100 μL), and the plate was returned to the incubator for 1 h. After 1 h, 100 μL of luciferase substrate reagent (75 mM tricine at pH7.8, 24 mM $MgSO_4$, 0.3 mM EDTA, 2 mM DTT, 0.313 D-luciferin, 0.64 mM coenzyme A, 0.66 mM ATP, 150 mM KCl, 10% Triton-X, 20% glycerol, and 3.5% DMSO) was added to wells, and the bioluminescence was immediately read (0.5 s integration time). Cells that were incubated in 1% DMSO were used as 100% bioluminescence (*i.e.*, DMSO = 100% refolding), and the relative refolding for each compound concentration was compared to that in 1% DMSO. The concentrations for each compound were in triplicate, and dose–response curves were generated using GraphPad Prism 5.0.

Mitochondrial Bioenergetics Assessment

The oxygen consumption rate (OCR) was measured by the XF96 Extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA) as previously described[1]. Briefly, 50B11 neuronal cell lines were treated with DMSO, or KU-32, or KU-32 analogs (5 μM concentrations each) 5A-I for 24 h and were subjected to mitochondrial bioenergetics assessment. The oxygen consumption rate was in the absence or presence of multiple mitochondrial respiratory chain poisons.

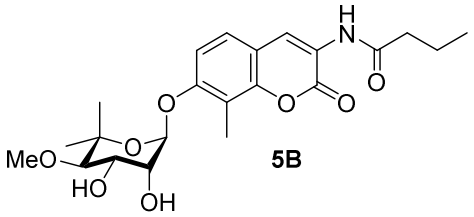
2. Experimental Details

N-(7-(((2R,3R,4S)-3,4-dihydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-8-methyl-2-oxo-2H-chromen-3-yl)propionamide (5A) ^{1}H NMR (500 MHz, Chloroform-d) δ 8.65 (s, 1H), 7.98 (s, 1H), 7.30 (d, *J* = 8.7 Hz, 1H), 7.19 (d, *J* = 8.7 Hz, 1H), 5.61 (d, *J* = 1.9 Hz, 1H), 4.31 – 4.21 (m, 2H), 3.62 (s, 3H), 3.42 – 3.35 (m, 1H), 2.47 (q, *J* = 7.5 Hz, 2H), 2.28 (s, 3H), 1.39 (s, 3H), 1.30 – 1.25 (m, 3H), 1.15 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.16, 159.35, 155.98, 149.12, 125.80, 124.37, 121.74, 114.31, 114.13, 111.25, 97.80, 84.42, 78.67, 71.33, 68.70, 62.12, 30.93, 29.40, 22.57, 9.54, 8.50. HRMS (ESI^+) m/z: [M + Cl^-] calcd for $C_{21}H_{27}ClNO_8$ 456.1425; found, 456.0056.



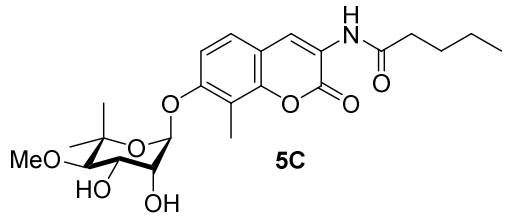
5A

N-(7-(((2R,3R,4S)-3,4-dihydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-8-methyl-2-oxo-2H-chromen-3-yl)butyramide (5B) ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.67 (s, 1H), 7.98 (s, 1H), 7.31 (d, *J* = 8.7 Hz, 1H), 7.19 (d, *J* = 8.7 Hz, 1H), 5.62 (d, *J* = 1.9 Hz, 1H), 4.35 – 4.11 (m, 2H), 3.62 (s, 3H), 3.41 – 3.35 (m, 1H), 2.41 (t, *J* = 7.5 Hz, 2H), 2.28 (s, 3H), 1.82 – 1.74 (m, *J* = 7.4 Hz, 2H), 1.40 (s, 3H), 1.15 (s, 3H), 1.03 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.46, 159.36, 155.98, 149.13, 125.81, 124.39, 121.72, 114.31, 114.13, 111.25, 97.79, 84.42, 78.67, 71.33, 68.70, 62.12, 39.75, 29.86, 29.40, 19.02, 13.84, 8.50. HRMS (ESI^+) m/z: [M + Cl^-] calcd for $C_{22}H_{29}ClNO_8$ 470.1582; found, 470.0058.



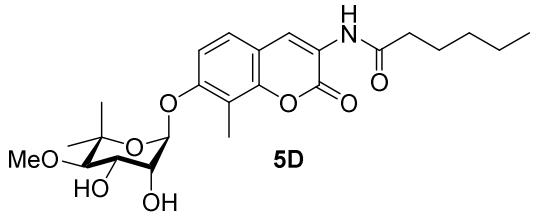
5B

N-(7-(((2R,3R,4S)-3,4-dihydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-8-methyl-2-oxo-2H-chromen-3-yl)pentanamide (5C) ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.65 (s, 1H), 7.98 (s, 1H), 7.29 (d, *J* = 8.6 Hz, 1H), 7.18 (d, *J* = 8.7 Hz, 1H), 5.61 (d, *J* = 1.9 Hz, 1H), 4.29 – 4.20 (m, 2H), 3.61 (s, 3H), 3.38 (d, *J* = 8.9 Hz, 1H), 2.43 (dd, *J* = 8.1, 7.1 Hz, 2H), 2.27 (s, 3H), 1.78 – 1.68 (m, 2H), 1.46 – 1.39 (m, 2H), 1.38 (s, 3H), 1.14 (s, 3H), 0.96 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.67, 159.35, 155.97, 149.11, 125.80, 124.41, 121.71, 114.29, 114.10, 111.24, 97.82, 84.41, 78.67, 71.33, 68.70, 62.11, 37.62, 29.38, 27.59, 22.56, 22.47, 13.93, 8.48. HRMS (ESI^+) m/z: [M + Cl^-] calcd for $C_{23}H_{31}ClNO_8$ 484.1738; found, 486.0199.



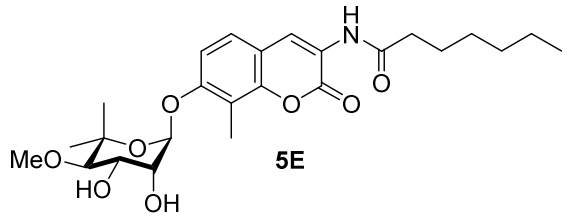
5C

N-(7-(((2R,3R,4S,5R)-3,4-dihydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-8-methyl-2-oxo-2H-chromen-3-yl)hexanamide (5D) ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.66 (s, 1H), 7.97 (s, 1H), 7.30 (d, *J* = 8.7 Hz, 1H), 7.19 (d, *J* = 8.7 Hz, 1H), 5.61 (d, *J* = 1.8 Hz, 1H), 4.29 – 4.22 (m, 2H), 3.62 (s, 3H), 3.39 (dd, *J* = 10.2, 1.5 Hz, 1H), 2.42 (t, *J* = 7.5 Hz, 2H), 2.28 (s, 3H), 1.77 – 1.69 (t, *J* = 7.5 Hz, 2H), 1.41– 1.33 (m, 7H), 1.14 (s, 3H), 0.97 – 0.89 (m, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.63, 159.36, 155.97, 149.13, 125.81, 124.36, 121.75, 114.32, 114.14, 111.25, 97.78, 84.42, 78.67, 71.33, 68.70, 62.13, 37.88, 31.48, 29.86, 29.41, 25.24, 22.56, 14.08, 8.50. HRMS (ESI^+) m/z: [M + Cl^-] calcd for $C_{24}H_{33}ClNO_8$ 498.1895; found, 498.1688.



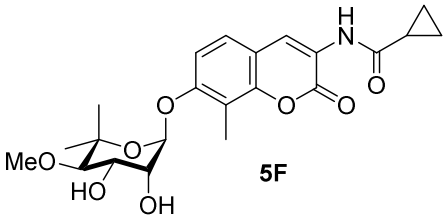
5D

N-(7-(((2R,3R,4S)-3,4-dihydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-8-methyl-2-oxo-2H-chromen-3-yl)heptanamide (5E) ^{1}H NMR (500 MHz, Chloroform-d) δ 8.66 (s, 1H), 7.97 (s, 1H), 7.30 (d, *J* = 8.6 Hz, 1H), 7.18 (d, *J* = 8.7 Hz, 1H), 5.61 (d, *J* = 1.9 Hz, 1H), 4.30 – 4.21 (m, 2H), 3.62 (s, 3H), 3.39 (d, *J* = 8.8 Hz, 1H), 2.48 – 2.38 (m, 2H), 2.28 (s, 3H), 1.80 – 1.69 (m, 2H), 1.43 – 1.35(m, 4H), 1.34 – 1.31 (m, 2H), 1.14 (s, 3H), 0.93 – 0.88 (m, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.65, 159.36, 155.97, 149.13, 125.81, 124.38, 121.74, 114.31, 114.13, 111.25, 97.79, 84.42, 78.67, 71.33, 68.70, 62.12, 37.92, 31.66, 29.41, 29.01, 25.51, 22.64, 22.57, 14.19, 8.50. HRMS (ESI^+) m/z: [M + Cl^-] calcd for $C_{25}H_{35}ClNO_8$ 512.2051; found, 512.1925.



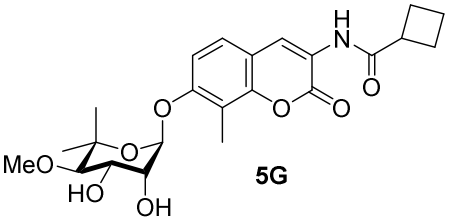
5E

N-(7-(((2R,3R,4S)-3,4-dihydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-8-methyl-2-oxo-2H-chromen-3-yl)cyclopropanecarboxamide (5F) ^{1}H NMR (500 MHz, Chloroform-d) δ 8.60 (s, 1H), 8.21 (s, 1H), 7.28 – 7.25 (m, 1H), 7.17 (d, *J* = 8.7 Hz, 1H), 5.60 (d, *J* = 1.9 Hz, 1H), 5.31 (s, 1H), 4.30 – 4.22 (m, 2H), 3.61 (s, 3H), 3.42 – 3.35 (m, 1H), 2.27 (s, 3H), 1.66 – 1.61 (m, 1H), 1.38 (s, 3H), 1.14 (s, 3H), 1.11 (dd, *J* = 4.4, 3.0 Hz, 2H), 0.92 (dd, *J* = 7.8, 3.1 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.08, 159.36, 155.93, 149.07, 125.73, 124.29, 121.88, 114.29, 114.14, 111.23, 97.82, 84.41, 78.67, 71.33, 68.70, 29.37, 22.57, 16.08, 8.71, 8.49. HRMS (ESI^+) m/z: [M + Cl^-] calcd for $C_{22}H_{27}ClNO_8$ 468.1425; found, 468.1684.



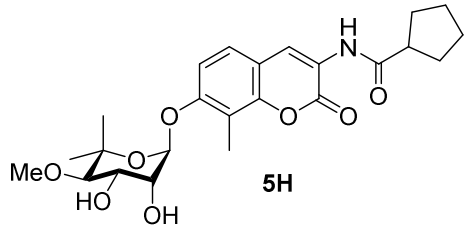
5F

N-(7-(((2R,3R,4S)-3,4-dihydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-8-methyl-2-oxo-2H-chromen-3-yl)cyclobutanecarboxamide (5G) ^{1}H NMR (500 MHz, Chloroform-d) δ 8.66 (s, 1H), 7.89 (s, 1H), 7.29 (d, *J* = 8.7 Hz, 1H), 7.17 (d, *J* = 8.7 Hz, 1H), 5.61 (d, *J* = 1.9 Hz, 1H), 4.33 – 4.22 (m, 2H), 3.61 (s, 3H), 3.42 – 3.35 (m, 1H), 3.23 (pd, *J* = 8.4, 1.0 Hz, 1H), 2.46 – 2.35 (m, 2H), 2.32 – 2.21 (m, 6H), 2.05 (dq, *J* = 11.1, 8.9 Hz, 1H), 2.00 – 1.90 (m, 1H), 1.38 (s, 3H), 1.14 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.52, 159.36, 155.97, 149.11, 125.79, 124.41, 121.73, 114.29, 114.12, 111.24, 97.85, 84.42, 78.68, 71.34, 68.71, 62.12, 40.97, 29.38, 25.42, 22.58, 18.24, 8.49. HRMS (ESI^+) m/z: [M + Cl^-] calcd for $C_{23}H_{29}ClNO_8$ 482.1582; found, 482.1323.



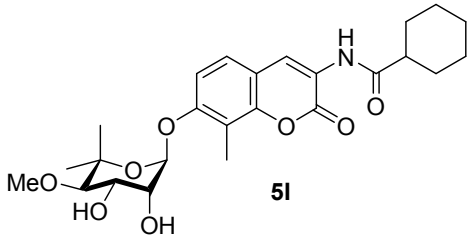
5G

N-(7-(((2R,3R,4S)-3,4-dihydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-8-methyl-2-oxo-2H-chromen-3-yl)cyclopentanecarboxamide (5H) ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.65 (s, 1H), 8.01 (s, 1H), 7.28 (d, *J* = 8.7 Hz, 1H), 7.18 (d, *J* = 8.7 Hz, 1H), 5.61 (d, *J* = 1.9 Hz, 1H), 4.31 – 4.21 (m, 2H), 3.61 (s, 3H), 3.38 (d, *J* = 8.8 Hz, 1H), 2.78 (q, *J* = 8.0 Hz, 1H), 2.27 (s, 3H), 2.03 – 1.92 (m, 1H), 1.90 (ddd, *J* = 7.7, 5.2, 2.5 Hz, 1H), 1.85 – 1.76 (m, 2H), 1.73 – 1.60 (m, 2H), 1.39 (s, 3H), 1.14 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 175.95, 159.40, 155.93, 149.09, 125.76, 124.30, 121.88, 114.29, 114.18, 111.23, 97.80, 84.42, 78.66, 71.33, 68.70, 62.12, 46.98, 30.63, 29.39, 26.12, 22.57, 8.50. HRMS (ESI^+) m/z: [M + Cl^-] calcd for $C_{24}H_{31}ClNO_8$ 496.1738; found, 496.1740.



5H

N-(7-(((2R,3R,4S)-3,4-dihydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-8-methyl-2-oxo-2H-chromen-3-yl)cyclohexanecarboxamide (5I)) ^{1}H NMR (500 MHz, Chloroform-d) δ 8.65 (s, 1H), 8.03 (s, 1H), 7.29 (d, *J* = 8.7 Hz, 1H), 7.17 (d, *J* = 8.8 Hz, 1H), 5.60 (d, *J* = 1.9 Hz, 1H), 4.30 – 4.24 (m, 2H), 3.61 (s, 3H), 3.43 – 3.34 (m, 1H), 2.37 – 2.27 (m, 1H), 2.26 (s, 3H), 2.01 – 1.95 (m, 2H), 1.85 (dt, *J* = 12.6, 3.2 Hz, 2H), 1.59 – 1.48 (m, 2H), 1.38 (s, 4H), 1.38 – 1.24 (m, 3H), 1.14 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 175.69, 159.41, 155.94, 149.08, 125.76, 124.38, 121.78, 114.27, 114.14, 111.22, 97.84, 84.41, 78.67, 71.32, 68.70, 62.10, 46.54, 29.65, 29.64, 29.36, 25.73, 25.67, 22.57, 8.48.
HRMS (ESI^+) m/z: [M + Cl^-] calcd for $C_{25}H_{33}ClNO_8$ 510.1895; found, 509.1749.



5I