Supporting Information

Development of ergosterol peroxide derivatives for cellular localisation studies

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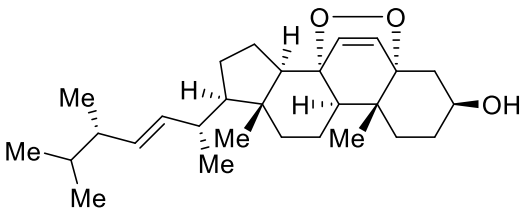
Contents

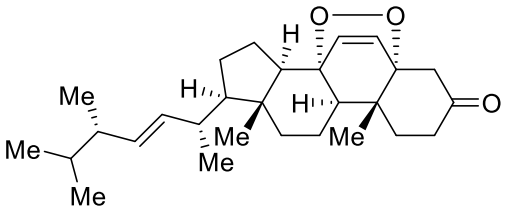
Experimental chemistry procedures

General Information: Chemical manipulations were carried out under inert gas atmosphere unless otherwise noted. Anhydrous tetrahydrofuran (THF), diethyl ether (Et_2O), dichloromethane (CH_2Cl_2), toluene ($PhCH_3$), acetonitrile (CH_3CN), and methanol (MEOH) were obtained from solvent drying system. Reagents of the highest available quality were purchased commercially and used without further purification unless otherwise stated. Title compounds were purified by flash column chromatography using E. Merck silica gel (60, particle size 0.040–0.063 mmol) or Biotage Isolera Four with normal-phase silica gel. Reactions were monitored by thin-layer chromatography (TLC) on 0.25 mmol E. Merck silica gel plates (60F-254), using UV light for visualization and an ethanolic solution of anisaldehyde, or PMA, CAM solutions and heat as developing agent. Reactions of ergosterol peroxide (EP) were monitored by using Agilent 1100 series LCMS and low-resonance electrospray ionization (ESI) model with UV detection at 254 nm. The structures of the compounds were confirmed by ^{1}H and ^{13}C NMR that were recorded on 400 or 500 MHz Bruker AVANCE III HD NMR. Chemical shifts were reported as ppm relative to the solvent residual peak ($CHCl_3$: 7.26 ppm for ^{1}H, 77.2 ppm for ^{13}C; acetone-d6: 2.05 ppm for ^{1}H, 29.9 ppm for ^{13}C; DMSO d6: 2.50 ppm for ^{1}H, 39.5 ppm for ^{13}C). Data are reported as follows: chemical shifts, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, br = broad), coupling constant (Hz), and integration. Optical rotation was measured on DCIF polarimeter (JASCO P- 1010) using a 2-mL cell with a 100-mm path length. High resolution mass spectra (HRMS) were recorded on an Agilent ESI-TOF (time of flight) mass spectrometer using MALDI (matrix-assisted laser desorption ionization) or ESI (electrospray ionization) or on a Waters Xevo G2 Q-ToF mass spectrometer. Compounds were analyzed by using electrospray ionization in positive-ion mode. The purity of the synthesized compounds was determined on a Waters ACQUITY UPLC-PDA-ELSD-MS system using a C_{18} reverse phase column and 0.1% formic acid/water - 0.1% formic acid/acetonitrile as the solvents. All compounds were at least 95% pure based on analytical HPLC and NMR.

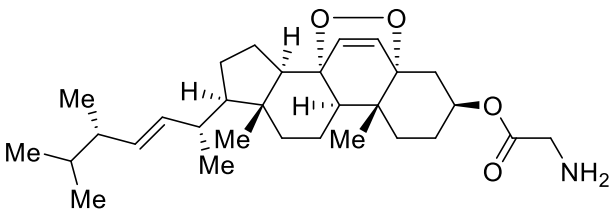
1.2 Compound characterization



Compound **2**: A two-neck round bottom flask (2.0 L) equipped with water-cooled condenser was charged with a solution of ergosterol (7.0 g, 17.66 mmol) and phloxine B (60.0 mg, 0.07 mmol) in dry MeOH (1.50 L), the reaction mixture was stirred at 5 °C in water-cooled bath using a well-ventilated hood, while a steady stream of oxygen was bubbling through the reaction mixture during the irradiation with iodine tungsten lamp (120V, 300 W) for 3 hours (h). The reaction mixture was then directly concentrated under reduced pressure, the crude product was subjected to silica gel column chromatography (hexane: EtOAc, 1:9 v/v) to provide compound 3 (7.0 g, 16.40 mmol, 93% yield). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 6.50 (d, *J* = 8.4 Hz, 1H), 6.24 (d, *J* = 8.5 Hz, 1H), 5.22 (dd, *J* = 15.3, 7.6 Hz, 1H), 5.14 (dd, *J* = 15.3, 8.3 Hz, 1H), 3.96 (dq, *J* = 11.1, 5.6, 5.0 Hz, 1H), 2.20-1.05 (m, 30H), 1.00 (dd, *J* = 6.7, 2.2 Hz, 3H), 0.91 (d, *J* = 6.8 Hz, 3H), 0.88 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 135.39, 135.18, 132.28, 130.72, 82.13, 79.40, 66.45, 56.17, 51.66, 51.06, 44.54, 42.76, 39.72, 39.32, 36.94, 36.91, 34.67, 33.05, 30.10, 28.64, 23.39, 20.87, 20.62, 19.94, 19.63, 18.16, 17.55, 12.86. HRMS (ESI-TOF) calculated for $C_{28}H_{44}N_aO_3$ ([M + Na]$^+$): 451.3188, found: 451.3191.

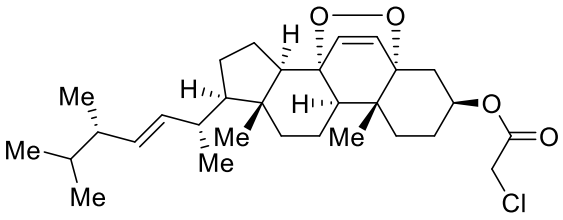


Compound **3a:** To a solution of compound ergosterol peroxide (21.40 mg, 0.05 mmol) in dry CH_2Cl_2 (1.0 mL) was added *N*-methylmorpholine *N*-oxide (NMO, 18.0 mg, 0.15 mmol) at 25 °C, followed by catalytic amount of tetrapropylammonium perruthenate (TPAP, 1.0 mg, 3.0 µmmol), the reaction mixture was stirred at this temperature for 1 h. The reaction mixture was then directly filtered through a short silica-pad, which was rinsed with CH_2Cl_2 (2 x 5.0 mL). The combined organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was subjected to silica gel column chromatography (hexane: EtOAc, 1:9 v/v) to provide compound **3a** (17.0 mg, 0.04 mmol, 80% yield). Compound **3a**: ^{1}H NMR (500 MHz, Acetonitrile-d_3) δ 6.69 (d, *J* = 9.3 Hz, 1H), 5.91 (d, *J* = 9.4 Hz, 1H), 5.27 (dd, *J* = 15.3, 7.5 Hz, 1H), 5.20 (dd, *J* = 15.4, 8.1 Hz, 1H), 2.44 – 2.37 (m, 1H), 2.35 – 2.20 (m, 2H), 2.08 – 2.00 (m, 1H), 1.98 – 1.82 (m, 5H), 1.78 – 1.60 (m, 4H), 1.56 – 1.13 (m, 6H), 0.99 (d, *J* = 6.6 Hz, 3H), 0.95 (s, 3H), 0.92 (d, *J* = 6.8 Hz, 3H), 0.84 (dd, *J* = 8.9, 6.8 Hz, 6H), 0.72 (s, 3H); ^{13}C NMR (126 MHz, CD_3CN) δ 207.85, 143.10, 136.58, 132.87, 128.40, 73.04, 68.80, 65.19, 59.23, 57.42, 55.12, 43.67, 43.54, 40.68, 40.36, 37.19, 34.38, 33.89, 33.47, 28.94, 25.87, 21.62, 21.19, 21.11, 20.26, 19.93, 18.00, 12.44. HRMS (ESI-TOF) calculated for $C_{28}H_{43}O_3$ ([M + H]$^+$): 427.3212, found: 427.3223.

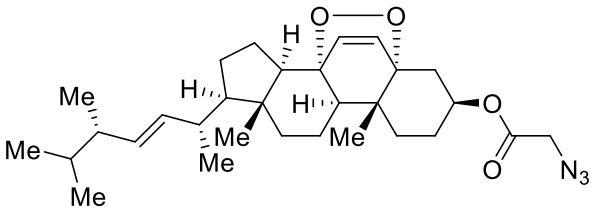


Compound **3b:** To a solution of Fmoc-glycine (48.50 mg, 0.10 mmol) and triethylamine (42.0 µL, 0.30 mmol) in dry CH_2Cl_2 (1.0 mL) was added 2,4,6-trichloro-benzoyl chloride (20.70 µL, 0.11 mmol) at 25 °C, the reaction mixture was stirred at this temperature for 10 mins. Then, ergosterol peroxide (42.80 mg, 0.10 mmol) was added to the above reaction mixture, followed by addition of 4-dimethylaminopyridine (DMAP, 1.0 mg, 0.01 mmol), the reaction mixture was stirred at 25 °C for 10 h. The reaction mixture was then diluted

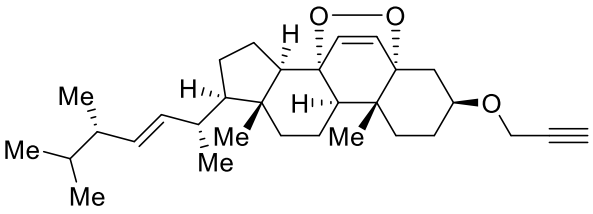
with brine (2.0 mL) and extracted with CH_2Cl_2 (3 x 2.0 mL). The combined organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was subjected to silica gel column chromatography (hexane: EtOAc, 1:9 v/v) to provide compound **3b** (35.10 mg, 0.082 mmol, 82% yield). Compound **3b**: ^{1}H NMR (500 MHz, Methanol-*d*$_4$) δ 6.56 (d, *J* = 8.5 Hz, 1H), 6.27 (d, *J* = 8.5 Hz, 1H), 5.25 (dd, *J* = 15.3, 7.5 Hz, 1H), 5.19 (dd, *J* = 15.3, 8.1 Hz, 1H), 4.98 (dq, *J* = 11.3, 5.5 Hz, 1H), 2.15 – 1.20 (m, 24H), 1.02 (d, *J* = 6.6 Hz, 3H), 0.96 – 0.92 (m, 6H), 0.88 – 0.82 (m, 9H); ^{13}C NMR (126 MHz, MeOD) δ 170.23, 136.80, 136.31, 133.48, 131.94, 83.19, 80.83, 71.67, 57.55, 53.06, 52.69, 45.78, 44.33, 43.49, 41.12, 40.64, 38.17, 35.51, 34.37, 34.07, 29.80, 27.29, 24.39, 23.56, 21.54, 21.38, 20.45, 20.09, 18.44, 18.18, 13.28. HRMS (ESI-TOF) calculated for $C_{30}H_{48}NO_4$ ([M + H]$^+$): 486.3583, found: 486.3583.



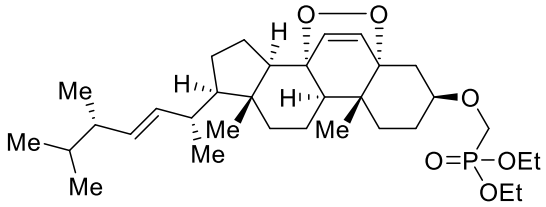
Compound **3c**: To a solution of ergosterol peroxide (42.80 mg, 0.10 mmol) and triethylamine (42.0 µL, 0.30 mmol) in dry CH_2Cl_2 (1.0 mL) was added chloro-acetyl chloride (8.0 µL, 0.10 mmol) at 0 °C, the reaction mixture was stirred at this temperature for 30.0 mins. The reaction mixture was then diluted with brine (3.0 mL) and extracted with CH_2Cl_2 (3 x 3.0 mL). The combined organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was subjected to silica gel column chromatography (hexane: EtOAc, 1:9 v/v) to provide compound **3c** (35.30 mg, 0.07 mmol, 71% yield). Compound **3c**: ^{1}H NMR (500 MHz, Chloroform-*d*) δ 6.51 (d, *J* = 8.5 Hz, 1H), 6.23 (d, *J* = 8.5 Hz, 1H), 5.21 (dd, *J* = 15.3, 7.5 Hz, 1H), 5.14 (dd, *J* = 15.3, 8.3 Hz, 1H), 5.11 – 5.03 (m, 1H), 4.01 (s, 2H), 2.19 – 2.14 (m, 1H), 2.10 – 1.91 (m, 5H), 1.84 (q, *J* = 6.7 Hz, 1H), 1.73 (ddq, *J* = 15.0, 8.7, 5.1 Hz, 2H), 1.65 – 1.31 (m, 7H), 1.29 – 1.18 (m, 4H), 0.99 (d, *J* = 6.6 Hz, 3H), 0.91 – 0.89 (m, 6H), 0.84 – 0.79 (m, 9H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.33, 135.13, 134.83, 132.30, 131.01, 81.68, 79.44, 71.77, 56.14, 51.55, 50.95, 44.54, 42.74, 41.05, 39.69, 39.24, 36.90, 34.17, 33.03, 32.92, 28.60, 26.05, 23.34, 20.84, 20.57, 19.92, 19.61, 18.02, 17.54, 12.85. HRMS (ESI-TOF) calculated for $C_{30}H_{46}ClO_4$ ([M + H]$^+$): 505.3085, found: 505.3094.



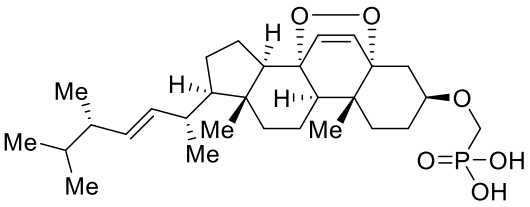
Compound **3d**: To a solution of compound **3c** (25.0 mg, 0.05 mmol) in dry DMSO (1.0 mL) was added sodium azide (10.0 mg, 0.15 mmol) at 25 °C, the reaction mixture was stirred at this temperature for 12 h. The reaction mixture was then diluted with brine (5.0 mL) and extracted with EtOAc (3 x 5.0 mL). The combined organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was subjected to silica gel column chromatography (hexane: EtOAc, 1:9 v/v) to provide compound **3d** (22.0 mg, 0.043 mmol, 86% yield). Compound **3d**: ^{1}H NMR (500 MHz, Chloroform-*d*) δ 6.50 (dd, *J* = 8.8, 3.2 Hz, 1H), 6.22 (dd, *J* = 8.6, 3.2 Hz, 1H), 5.20 (ddd, *J* = 10.7, 7.5, 3.7 Hz, 1H), 5.16 – 5.06 (m, 2H), 3.81 (d, *J* = 3.2 Hz, 2H), 2.18 – 2.11 (m, 1H), 2.10 – 0.70 (m, 37H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.27, 135.10, 134.78, 132.25, 130.96, 81.62, 79.39, 71.46, 56.11, 51.52, 50.92, 50.41, 44.50, 42.71, 39.66, 39.21, 36.86, 34.13, 32.99, 28.57, 26.16, 23.30, 20.81, 20.54, 19.89, 19.58, 17.99, 17.51, 12.82. HRMS (ESI-TOF) calculated for $C_{30}H_{46}N_3O_4$ ([M + H]$^+$): 512.3488, found: 512.3506.



Compound **3e**: To a suspension of compound ergosterol peroxide (21.40 mg, 0.05 mmol) and potassium carbonate (21.0 mg, 0.15 mmol) in dry DMF (1.0 mL) was added propargyl bromide (5.0 µL, 0.06 mmol) and stirred at 50 °C for 10 h. The reaction mixture was then diluted with brine (3.0 mL) and extracted with EtOAc (3 x 3.0 mL). The combined organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was subjected to silica gel column chromatography (hexane: EtOAc, 1:9 v/v) to provide compound **3e** (18.60 mg, 0.040 mmol, 80% yield). Compound **3e**: ^{1}H NMR (500 MHz, Chloroform-*d*) δ 6.50 (d, *J* = 8.6 Hz, 1H), 6.24 (d, *J* = 8.4 Hz, 1H), 5.22 (dd, *J* = 15.3, 7.6 Hz, 1H), 5.14 (dd, *J* = 15.2, 8.2 Hz, 1H), 2.23 – 2.18 (m, 1H), 2.10 – 0.75 (m, 41H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 135.38, 135.18, 132.29, 130.73, 81.98, 79.43, 74.01, 73.49, 56.19, 55.70, 51.66, 51.12, 44.55, 42.76, 39.72, 39.32, 37.11, 34.57, 33.95, 33.05, 29.69, 28.64, 26.73, 23.38, 20.86, 20.59, 19.94, 19.63, 18.07, 17.55, 12.87. HRMS (ESI-TOF) calculated for $C_{31}H_{47}O_3$ ([M + H]$^+$): 467.3525, found: 467.3531.

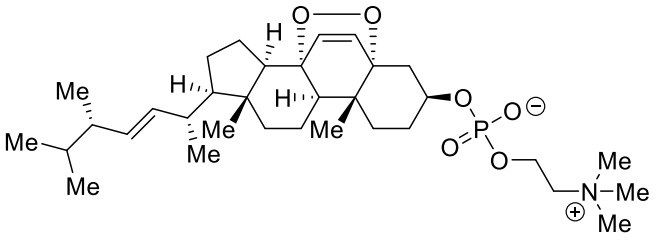


Compound **3f:** To a suspension of ergosterol peroxide (21.40 mg, 0.05 mmol) and potassium carbonate (14.0 mg, 0.10 mmol) in dry THF (1.0 mL) was added diethyl chloro-methylphosphonate (9.0 µL, 0.06 mmol) and stirred at 25 °C for 10 h. The reaction mixture was then diluted with brine (3.0 mL) and extracted with EtOAc (3 x 3.0 mL). The combined organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was subjected to silica gel column chromatography (hexane: EtOAc, 1:9 v/v) to provide compound **3f** (26.0 mg, 0.045 mmol, 90% yield). Compound **3f**: ^{1}H NMR (500 MHz, Chloroform-*d*) δ 6.49 (d, *J* = 8.3 Hz, 1H), 6.22 (d, *J* = 8.4 Hz, 1H), 5.22 (dd, *J* = 15.3, 7.5 Hz, 1H), 5.14 (dd, *J* = 15.2, 8.3 Hz, 1H), 3.91 (tt, *J* = 10.9, 5.1 Hz, 1H), 2.05 – 0.75 (m, 50H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 135.54, 135.19, 132.26, 130.60, 82.36, 79.42, 66.77, 56.18, 51.69, 51.15, 44.53, 42.74, 39.72, 39.32, 37.43, 36.91, 34.93, 33.04, 31.57, 30.48, 29.69, 28.66, 23.38, 22.64, 20.84, 20.56, 19.93, 19.62, 18.09, 17.52, 14.11, 12.85. HRMS (ESI-TOF) calculated for $C_{33}H_{56}O_6P$ ([M + H]$^+$): 579.3815, found: 579.3821.

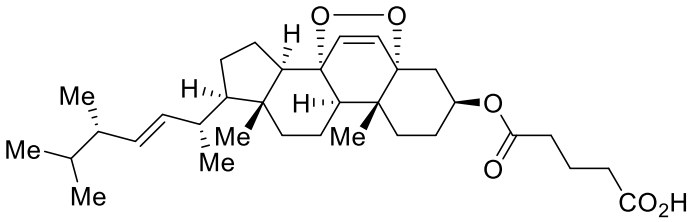


Compound **3g**: To a solution of compound **3f** (13.0 mg, 0.022 mmol) in THF: H2O (1.0 mL, 1:1, v/v) was added NaOH (0.1 mL, aqueous 0.5N, 0.05 mmol) at 25 °C and the reaction mixture was vigorously stirred for 6 h. The reaction mixture was then diluted with brine (1.0 mL) and cooled to 0 °C, the pH value of the reaction mixture was carefully adjusted to 7.0 by aqueous HCl (0.1N) titration. The reaction mixture was extracted with EtOAc (3 x 3.0 mL). The combined organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was subjected to silica gel column chromatography (hexane: EtOAc, 1:9 v/v) to provide compound **3g** (8.40 mg, 0.016 mmol, 73% yield). Compound **3g**: ^{1}H NMR (500 MHz, Methanol-d_4) δ 6.53 (d, *J* = 8.3 Hz, 1H), 6.26 (d, *J* = 8.6 Hz, 1H), 5.25 (dd, *J* = 15.2, 7.6 Hz, 1H), 5.18 (dd, *J* = 15.2, 8.2 Hz, 1H), 3.77 (dt, *J* = 11.4, 6.0 Hz, 1H), 2.35 – 0.75 (m, 40H); ^{13}C NMR (126 MHz, MeOD) δ 136.80, 133.46, 131.72,

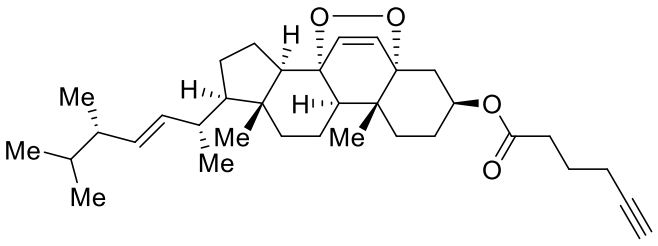
83.47, 80.75, 66.99, 57.56, 53.11, 52.75, 45.75, 44.33, 41.12, 40.68, 38.16, 37.77, 35.91, 34.37, 30.87, 30.68, 29.80, 24.40, 21.56, 21.39, 20.46, 20.09, 18.60, 18.18, 13.28. HRMS (ESI-TOF) calculated for $C_{29}H_{48}O_6P$ ([M + H]$^+$): 523.3189, found: 523.3193.



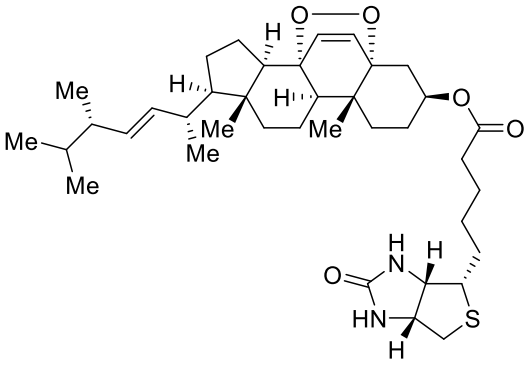
Compound **3h**: To a solution of compound **3h** (21.0 mg, 0.05 mmol) and triethylamine (20.0 µL, 0.15 mmol) in dry benzene (1.0 mL) was added 2-chloro-1,3,2-dioxaphospholane-2-oxide (9.0 µL, 0.10 mmol) at 0 °C, the reaction mixture was warmed up to 25 °C and stirred at this temperature for 10 h. The reaction mixture was then filtered through a short cotton plug, the filtrate was directly concentrated to dryness, diluted with dry CH_3CN (1.0 mL) and transferred into a small pressure bottle. The above reaction mixture was cooled to -78 °C and treated with excess trimethylamine (TMA, 0.10 mL) at the same temperature under nitrogen. The reactor was closed, and the reaction mixture was stirred at 60 °C for 10 h before cooling back to 25 °C. The solvent and excess TMA were evaporated under reduced pressure, the residual crude product was then subjected to silica gel column chromatography (MeOH: EtOAc, 1:1 v/v) to provide compound **3h** (23.0 mg, 0.039 mmol, 77% yield). ^{1}H NMR (500 MHz, Methanol-d_4) δ 6.53 (d, *J* = 8.5 Hz, 1H), 6.28 (d, *J* = 8.5 Hz, 1H), 5.25 (dd, *J* = 15.3, 7.5 Hz, 1H), 5.18 (dd, *J* = 15.3, 8.2 Hz, 1H), 4.33 (ddd, *J* = 11.1, 7.7, 5.9 Hz, 1H), 3.90 (dt, *J* = 6.9, 4.9 Hz, 2H), 3.69 (t, *J* = 5.0 Hz, 2H), 2.21 (ddd, *J* = 13.9, 5.2, 1.8 Hz, 1H), 2.15 – 1.20 (m, 27H), 1.02 (d, *J* = 6.6 Hz, 3H), 0.95 – 0.91 (m, 6H), 0.88 – 0.83 (m, 9H); ^{13}C NMR (126 MHz, MeOD) δ 136.79, 136.67, 133.44, 131.69, 83.42, 80.68, 72.18, 68.04, 65.99, 62.98, 57.55, 53.09, 52.74, 49.85, 47.75, 45.74, 45.36, 44.30, 41.10, 40.66, 38.05, 35.82, 34.34, 29.80, 24.39, 21.57, 21.39, 20.46, 20.10, 18.55, 18.18, 13.30, 9.19. HRMS (ESI-TOF) calculated for $C_{33}H_{57}NO_6P$ ([M + H]$^+$): 594.3924, found: 594.3931.



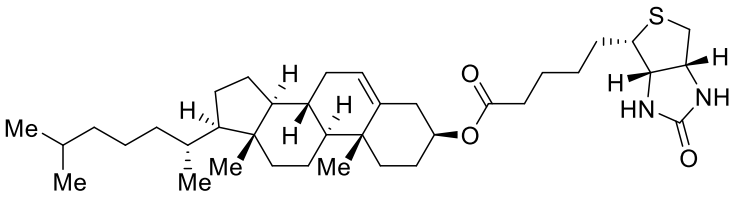
Compound **3i**: To a solution of compound ergosterol peroxide (21.40 mg, 0.05 mmol) and 4-dimethylaminopyridine (DMAP, 6.0 mg, 0.05 mmol) in dry CH_2Cl_2 (1.0 mL) was added glutaric anhydride (7.0 mg, 0.06 mmol) at 25 °C, the reaction mixture was stirred at this temperature for 10 h. The reaction mixture was then diluted with brine (3.0 mL) and extracted with CH_2Cl_2 (3 x 3.0 mL). The combined organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was subjected to silica gel column chromatography (hexane: EtOAc, 1:9 v/v) to provide compound **3l** (25.0 mg, 0.046 mmol, 93% yield). Compound **3l**: ^{1}H NMR (500 MHz, Chloroform-*d*) δ 6.51 (d, *J* = 8.4 Hz, 1H), 6.25 – 6.19 (m, 1H), 5.27 – 5.13 (m, 2H), 5.06 – 4.97 (m, 1H), 2.95 – 0.75 (m, 44H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 177.31, 171.86, 166.43, 135.17, 135.03, 132.29, 130.90, 81.72, 79.38, 69.60, 56.16, 51.58, 50.99, 44.54, 42.76, 39.71, 39.28, 36.93, 34.25, 33.40, 33.15, 33.05, 32.68, 29.97, 29.69, 28.62, 26.27, 23.35, 20.86, 20.60, 19.93, 19.84, 19.62, 18.06, 17.55, 16.34, 12.86. HRMS (ESI-TOF) calculated for $C_{33}H_{51}O_6$ ([M + H]$^+$): 543.3686, found: 543.3682.



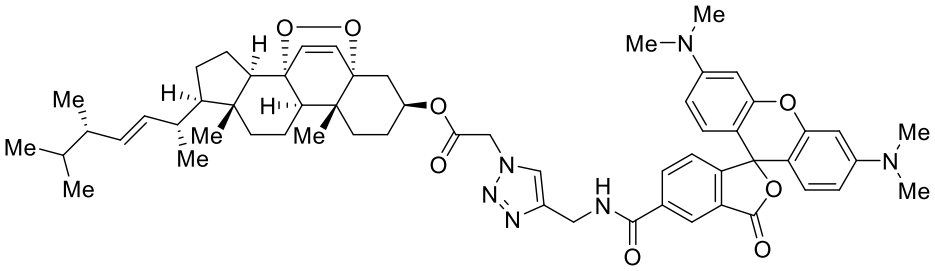
Compound **3j**: To a solution of 5-hexynoic acid (6.70 µL, 0.06 mmol) and triethylamine (25.0 µL, 0.18 mmol) in dry CH_2Cl_2 (1.0 mL) was added 2,4,6-trichloro-benzoyl chloride (11.0 µL, 0.07 mmol) at 25 °C, the reaction mixture was stirred at this temperature for 10 min. Then, ergosterol peroxide (21.0 mg, 0.05 mmol) was added to the above reaction mixture, followed by addition of 4-dimethylaminopyridine (DMAP, 1.0 mg, 0.01 mmol), the reaction mixture was stirred at 25 °C for 12 h. The reaction mixture was then diluted with brine (2.0 mL) and extracted with CH_2Cl_2 (3 x 2.0 mL). The combined organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was subjected to silica gel column chromatography (hexane: EtOAc, 1:9 v/v) to provide compound **3j** (22.50 mg, 0.043 mmol, 86% yield). Compound **3j**: ^{1}H NMR (500 MHz, Chloroform-*d*) δ 6.50 (d, *J* = 8.5 Hz, 1H), 6.22 (d, *J* = 8.5 Hz, 1H), 5.21 (dd, *J* = 15.3, 7.5 Hz, 1H), 5.14 (dd, *J* = 15.3, 8.2 Hz, 1H), 5.00 (tt, *J* = 11.3, 5.2 Hz, 1H), 2.40 (t, *J* = 7.4 Hz, 2H), 2.24 (td, *J* = 7.0, 2.7 Hz, 2H), 2.12 (ddd, *J* = 13.8, 5.3, 1.9 Hz, 1H), 2.07 – 1.90 (m, 6H), 1.88 – 1.80 (m, 3H), 1.78 – 1.30 (m, 10H), 1.29 – 1.15 (m, 3H), 0.99 (d, *J* = 6.5 Hz, 3H), 0.92 – 0.88 (m, 6H), 0.84 – 0.79 (m, 9H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.03, 135.16, 135.03, 132.27, 130.88, 83.30, 81.70, 79.35, 69.44, 69.04, 56.14, 51.57, 50.98, 44.52, 42.74, 39.70, 39.26, 36.92, 34.25, 33.16, 33.03, 28.60, 26.27, 23.61, 23.34, 20.85, 20.59, 19.92, 19.61, 18.05, 17.82, 17.54, 12.85. HRMS (ESI-TOF) calculated for $C_{34}H_{51}O_4$ ([M + H]$^+$): 523.3787, found: 523.3782.



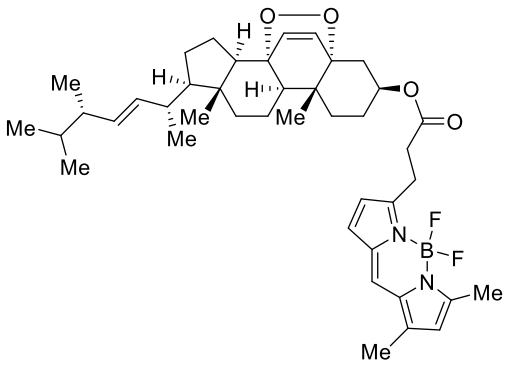
Compound **3k**: To a solution of biotin (15.0 mg, 0.06 mmol) and triethylamine (25.0 µL, 0.18 mmol) in dry CH_2Cl_2 (1.0 mL) was added 2,4,6-trichloro-benzoyl chloride (11.0µL, 0.07mmol) at 25 °C, the reaction mixture was stirred at this temperature for 10 min. Then, ergosterol peroxide (21.0 mg, 0.05 mmol) was added to the above reaction mixture, followed by addition of 4-dimethylaminopyridine (DMAP, 1.0 mg, 0.01 mmol), the reaction mixture was stirred at 25 °C for 10 h. The reaction mixture was then diluted with brine (3.0 mL) and extracted with CH_2Cl_2 (3 x 3.0 mL). The combined organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was subjected to silica gel column chromatography (hexane: EtOAc, 1:9 v/v) to provide compound **3k** (27.0 mg, 0.041 mmol, 82% yield). Compound **3k**: ^{1}H NMR (500 MHz, DMSO-d_6) δ 6.45 (d, *J* = 8.6 Hz, 1H), 6.40 (s, 1H), 6.35 (s, 1H), 6.24 (d, *J* = 8.5 Hz, 1H), 5.21 (dd, *J* = 15.3, 7.3 Hz, 1H), 5.14 (dd, *J* = 15.3, 8.2 Hz, 1H), 4.75 (dt, *J* = 11.8, 6.3 Hz, 1H), 4.31 (dd, *J* = 7.8, 5.0 Hz, 1H), 4.13 (t, *J* = 6.6 Hz, 1H), 3.09 (dt, *J* = 9.9, 5.3 Hz, 1H), 2.81 (dd, *J* = 12.5, 5.0 Hz, 1H), 2.57 (d, *J* = 12.5 Hz, 1H), 2.24 (t, *J* = 7.3 Hz, 2H), 2.11 – 1.89 (m, 3H), 1.89 – 1.80 (m, 2H), 1.75 (d, *J* = 10.6 Hz, 1H), 1.71 – 1.05 (m, 11H), 0.95 (d, *J* = 6.5 Hz, 3H), 0.86 (d, *J* = 6.8 Hz, 3H), 0.83 – 0.76 (m, 9H), 0.74 (s, 3H); ^{13}C NMR (126 MHz, DMSO) δ 172.67, 163.17, 135.47, 135.27, 131.84, 130.64, 81.51, 78.99, 69.07, 61.40, 59.53, 55.66, 55.62, 51.39, 50.97, 44.33, 42.25, 36.71, 34.24, 33.80, 32.90, 32.73, 28.56, 28.23, 26.15, 24.75, 23.04, 20.97, 20.48, 20.04, 19.71, 17.97, 17.57, 12.82. HRMS (ESI-TOF) calculated for $C_{38}H_{59}N_2O_5S$ ([M + H]$^+$): 655.4145, found: 655.4145.



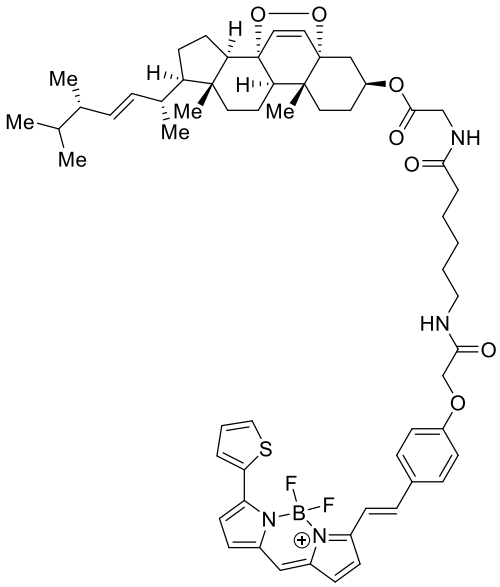
Cholesterol biotin ester (cholesterol control probe): To a solution of biotin (15.0 mg, 0.06 mmol) and triethylamine (25.0 µL, 0.18 mmol) in dry CH_2Cl_2 (1.0 mL) was added 2,4,6-trichloro-benzoyl chloride (11.0 µL, 0.07 mmol) at 25 °C, the reaction mixture was stirred at this temperature for 10 min. Then, cholesterol (19.0 mg, 0.05 mmol) was added to the above reaction mixture, followed by addition of 4-dimethylaminopyridine (DMAP, 1.0 mg, 0.01 mmol). The reaction mixture was stirred at 25 °C for 10 h, then diluted with brine (3.0 mL) and extracted with CH_2Cl_2 (3 x 3.0 mL). The combined organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was subjected to silica gel column chromatography (hexane: EtOAc, 1:9 v/v) to provide compound cholesterol-biotin ester (26.0 mg, 0.042 mmol, 85% yield). Compound cholesterol control probe: ^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.38 (d, *J* = 5.1 Hz, 1H), 4.71 (dd, *J* = 8.1, 4.8 Hz, 1H), 4.61 (dt, *J* = 11.6, 4.0 Hz, 1H), 4.49 (dd, *J* = 8.1, 4.5 Hz, 1H), 3.24 (t, *J* = 6.3 Hz, 1H), 2.97 (dd, *J* = 13.2, 4.8 Hz, 1H), 2.89 (d, *J* = 13.2 Hz, 1H), 2.37 – 2.27 (m, 4H), 2.00 (dd, *J* = 12.6, 3.5 Hz, 1H), 1.90 – 1.64 (m, 4H), 1.64 – 1.41 (m, 3H), 1.34 (d, *J* = 9.8 Hz, 2H), 1.29 – 1.22 (m, 3H), 1.19 – 1.05 (m, 5H), 1.02 (s, 3H), 0.91 (d, *J* = 6.6 Hz, 3H), 0.87 (d, *J* = 2.3 Hz, 3H), 0.86 (d, *J* = 2.3 Hz, 3H), 0.68 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.30, 171.17, 143.22, 139.47, 74.22, 60.40, 56.66, 56.12, 54.94, 50.00, 42.68, 42.30, 39.71, 39.50, 38.09, 36.95, 36.58, 36.17, 35.78, 31.84, 29.69, 28.22, 28.00, 27.79, 24.56, 24.27, 23.82, 22.81, 22.55, 21.02, 19.31, 18.70, 18.65, 14.19, 11.85. HRMS (ESI-TOF) calculated for $C_{37}H_{61}N_2O_3S$ ($[M + H]^+$): 613.4403, found: 613.4401.



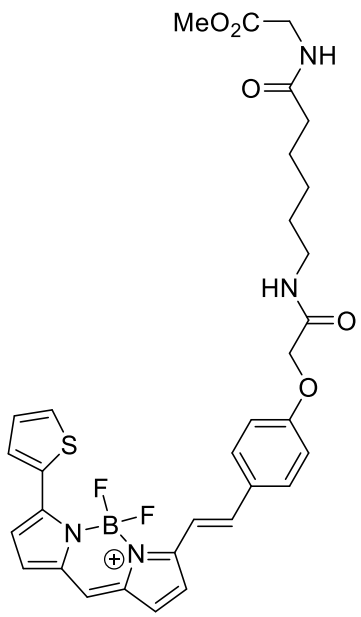
Compound 3l: To a solution of the compound **3d** (3.0 mg, 0.006 mmol) and FITC propargyl amide (3.0 mg, 0.006 mmol) in H_2O:*tert*-butanol (2.0 mL, 1:1, v/v) was added sodium ascorbate (2.0 mg, 0.01 mmol) and copper (II) sulfate pentahydrate (1.0 mg, 0.005 mmol), the heterogeneous reaction mixture was vigorously stirred at 25 °C for 10 h. The reaction mixture was then diluted with brine (2.0 mL) and extracted with EtOAc (3 x 3.0 mL). The combined organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was subjected to silica gel column chromatography (hexane: EtOAc, 1:9 v/v) to provide compound **3l** (4.0 mg, 0.004 mmol, 68% yield). Compound **3l**: ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.20 (d, *J* = 8.4 Hz, 1H), 8.16 (d, *J* = 8.4 Hz, 1H), 8.05 (dd, *J* = 13.6, 8.4 Hz, 1H), 7.97 (dd, *J* = 11.3, 8.3 Hz, 2H), 7.79 (t, *J* = 8.0 Hz, 1H), 7.71 (t, *J* = 7.8 Hz, 1H), 7.59 (t, *J* = 7.6 Hz, 1H), 7.53 (d, *J* = 7.4 Hz, 1H), 7.51 – 7.46 (m, 1H), 6.43 (d, *J* = 8.3 Hz, 1H), 6.17 (d, *J* = 8.3 Hz, 1H), 5.20 – 5.10 (m, 2H), 4.50 – 0.50 (m, 53H); ^{13}C NMR (126 MHz, MeOD) δ 172.29, 159.04, 155.44, 147.87, 142.03, 137.02, 134.25, 130.54, 129.45, 128.87, 127.97, 126.94, 126.65, 125.80, 120.82, 120.52, 118.84, 116.38, 116.23, 111.72, 109.93, 97.51, 79.54, 76.14, 75.19, 58.85, 56.19, 55.24, 44.35, 43.83, 34.37, 30.76, 30.07, 27.33, 26.77, 26.02, 23.78, 23.00, 21.69, 21.43, 20.79, 20.47, 20.09, 19.70, 19.32, 18.74, 18.22, 17.30, 13.20. HRMS (ESI-TOF) calculated for $C_{58}H_{71}N_6O_8$ ($[M + H]^+$): 979.5333, found: 979.5328.



Compound **3m**: To a solution of BODIPY™ FL [C_5 (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoic acid)] (3.0 mg, 0.01 mmol) and triethylamine (3.0 µL, 0.02 mmol) in dry CH_2Cl_2 (1.0 mL) was added 2,4,6-trichloro-benzoyl chloride (2.0 µL, 0.01 mmol) at 25 °C, the reaction mixture was stirred at this temperature for 10 min. Then, ergosterol peroxide (4.0 mg, 0.01 mmol) was added to the above reaction mixture, followed by addition of 4-dimethylaminopyridine (DMAP, 1.0 mg, 0.01 mmol), the reaction mixture was stirred at 25 °C for 10 h. The reaction mixture was then diluted with brine (2.0 mL) and extracted with CH_2Cl_2 (3 x 2.0 mL). The combined organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was subjected to silica gel column chromatography (hexane: EtOAc, 1:9 v/v) to provide compound **3m** (5.0 mg, 0.007 mmol, 70% yield). Compound **3m**: ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.07 (s, 1H), 6.87 (s, 1H), 6.50 (d, *J* = 8.6 Hz, 1H), 6.26 (d, *J* = 4.1 Hz, 1H), 6.21 (d, *J* = 8.6 Hz, 1H), 6.11 (s, 1H), 5.26 – 5.10 (m, 2H), 5.01 (dt, *J* = 11.8, 6.3 Hz, 1H), 4.12 (d, *J* = 7.1 Hz, 4H), 3.27 (s, 2H), 2.72 (s, 2H), 2.60 – 0.75 (m, 39H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.15, 160.23, 157.39, 143.69, 135.19, 135.12, 133.29, 132.28, 130.83, 128.10, 123.77, 120.35, 116.85, 81.75, 79.35, 69.67, 60.39, 56.16, 53.41, 51.59, 50.99, 44.54, 42.76, 39.72, 39.29, 36.94, 34.27, 33.68, 33.11, 33.05, 28.63, 26.22, 23.96, 23.35, 21.05, 20.86, 20.61, 19.94, 19.63, 18.05, 17.56, 14.93, 14.19, 12.86, 11.29. HRMS (ESI-TOF) calculated for $C_{42}H_{58}BF_2N_2O_4$ ($[M + H]^+$): 703.4458, found: 703.4453.



Compound **3n**: To a suspension of compound **3b** (2.0 mg, 0.005 mmol) and triethylamine (1.0 µL, 0.01 mmol) in dry CH_2Cl_2 (0.50mL) was added diethyl BODIPY™ 630/650-X NHS Ester (Succinimidyl Ester) (3.0 mg, 0.005 mmol) and stirred at 25 °C for 10 h. The reaction mixture was then diluted with brine (1.0 mL) and extracted with EtOAc (3 x 1.0 mL). The combined organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was subjected to silica gel column chromatography (hexane: EtOAc, 1:9 v/v) to provide **3n** (3.0 mg, 0.003 mmol, 60% yield). Compound **3n**: ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.67 – 7.44 (m, 3H), 7.08 – 6.93 (m, 4H), 6.62 (s, 1H), 6.51 (d, *J* = 8.6 Hz, 3H), 6.24 (t, *J* = 8.0 Hz, 3H), 6.05 (s, 1H), 5.20 – 4.99 (m, 2H), 4.54 (d, *J* = 3.0 Hz, 2H), 4.29 – 3.91 (m, 6H), 3.37 (d, *J* = 7.0 Hz, 3H), 2.45 – 0.65 (m, 43H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.14, 167.27, 166.83, 147.41, 135.14, 134.76, 132.39, 132.33, 131.08, 130.99, 130.70, 129.49, 115.09, 81.86, 81.68, 79.51, 79.47, 79.41, 60.39, 56.16, 53.41, 51.56, 51.00, 50.97, 44.58, 44.56, 42.83, 42.76, 39.71, 39.25, 36.91, 36.87, 34.18, 33.05, 30.93, 29.69, 28.62, 26.18, 26.16, 23.36, 21.05, 20.98, 20.86, 20.59, 19.96, 19.94, 19.66, 19.62, 18.10, 18.03, 17.67, 17.55, 14.19, 12.87. HRMS (ESI-TOF) calculated for $C_{59}H_{74}BF_2N_4O_7S$ ($[M + H]^+$): 1031.5334, found: 1031.5342.



BDY630X-glycine methyl ester control: To a suspension of compound glycine methyl ester hydrochloride (1.0 mg, 0.01 mmol) and triethylamine (5.0 µL, 0.03 mmol) in dry DMF (0.20 mL) was added diethyl BODIPY™ 630/650-X NHS Ester (Succinimidyl Ester) (1.0 mg, 0.002 mmol) and stirred at 25 ^{o}C for 10 h. The reaction mixture was then diluted with brine (1.0 mL) and extracted with EtOAc (3 x 1.0 mL). The combined organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was subjected to silica gel column chromatography (hexane: EtOAc, 1:9 v/v) to provide compound BDY630X-glycine methyl ester (0.90 mg, 0.001 mmol, 50% yield). 1H NMR (500 MHz, MeOD) δ 7.75 – 6.70 (m, 14H), 4.52 (s, 2H), 3.87 (s, 2H), 3.68(s, 3H), 2.35 – 0.75 (m, 10H); 13C NMR (126 MHz, MeOD) δ 176.59, 171.86, 133.80, 133.05, 130.04, 129.19, 128.73, 126.94, 116.11, 115.51, 52.62, 41.76, 36.49, 33.16, 30.81, 30.05, 27.28, 26.44. HRMS (ESI-TOF) calculated for $C_{32}H_{34}BF_2N_4O_5S$ ($[M + H]^+$): 635.2306, found: 635.2311.

2. Cytotoxicity assay

All cell lines were incubated at 37 °C and maintained in a 5% CO_2 atmosphere according to proper sterile cell culture practices.[1] Cells were tested for mycoplasma contamination with Mycoplasma Detection Kit (# LT07-318, Lonza) using the manufacturer's conditions and were deemed negative. Cell lines were purchased from American Type Culture Collection (ATCC®) and cultured without antibiotics or as specified by provider (SUM149, provided by Dr. Martinez). Adherent cells MDA-MB-231, T-47D (ATCC® HTB-133), BJ and HMEC were grown to 80%-90% confluence to densities recommended by ATCC before use. Cells were cultured in DMEM, (ATCC®, 30-2002), F10 Nutrient mixture (Gibco 12390-035), EMEM (ATCC® 30-2003), or other specific medium supplemented with 10% fetal bovine serum (FBS, Hyclone), unless otherwise stated. The human breast cancer cell lines (MDA-MB-231, SUM149, T-47D, BJ, and HMEC) to test cytotoxicity by the methods described have been previously reported by us.[2b] BJ (CRL-2522, normal human foreskin fibroblast) cells were used to test for general cytotoxicity and are typically sensitive to necrosis- or apoptosis–inducing compounds. HMEC cells (primary mammary epithelial normal, HMEC (ATCC® PCC-600-010, 3000 cells/well) were grown in 96 well plates in Mammary epithelial cell basal medium (ATCC® PCS-600-030) supplemented with growth Kit (ATCC® PCS-600-040). The kit includes all the required additives for healthy growth under FBS free conditions up to passage 15 as recommended by ATCC®. Cytotoxicity assay was performed using the CellTiterGlo Luminescent Cell Viability Assay kit (G7570, Promega, Madison, WI), performed according to the manufacturer's instructions. Luminescence was recorded with an Envision plate reader (Perkin Elmer). Cells were then seeded in 96-well white polystyrene flat-bottomed plates (Corning #3917 at 5000 cells per well in 100uL/well) at concentrations experimentally determined to ensure logarithmic growth during the duration of the experiment and prevent adverse effects on cell growth by DMSO exposure. The plates were incubated at 37 °C in a 5% CO_2 atmosphere for 24 hours (h) before treatment.

Stock solutions of test compounds (10 mmol in DMSO) in nine 3-fold serial dilutions were dispensed via pintool. The final concentration of DMSO was 0.3 % (v/v) in each well. Positive controls included staurosporine (25µM), gambogic acid (10µM) and a toxic quinoline generated in-house. The plates were incubated for 72 h at 37 °C in a 5 % CO_2 atmosphere, and then quenched with CellTiterGlo (CTG, 30 µL per well) at RT. Plates were then incubated at RT for 20 min and centrifuged at 1000 rpm for 1 min. Luminescence was read on an Envision plate reader (Perkin Elmer). As a complement, a Propidium iodide (PI) cell viability assay was performed. Cells were seeded $6x10^4$-$2x10^5$ cells/well and cultured for 24 h. Then, cells were treated in 2 technical duplicates with 2-fold serial dilutions of each compound for 48 h. Three independent replicates were performed of each experiment. Cells were fixed (cold methanol), and stained for visualization of nuclei [0.4% PI, Sigma-Aldrich). Fluorescence was measured with a Lionheart FX plate reader (BioTek, Winooski, VT). Cell viability was calculated as percent of surviving cells after treatment relative to negative control wells.

Statistical analysis: For the CellTiterGlo (CTG) assay, 3 or 4 technical replicates were conducted for each experimental condition, and a minimum of 3 independent experiments were conducted for cellular assays. The mean luminescence of each experimental treatment group was normalized as a percentage of the mean intensity of untreated controls. EC_{50} values (µM) were calculated by Pipeline Pilot Software

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1[a] Hay, R.J., Caputo, J.L., Macy, M.L., Eds. ATCC Quality Control Methods for Cell Lines. 2nd edition, Published by ATCC, **1992**. 1[b] Ling, T.; Maier, J.; Das, S.; Budhraja, A.; Bassett, R.; Potts, M.B; Shelat, A.; Rankovic Z.; Opferman, J.T.; Rivas, F. Identification of substituted 5-membered heterocyclic compounds as potential anti-leukemic agents. *Eur J Med Chem.* **2018**, *164*, 391-398.

(Accelrys, Enterprise Platform, CA, USA) and from dose response curve-fitting via non-linear regression by GraphPad Prism (Version 7.0 San Diego, CA).

Number	MDA-MB-231 EC50 (μM)	SUM149 EC50 (μM)	T47D EC50 (μM)	HMEC EC50 (μM)	BJ EC50 (μM)	TI (HMEC/ SUM149)
Phosphoramide	>11	>12	ND	>10	12	>1
Doxorubicin	1	1	ND	>10	15	>11
Taxol	2	0.01	ND	>10	23	>800
Capecitabine	5	5	ND	10	10	>2
2	18	9	19	>20	>20	>3
3a	7	5	10	>13	>20	>3
3b	16	3	>20	>20	>20	>4
3c	>20	5	>20	>20	>20	>4
3d	>20	10	>20	>20	>20	>2
3e	16	10	>20	>20	>20	>2
3f	9	5	>20	>20	>21	>4
3g	37	5	>20	>20	>21	>4
3h	25	21	17	>20	>20	>1
3i	33	5	18	>20	>20	>4
3j	8	4	11	>13	>21	>4
3k	10	4	>10	>14	>21	>6
3l	20	18	>20	>20	>21	>1
3m	18	2	18	>15	>21	>6
3n	7	2	16	>15	>21	>6

Table S1. EC_{50} values determined by CTG assay. Each value represents the average of 3 independent CTG assays with each data point done in triplicates. Normal breast epithelial cells (HMEC) and BJ cells were evaluated for comparison purposes. Therapeutic Index (TI) was calculated from HMEC/SUM149.

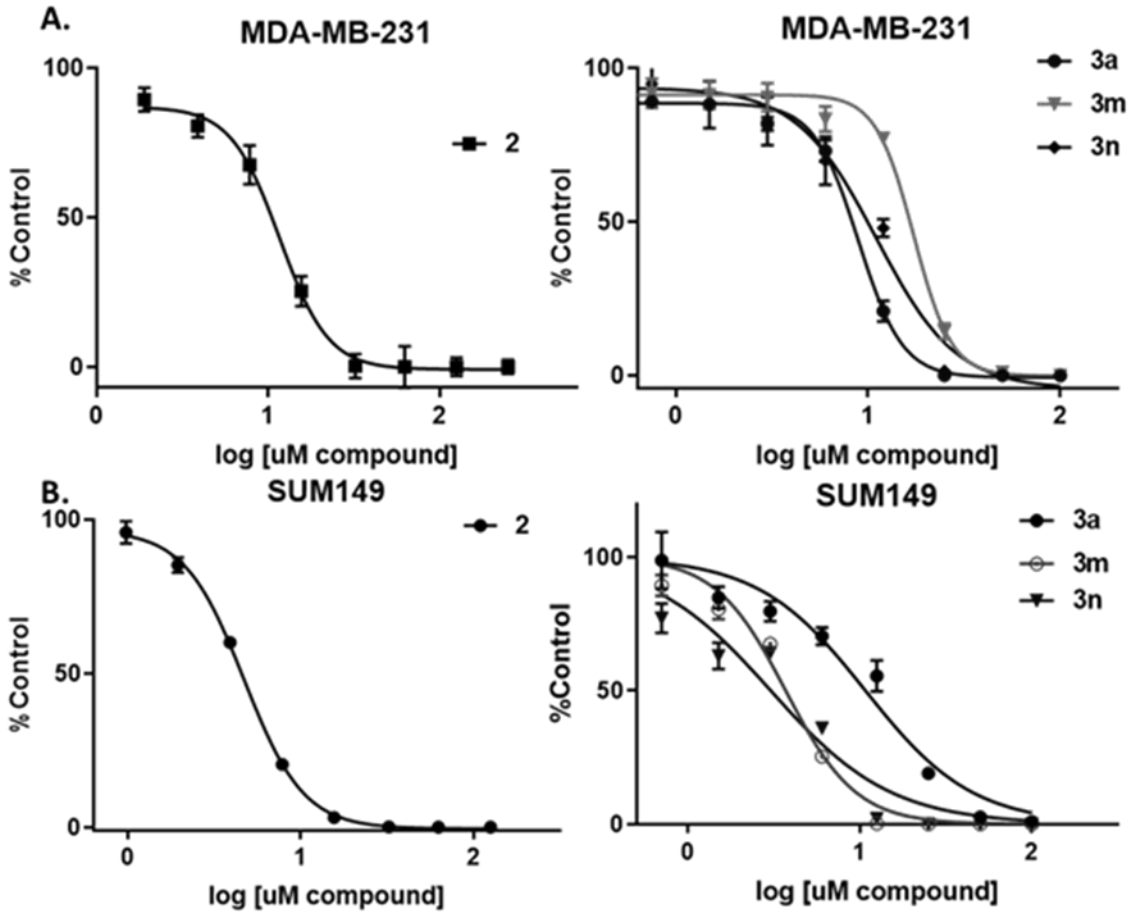

Figure S1.1. Representative graphs of cell viability experiments in breast cancer cell models using CTG assay (72 h). **A.** MDA-MB-231. **B.** SUM149.

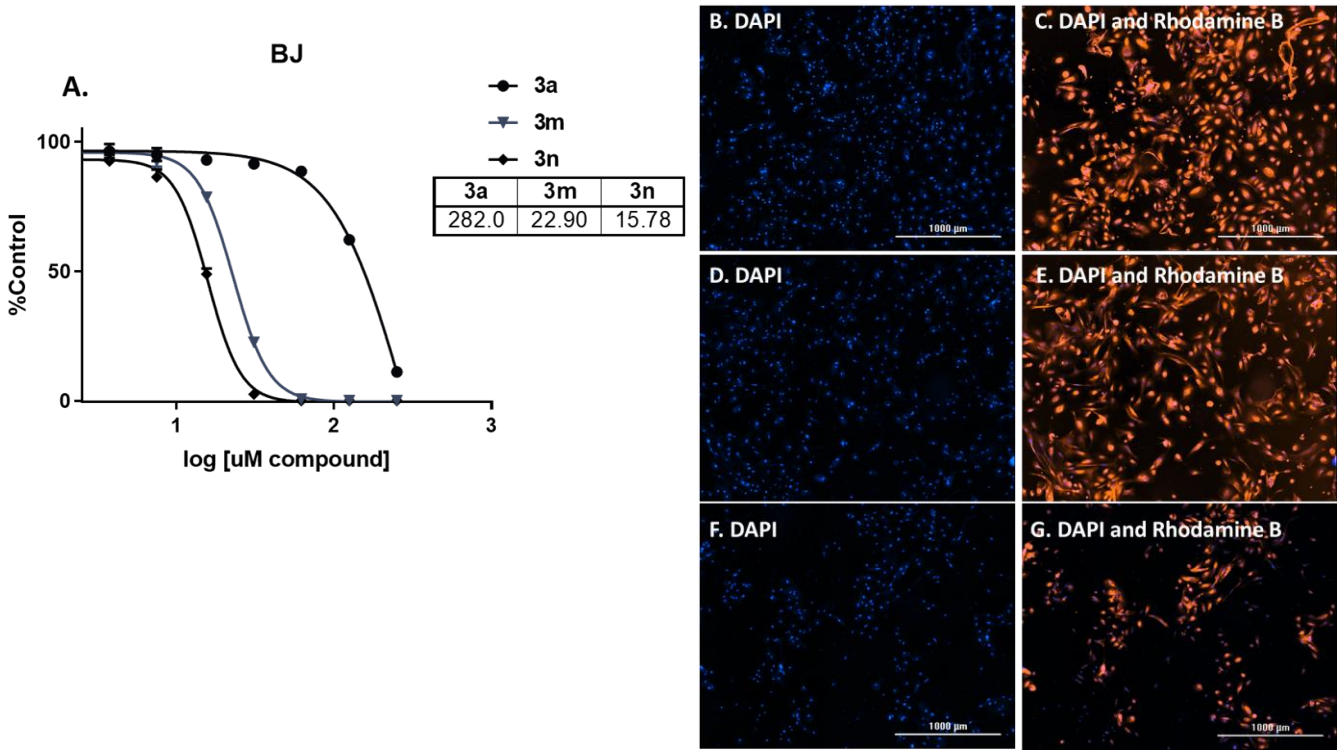

Figure S1.2. A. Representative graph of CTG viability assay (72 h) against non-cancerous cell line BJ. **B.-E.** Images of HMEC cells after staining with DAPI (blue) and Rhodamine B (red) after 72 h treatment with compounds. **B-C.** Cells treated with vehicle (DMSO). **D-E.** Cells treated with compound **3** at 30 µM. **F-G.** Cells treated with positive control, staurosporine (STS) at 2 µM.

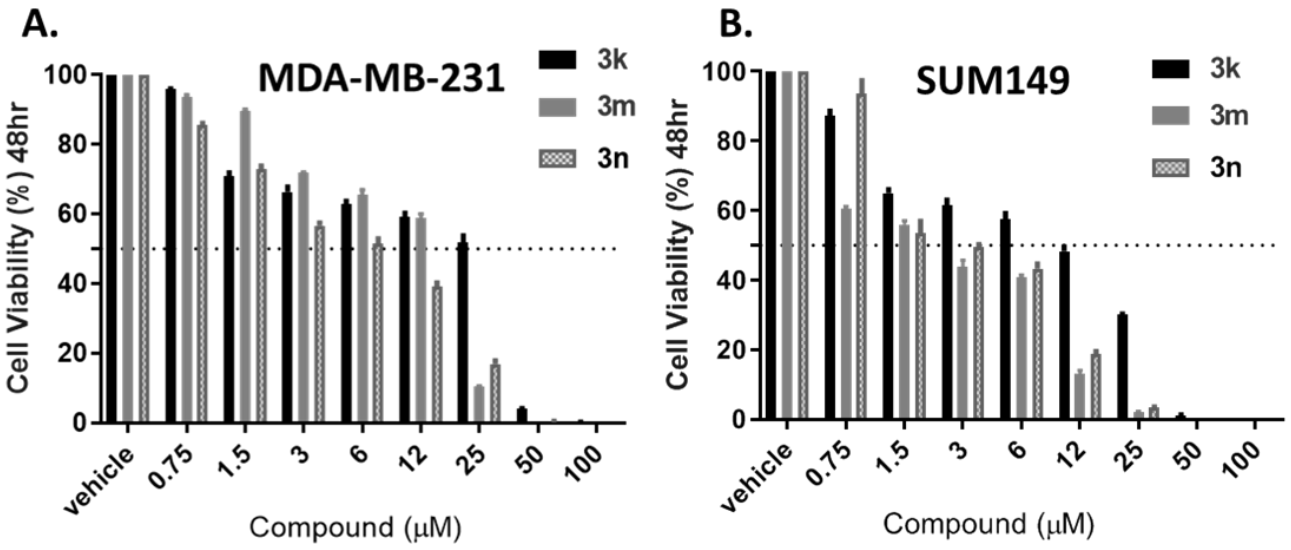

Figure S1.3. Representative graphs of viability/apoptosis assays (48 h) using the Propidium Iodide assay for compound **3k**, **3n**-**3m**. **A.** MDA-MB-231 cell line. **B.** SUM149 cell line. Bars represent mean +/- SEM of at least three biological replicates.

3. Microscopy and Proteomic studies

Microscopy with MDA-MB-231 and SUM149:

Cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and maintained according to proper sterile cell culture practices.[1] Human triple negative breast cancer cell lines MDA-MB-231 and SUM149 were cultured in Dulbecco's modified Eagle's medium phenol free (DMEM; Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum (FCS) and 1 mg/mL penicillin–streptomycin (Invitrogen, Paisley, UK) and F10 Nutrient mixture (Gibco 12390-035) supplemented with 10% fetal bovine serum (FBS), 1 µg/mL insulin, 1% penicillin–streptomycin (Invitrogen, Paisley, UK) and 2 µM cortisol, respectively. For plasmid transfection, cells were plated in 6-well flat bottom cell culture dishes (Corning™ Costar) at a density of 1.5×10^5 cells per well for MDA-MB-231 and 2.5×10^5 cells for SUM149. Cells were transfected with 150 ng/µL mRuby-peroxisomes-2 plasmid DNA (addgene # 54840) mediated by FugeneHD transfecting reagent according to the manufacturer's instructions (Promega USA) and incubated for 12 h before replacing the culture medium with fresh one. After 24h, the transfected cells were subcultured 24 h in 60-mm dishes and maintained in conditioned medium supplemented with G418 (50-250 µg/mL; Geneticin®, Life technology, USA). After three passages cells were sorted by Flow cytometry and maintained in conditioned medium. For RFP-Golgi and GFP-peroxisomes transiently transfected cells (MDA-MB-231 or SUM149) were generated via BacMam technology, namely CellLight™ Golgi-RFP, BacMam 2.0 (Cat # C10593, Invitrogen, USA) and CellLight™ Peroxisome-GFP, BacMam 2.0 (Cat# C10604, Invitrogen, USA) according to the manufacturer.

Confocal microscopy co-localisation studies were conducted in 8 well chambered coverslips (ibidi GmbH µ-slide # 80826, Martinsried, Germany) for MDA-MB-231 and SUM149. Coverslips were first coated with 0.1 % Gelatin for 30 minutes for better cell adherence. Cells were then plated in phenol red free medium at a density of 4×10^4 cells per well and incubated at 37 °C overnight. Then the cells were treated with 1-10 µM of EP-probe and/or organelle tracker for 1 h at the following final concentrations: 1 µM ER-Tracker™ Blue-White DPX (E12353, Invitrogen); 100 nM LysoTracker™ Red (L7528, Invitrogen); 250 nM MitoTracker™ red (M22425, Invitrogen); 250 nM MitoTracker™ Deep Red FM (M22426, Invitrogen); 1 µM MitoTracker™ Green FM (M7514, Invitrogen). Thirty minutes after addition of the probe and tracking dyes, nuclear stain Hoechst33342 (H3570, Invitrogen) was added to a final concentration of 500 nM. After incubation for a total of 1 h, cells were washed twice with fresh medium, followed by live imaging on a confocal imaging system (Marianas CSU-X spinning disk configured with a Zeiss Axio Observer microscope with 6 diode lasers, at 63x magnification and resolution of 512x512 pixels). Representative images of results obtained with SUM149 cells generated by confocal microscopy are shown in Figure **S1.4**.

To verify that the Bodipy labelled reagents are not directly responsible for the observed results, live-cell-washout experiments using Ergosterol peroxide-BodipyFL (**3m** green) and Ergosterol peroxide-Bodipy630/650-X (**3n** Deep Red) in MDA-MB-231 were conducted along with their respective control compounds (esters of BodipyFL and Bodipy630/650-X). Cells were treated with probes and control compounds for 30 minutes at a final concentration of 10 µM, followed by addition of the nuclear stain Hoechst33342. After 30 min further incubation cells were washed and imaged by confocal microscopy. Results are shown in Figures **S1.41 and S1.42**. Control compounds did not accumulate inside the cells (Figures **S1.41A and S1.42A**), whereas staining with compounds **3m** and **3n** is seen inside the cells (Figures **S1.41B and S1.42B**) respectively. Overall the data indicated that compound **3** did not accumulate in the peroxisome as expected due to its peroxide nature.

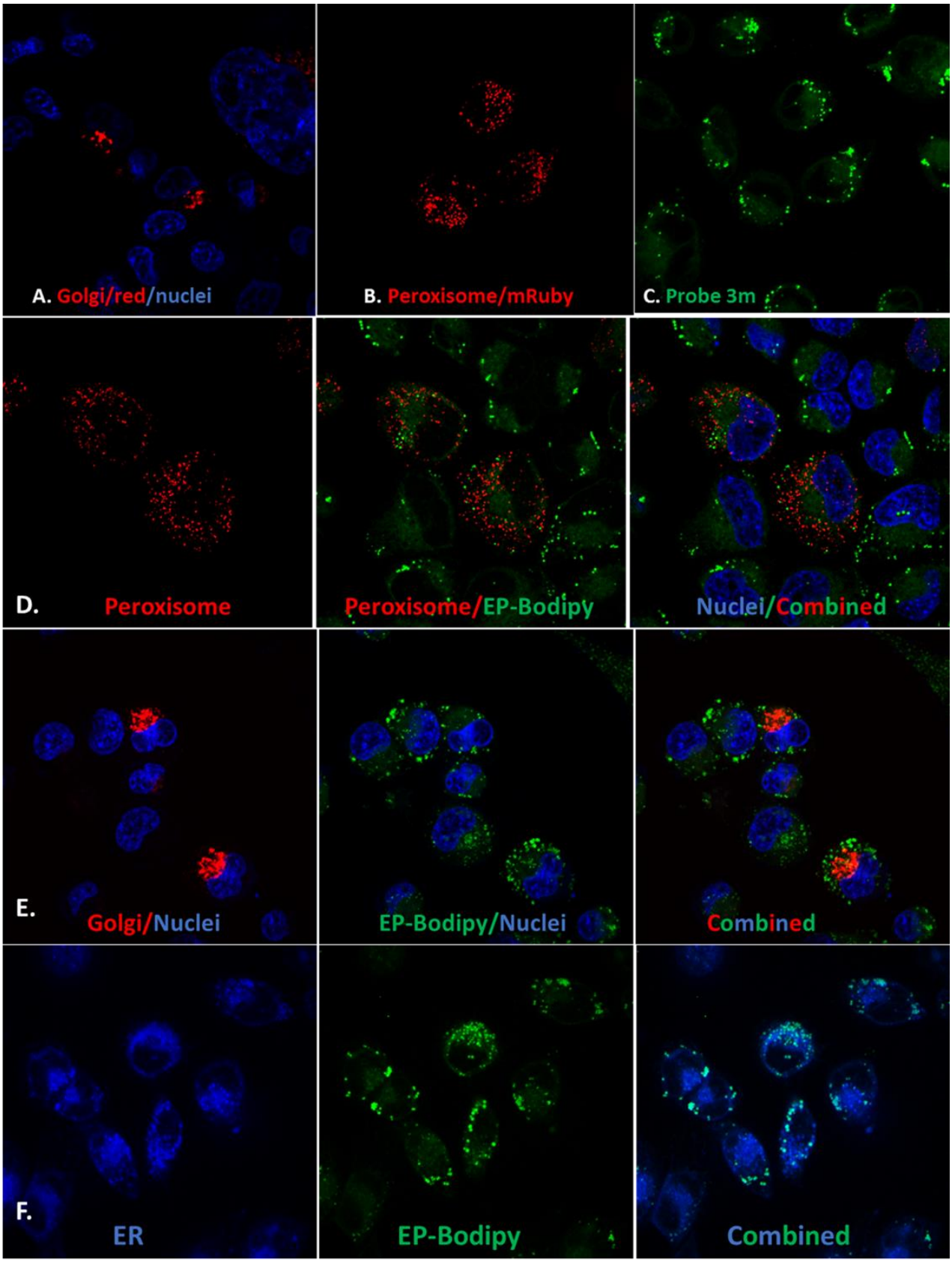

Figure S1.4. Representative images of co-localisation studies in SUM149 cells with probe **3m** (EP-

BodipyFL). **A.** Transiently transfected Golgi-RFP SUM149 cells. **B.** Stable mRuby-peroxisomes-2 SUM149. **C. 3m** BodipyFL. **D.** Peroxisome stable transfected cells, co-localisation with **3m**. **E.** RFP-Golgi transfected cells co-localisation with **3m**. **F.** Endoplasmic reticulum Tracker, co-localisation with **3m**.

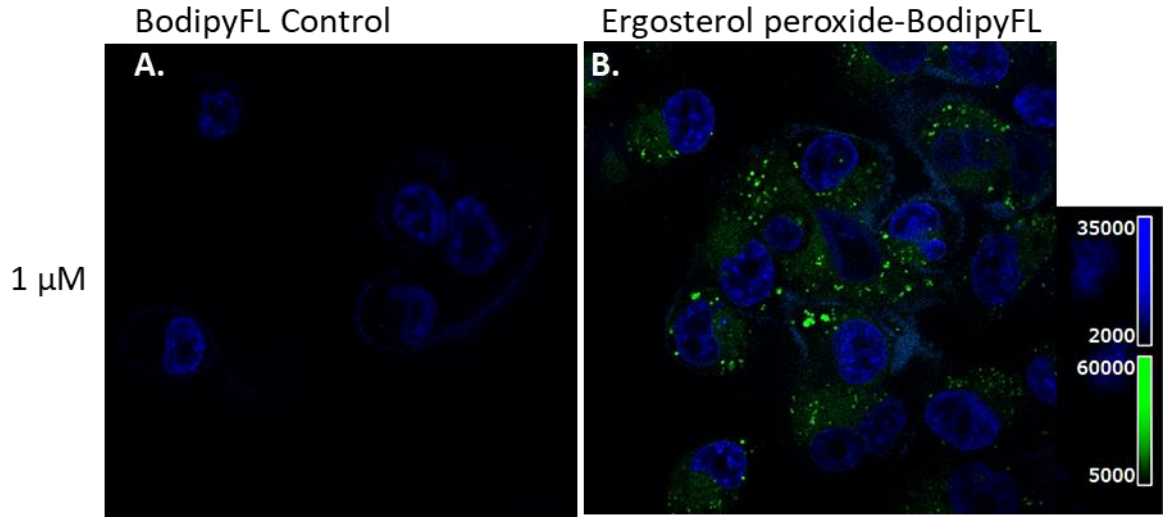

Figure S1.41. Representative images of washout experiment of BodipyFL methyl ester control and Ergosterol peroxide-BodipyFL probe (**3m**) both at 1 µM using MDA-MB-231 cellular model and nuclear stain Hoechst33342. **A.** No fluorescence of the control compound is observed after washing. **B.** Fluorescence of compound **3m** is observed after washing indicating intracellular accumulation.

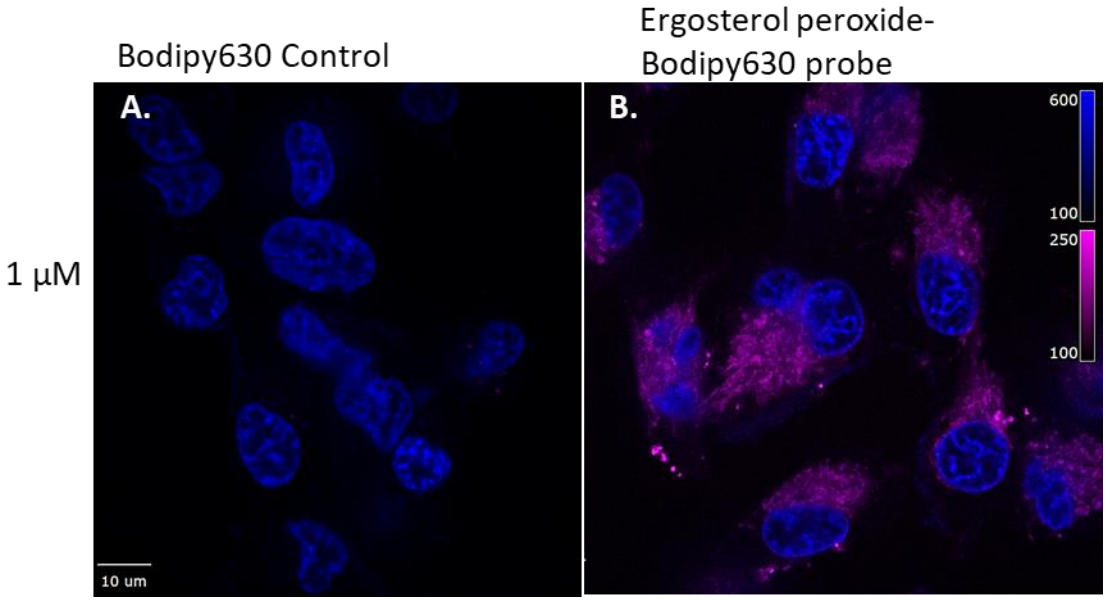

Figure S1.42. Representative washout experiment of BODIPY™ 630 ester control and Ergosterol peroxide-Bodipy630 probe (**3n**) both at 1 µM using MDA-MB-231 cellular model and nuclear stain Hoechst33342. **A.** No fluorescence of the ester control compound is observed after washing. **B.** Fluorescence of compound **3n** is observed after washing indicating intracellular accumulation.

Pulldown experiments with biotinylated EP in SUM149 and MDA-MB-231 lysates

Cell lysates from both cell lines were prepared with lysis buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate, and 10% (v/v) glycerol) supplemented with one protease inhibitor cocktail tablet (Roche, Indianapolis, IN, USA) and one PhosSTOP™ tablet (Roche) per 10 mL of buffer, and PMSF at 0.2 mM final concentration. Protein concentrations were determined by the BCA method (Pierce assay kit) and lysates were adjusted to a protein concentration of 1 mg/ml for pull downs.

Dynabeads™ M-280 Streptavidin (200 µL of resuspended beads per sample) were washed 2x with lysis buffer using a magnetic concentrator and resuspended in 200 µL lysis buffer. Biotinylated probes were then added to a final concentration of 1 µM and bound to the beads for 1 h at room temperature under rotation. Beads were then washed 3x with lysis buffer to remove excess probe using a magnetic concentrator. Beads were then resuspended in 200 µL of cell lysate and incubated on a rotator for 3 h at 4 °C. Beads were then concentrated with a magnetic concentrator for complete removal of the supernatant and washed once with 200 µL lysis buffer. After removal of the supernatant, 50 µL 2x LDS sample buffer (Invitrogen # NP0007) and 2 µL saturated biotin solution was added to the beads and after resuspension, proteins were eluted from the beads by heating to 95°C for 10 minutes. The eluates were then loaded on a 10% NuPage Bis-Tris polyacrylamide gel (Invitrogen) and electrophoresed until the samples had completely migrated into the gel. The gel was then stained with Simply Safe blue protein gel stain (Invitrogen) for visualization of the protein bands (see Figure **S1.5**) before cutting the bands out of the gel.

The proteins in the gel bands are reduced with dithiothreitol to break disulfide bonds followed by alkylation of cysteine residues by iodoacetamide to allow the recovery of cysteine-containing peptides. The gel bands are then washed, dried down in a speed vacuum, and rehydrated with a buffer containing a protease to allow the protease to enter the gel. For routine protein identification, trypsin is used for overnight proteolysis. The next day digested samples are acidified, and the peptides are extracted multiple times. The extracts are pooled, dried down and reconstituted in a small volume. The peptide samples are loaded on a nanoscale capillary reverse phase C_{18} column and separated by gradient elution (~80 min) on a Thermo EasynLC 1000 HPLC system. The eluted peptides are ionized by electrospray ionization and detected by an inline mass spectrometer (Thermo Elite). The MS spectra are collected first, and the 20 most abundant ions are sequentially isolated for MS/MS analysis. This process is cycled over the entire liquid chromatography gradient.

Database searches are performed using Sequest search engine in our in-house SPIDERS software package. All matched MS/MS spectra were filtered by mass accuracy and matching scores to reduce protein false discovery rate to ~1%. Finally, all proteins identified in one gel lane are combined. The total number of spectra, namely spectral counts (SC), matching to individual proteins may reflect their relative abundance in one sample after the protein size is normalized. Moreover, the SC are useful for comparing the level of the same protein across several samples (e.g. control and immunoprecipitated samples).

The spectral counts from the raw data obtained from SUM149 and MDA-MB-231 samples were processed as follows: The ratio SC 100C (specific probe)/ SC Cntrl (nonspecific probe) for both cell lines was calculated for all identified protein species. Proteins with a ratio (specific probe / nonspecific probe) below 3 and a p-value of 0.05 or higher were excluded from Table S2. The remaining proteins were sorted according to the ratio SC 100C/ SC Ctrl from highest to lowest (as shown in Table S2) and a scatter plot was generated by plotting SC 100C/ SC Ctrl vs -log(p-value) for each sample (shown in the manuscript, Figure 6). The sorting was performed using Microsoft Excel, and the scored data was plotted with GraphPad Prism 8.0.2 (GraphPad Software, Inc).

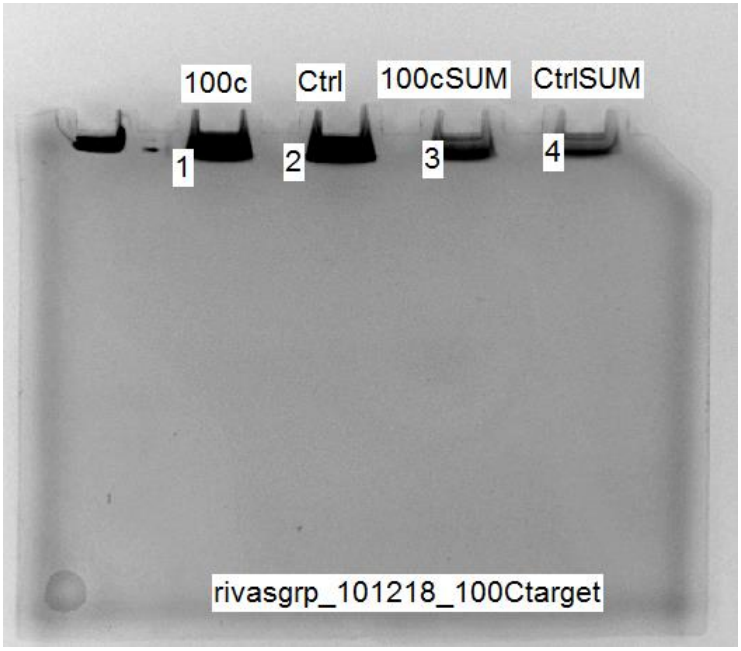

Figure S1.5. Stained SDS-PAGE Gel image of samples from pulldown assay submitted for Mass spectroscopy analysis: **1** (MDA-MB-231, **3k**), **2** (MDA-MB-231, cholesterol-biotin control), **3** (SUM149, **3k**), **4** (SUM149, cholesterol-biotin control). The numbers indicate excised gel bands for digestion and LC-MS/MS.

A.

Reference	Description	SC Cntrl	SC 100c	TP Cntrl	TP 100c	Mass (KD)	Abun-dance	p-value	SC 100C/ SC Ctrl
sp|P00338|LDHA_HUMAN	L-lactate dehydrogenase A chain OS=Hom	0.1	17	0	8	37	107.73	2.13E-06	170
sp|P78527|PRKDC_HUMAN	DNA-dependent protein kinase catalytic	0.1	16	0	10	469	3.84	4.35E-06	160
sp|P15311|EZRI_HUMAN	Ezrin OS=Homo sapiens GN=EZR PE=1 SV=4	0.1	15	0	7	69	43.97	8.92E-06	150
sp|Q5T4S7|UBR4_HUMAN	E3 ubiquitin-protein ligase UBR4 OS=Hom	0.1	14	0	10	573	2.09	1.83E-05	140
sp|P50395|GDIB_HUMAN	Rab GDP dissociation inhibitor beta OS=H	0.1	11	0	6	51	53.33	0.00016	110
sp|P23396|RS3_HUMAN	40S ribosomal protein S3 OS=Homo sapie	0.1	8	0	6	27	71.23	0.001442	80
sp|P04083|ANXA1_HUMAN	Annexin A1 OS=Homo sapiens GN=ANXA1	0.1	7	0	5	39	60.74	0.003026	70
sp|P22314|UBA1_HUMAN	Ubiquitin-like modifier-activating enzyme	0.1	7	0	6	118	27.17	0.003026	70
sp|O43175|SERA_HUMAN	D-3-phosphoglycerate dehydrogenase OS	0.1	7	0	5	57	6.18	0.003026	70
sp|P62258|1433E_HUMAN	14-3-3 protein epsilon OS=Homo sapiens	0.1	6	0	3	29	68.6	0.006394	60
sp|P31150|GDIA_HUMAN	Rab GDP dissociation inhibitor alpha OS=	0.1	6	0	4	51	19.78	0.006394	60
sp|P35241|RADI_HUMAN	Radixin OS=Homo sapiens GN=RDX PE=1 S	0.1	6	0	3	69	24.81	0.006394	60
sp|Q16658|FSCN1_HUMAN	Fascin OS=Homo sapiens GN=FSCN1 PE=1	0.1	6	0	4	54	5.5	0.006394	60
sp|O14980|XPO1_HUMAN	Exportin-1 OS=Homo sapiens GN=XPO1 PE	0.1	6	0	2	123	8.11	0.006394	60
sp|P07814|SYEP_HUMAN	Bifunctional glutamate/proline--tRNA liga	0.1	6	0	5	170	4.99	0.006394	60
sp|P08865|RSSA_HUMAN	40S ribosomal protein SA OS=Homo sapie	0.1	6	0	3	33	63.96	0.006394	60
sp|P30153|2AAA_HUMAN	Serine/threonine-protein phosphatase 2	0.1	5	0	2	65	19.15	0.013628	50
sp|P62826|RAN_HUMAN	GTP-binding nuclear protein Ran OS=Hom	0.1	5	0	4	24	32.78	0.013628	50
sp|P55884|EIF3B_HUMAN	Eukaryotic translation initiation factor 3	0.1	5	0	3	92	10.82	0.013628	50
sp|P07237|PDIA1_HUMAN	Protein disulfide-isomerase OS=Homo sa	0.1	5	0	4	57	35.04	0.013628	50
sp|Q9UQ80|PA2G4_HUMAN	Proliferation-associated protein 2G4 OS=	0.1	5	0	2	44	22.85	0.013628	50
sp|P50454|SERPH_HUMAN	Serpin H1 OS=Homo sapiens GN=SERPINH	0.1	5	0	5	46	32.32	0.013628	50
sp|P06744|G6PI_HUMAN	Glucose-6-phosphate isomerase OS=Hom	0.1	5	0	2	63	22.18	0.013628	50
sp|Q5EBL4|RIPL1_HUMAN	RILP-like protein 1 OS=Homo sapiens GN=	0.1	5	0	3	47	28.67	0.013628	50
sp|Q58FG0|HS905_HUMAN	Putative heat shock protein HSP 90-alpha	0.1	5	0	2	39	28.41	0.013628	50
sp|P06748|NPM_HUMAN	Nucleophosmin OS=Homo sapiens GN=NP	0.1	5	0	4	33	27.64	0.013628	50
sp|P63104|1433Z_HUMAN	14-3-3 protein zeta/delta OS=Homo sapie	0.1	4	0	3	28	63.11	0.029408	40
sp|Q02790|FKBP4_HUMAN	Peptidyl-prolyl cis-trans isomerase FKBP4	0.1	4	0	3	52	6.76	0.029408	40
sp|P40926|MDHM_HUMAN	Malate dehydrogenase, mitochondrial O	0.1	4	0	4	35	35.23	0.029408	40
sp|P30041|PRDX6_HUMAN	Peroxiredoxin-6 OS=Homo sapiens GN=PR	0.1	4	0	2	25	43.96	0.029408	40
sp|Q8NC51|PAIRB_HUMAN	Plasminogen activator inhibitor 1 RNA-bi	0.1	4	0	2	45	27.82	0.029408	40
sp|P52565|GDIR1_HUMAN	Rho GDP-dissociation inhibitor 1 OS=Hom	0.1	4	0	2	23	19.4	0.029408	40
sp|P49368|TCPG_HUMAN	T-complex protein 1 subunit gamma OS=H	0.1	4	0	3	60	26.45	0.029408	40
tr|F8W1N5|F8W1N5_HUMA	Nascent polypeptide-associated comple	0.1	4	0	2	8	89.64	0.029408	40
sp|P13010|XRCC5_HUMAN	X-ray repair cross-complementing protei	0.1	4	0	3	83	9.68	0.029408	40
sp|P36957|ODO2_HUMAN	Dihydrolipoyllysine-residue succinyltran	0.1	4	0	2	49	4.1	0.029408	40
sp|P04843|RPN1_HUMAN	Dolichyl-diphosphooligosaccharide--prot	0.1	4	0	4	69	15.32	0.029408	40
sp|O95373|IPO7_HUMAN	Importin-7 OS=Homo sapiens GN=IPO7 PE	0.1	4	0	4	119	7.54	0.029408	40
sp|P0DME0|SETLP_HUMAN	Protein SETSIP OS=Homo sapiens GN=SETS	0.1	4	0	3	35	28.68	0.029408	40
sp|P26038|MOES_HUMAN	Moesin OS=Homo sapiens GN=MSN PE=1 S	2	15	2	8	68	56.06	0.000795	7.5
sp|P00558|PGK1_HUMAN	Phosphoglycerate kinase 1 OS=Homo sap	2	11	2	7	45	78.5	0.008817	5.5
sp|P25705|ATPA_HUMAN	ATP synthase subunit alpha, mitochondri	4	18	4	10	60	57.77	0.001908	4.5
sp|P23528|COF1_HUMAN	Cofilin-1 OS=Homo sapiens GN=CFL1 PE=1	2	9	2	5	18	143.31	0.028161	4.5
sp|P61978|HNRPK_HUMAN	Heterogeneous nuclear ribonucleoprote	2	9	2	6	51	47.11	0.028161	4.5
sp|P27797|CALR_HUMAN	Calreticulin OS=Homo sapiens GN=CALR P	4	16	3	7	48	87.29	0.005492	4
sp|P04075|ALDOA_HUMAN	Fructose-bisphosphate aldolase A OS=Ho	4	15	3	8	39	91.38	0.009204	3.75
tr|J3QLN6|J3QLN6_HUMAN	Eukaryotic initiation factor 4A-I (Fragmen	3	11	3	4	18	122	0.027488	3.67
sp|P30101|PDIA3_HUMAN	Protein disulfide-isomerase A3 OS=Homo	3	11	3	6	57	30.84	0.027488	3.67
sp|Q15084|PDIA6_HUMAN	Protein disulfide-isomerase A6 OS=Homo	5	17	4	7	48	38.47	0.008542	3.4
sp|P49327|FAS_HUMAN	Fatty acid synthase OS=Homo sapiens GN	14	47	11	23	273	17.38	1.42E-05	3.36
sp|P60842|IF4A1_HUMAN	Eukaryotic initiation factor 4A-I OS=Homo	5	16	5	7	46	87.8	0.013832	3.2
sp|Q00610|CLH1_HUMAN	Clathrin heavy chain 1 OS=Homo sapiens	4	12	4	9	191	32.38	0.04076	3
sp|Q05639|EF1A2_HUMAN	Elongation factor 1-alpha 2 OS=Homo sap	4	12	3	6	50	123.91	0.04076	3

B.

Reference	Description	SC Cntrl	SC 100c	TP Cntrl	TP 100c	Mass (KD)	Abun-dance	p-value	SC 100C/ SC Ctrl
sp|Q5EBL4|RIPL1_HUMAN	RILP-like protein 1 OS=Homo sapiens GN=	0.1	22	0	12	47	28.67	6.02E-08	220
sp|P23588|IF4B_HUMAN	Eukaryotic translation initiation factor 4E	0.1	6	0	3	69	4.34	0.006394	60
sp|P62136|PP1A_HUMAN	Serine/threonine-protein phosphatase P	0.1	5	0	3	37	6.67	0.013628	50
sp|Q07960|RHG01_HUMAN	Rho GTPase-activating protein 1 OS=Hom	0.1	4	0	3	50	3.97	0.029408	40
sp|P35908|K22E_HUMAN	Keratin, type II cytoskeletal 2 epidermal (	0.1	4	0	1	65	4.59	0.029408	40
sp|Q96N66|MBOA7_HUMA	Lysophospholipid acyltransferase 7 OS=H	0.1	4	0	2	53	3.79	0.029408	40
sp|Q99733|NP1L4_HUMAN	Nucleosome assembly protein 1-like 4 OS	0.1	3	0	2	43	4.67	0.064647	30
sp|P50914|RL14_HUMAN	60S ribosomal protein L14 OS=Homo sapi	0.1	3	0	2	23	6.41	0.064647	30
sp|P15924|DESP_HUMAN	Desmoplakin OS=Homo sapiens GN=DSP P	0.1	3	0	3	332	1.81	0.064647	30
sp|Q5T4S7|UBR4_HUMAN	E3 ubiquitin-protein ligase UBR4 OS=Hom	2	8	2	5	573	2.09	0.049601	4

Table S2. Analysis of the pulldown experiment for 2 models of breast cancer. SC control refers to the cholesterol biotinylated control, 0.1 was included instead of 0 for calculation purposes. However, it means not detected under the evaluated conditions. **A**. Results for SUM149 cells treated with EP-biotin (**3k**). **B**. Results for MDA-MB-231 treated cells with EP-biotin (**3k**).

4. NMR Spectra

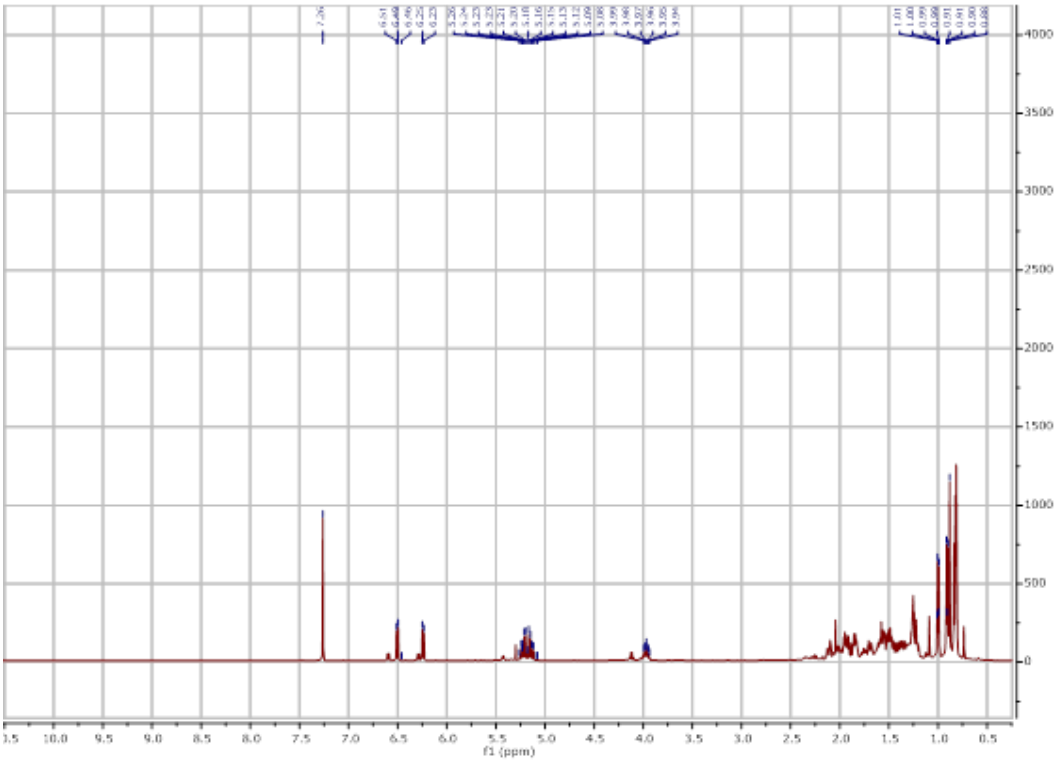

Figure S2.1 ^{1}H NMR Spectra of compound **2**.

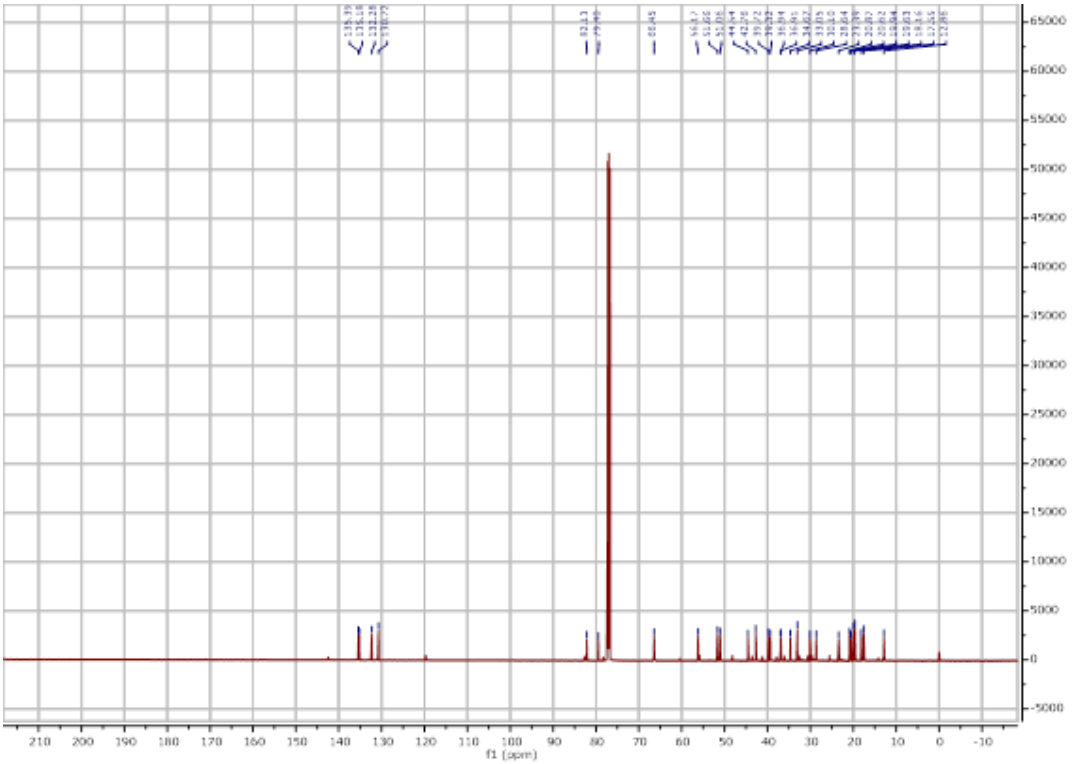

Figure S2.2 ^{13}C NMR Spectra of compound **2**.

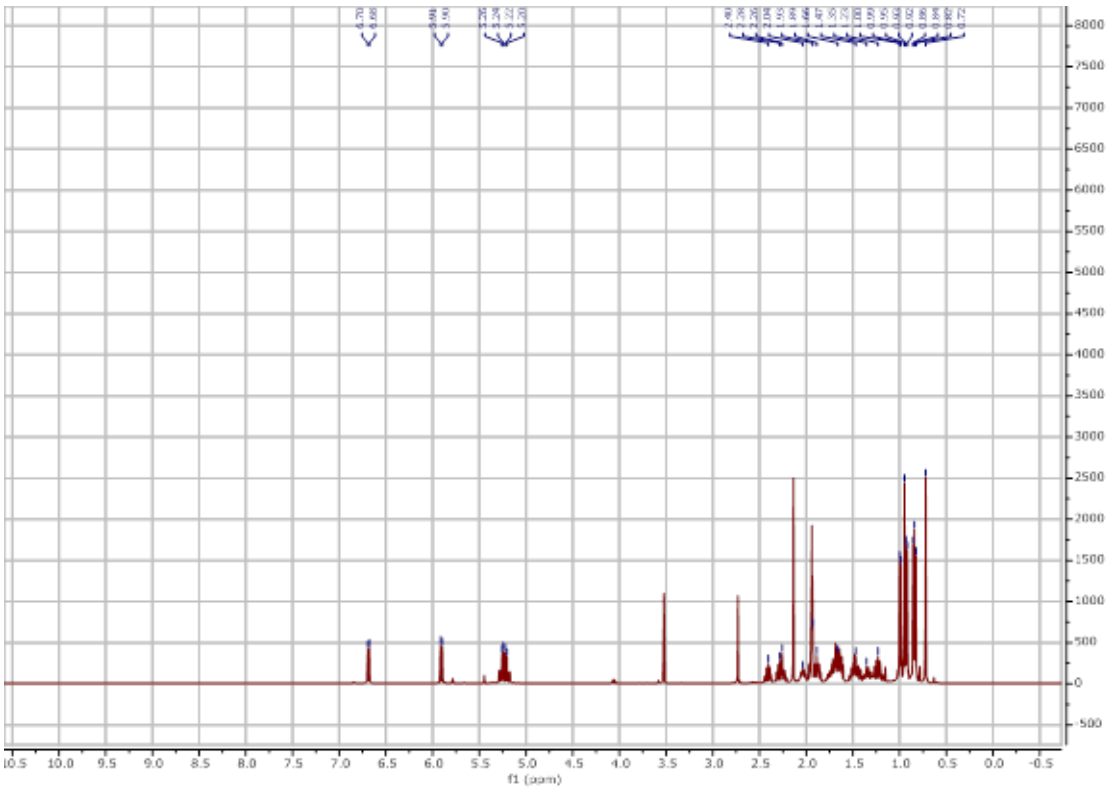

Figure S3.1 ^{1}H NMR Spectra of compound **3a**.

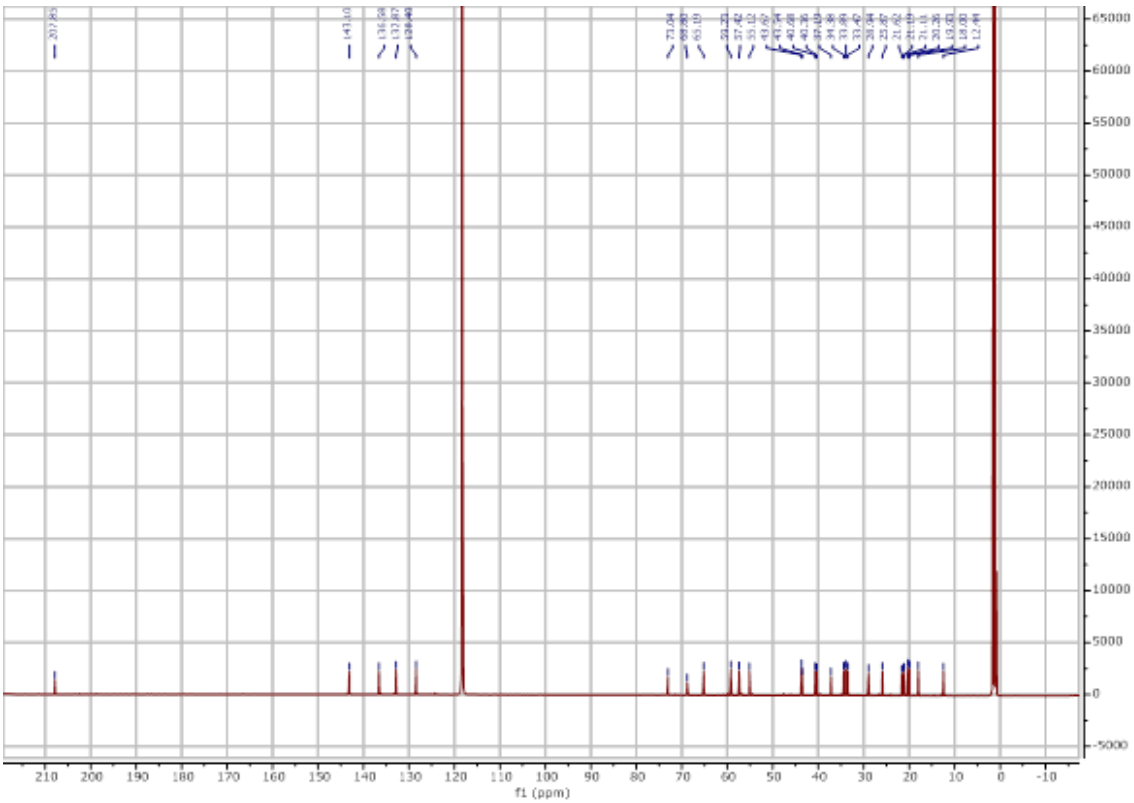

Figure S3.2 ^{13}C NMR Spectra of compound **3a**.

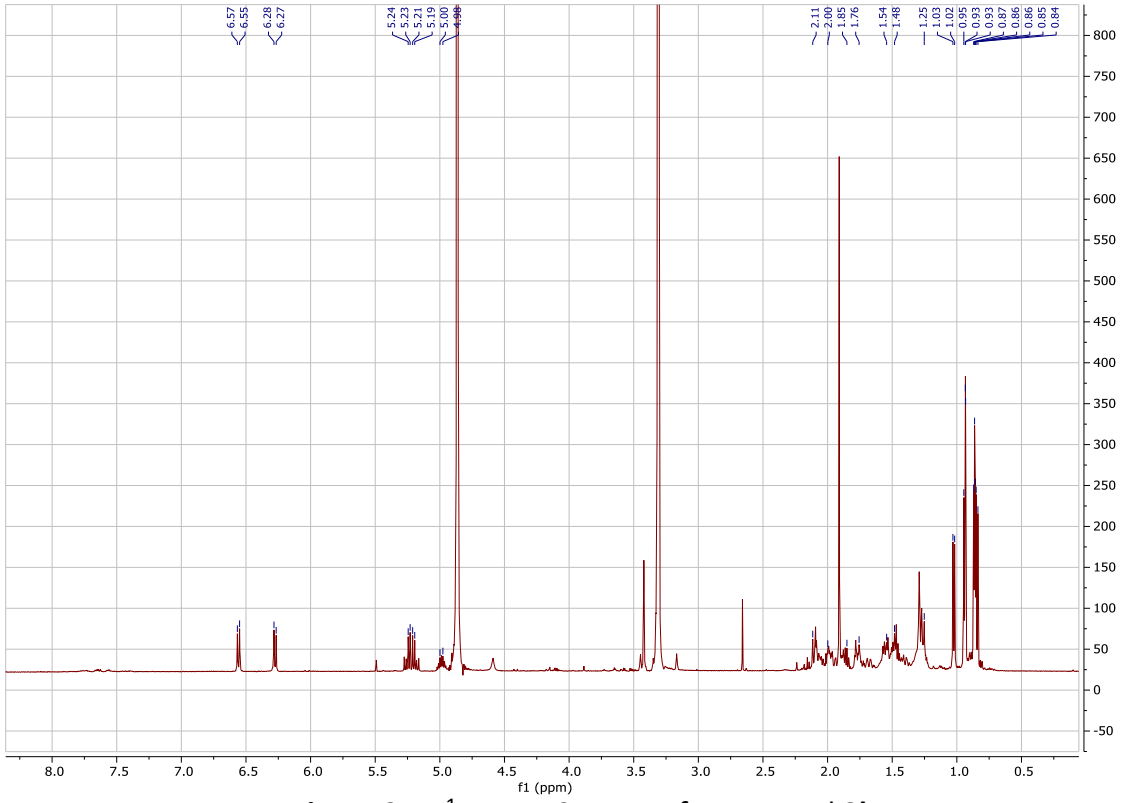

Figure S4.1 ^{1}H NMR Spectra of compound **3b**.

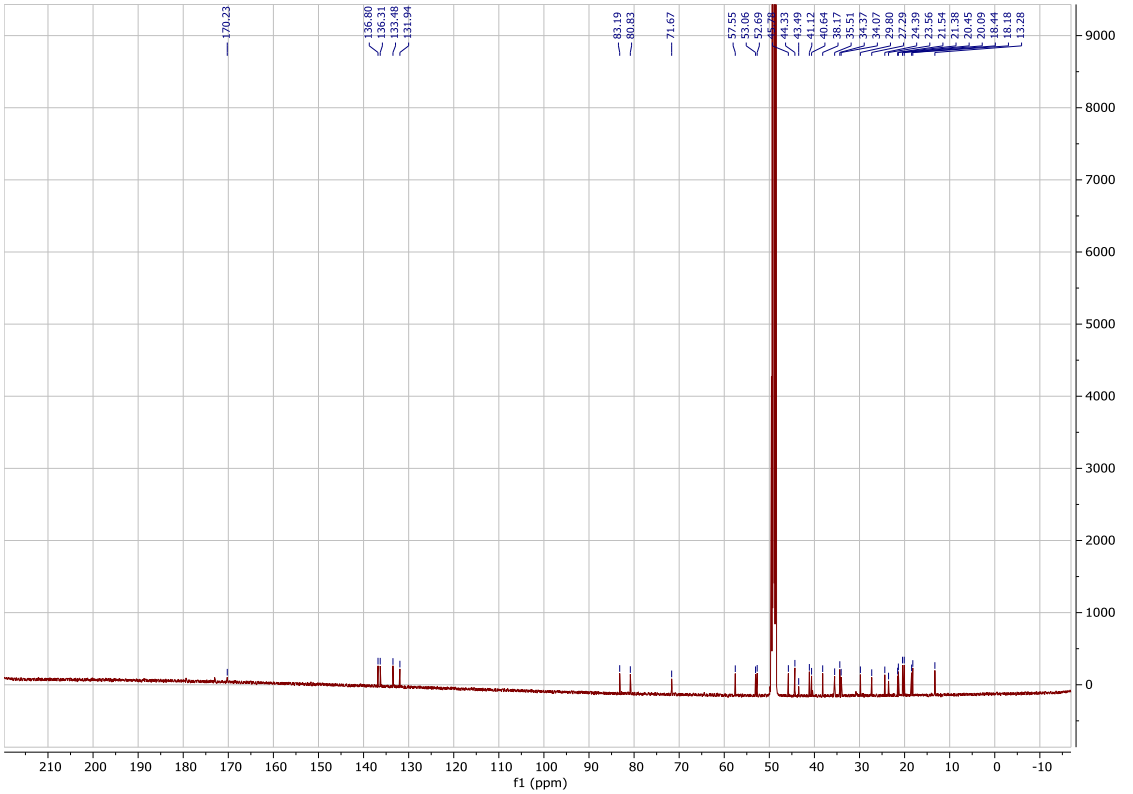

Figure S4.2 ^{13}C NMR Spectra of compound **3b**.

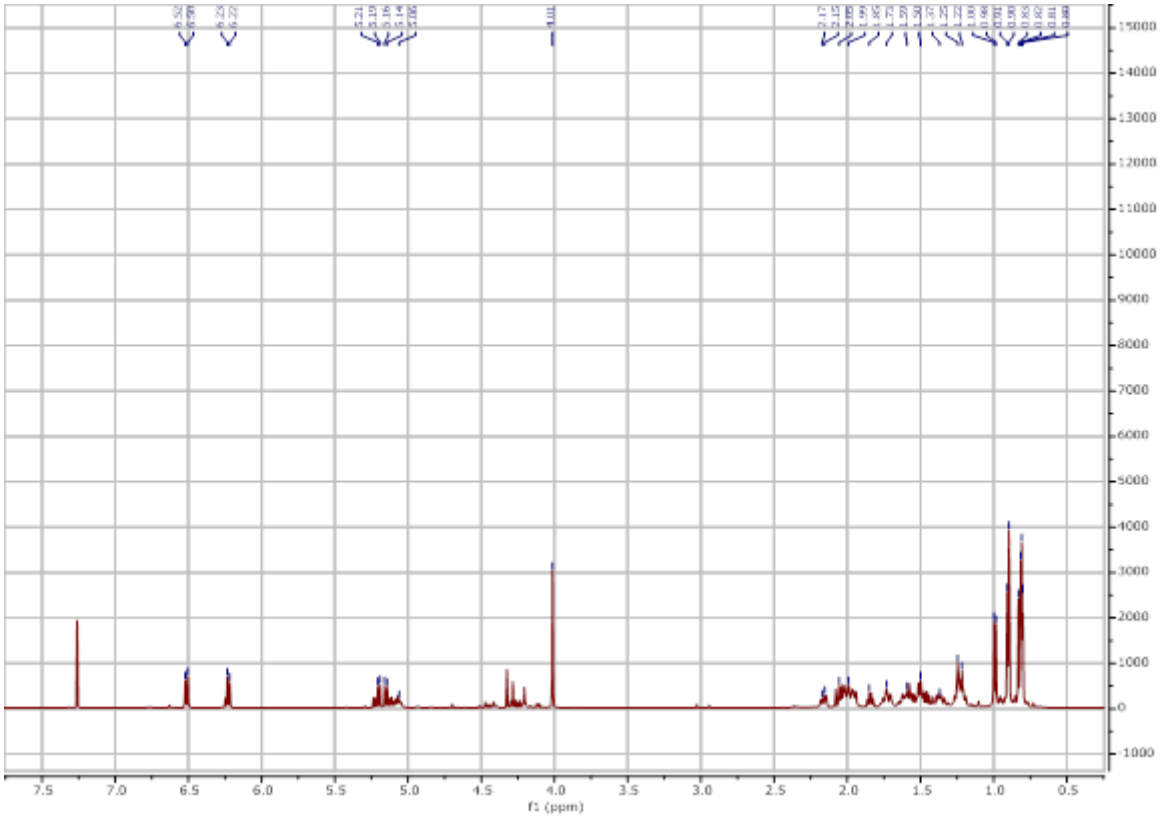

Figure S5.1 ^{1}H NMR Spectra of compound **3c**.

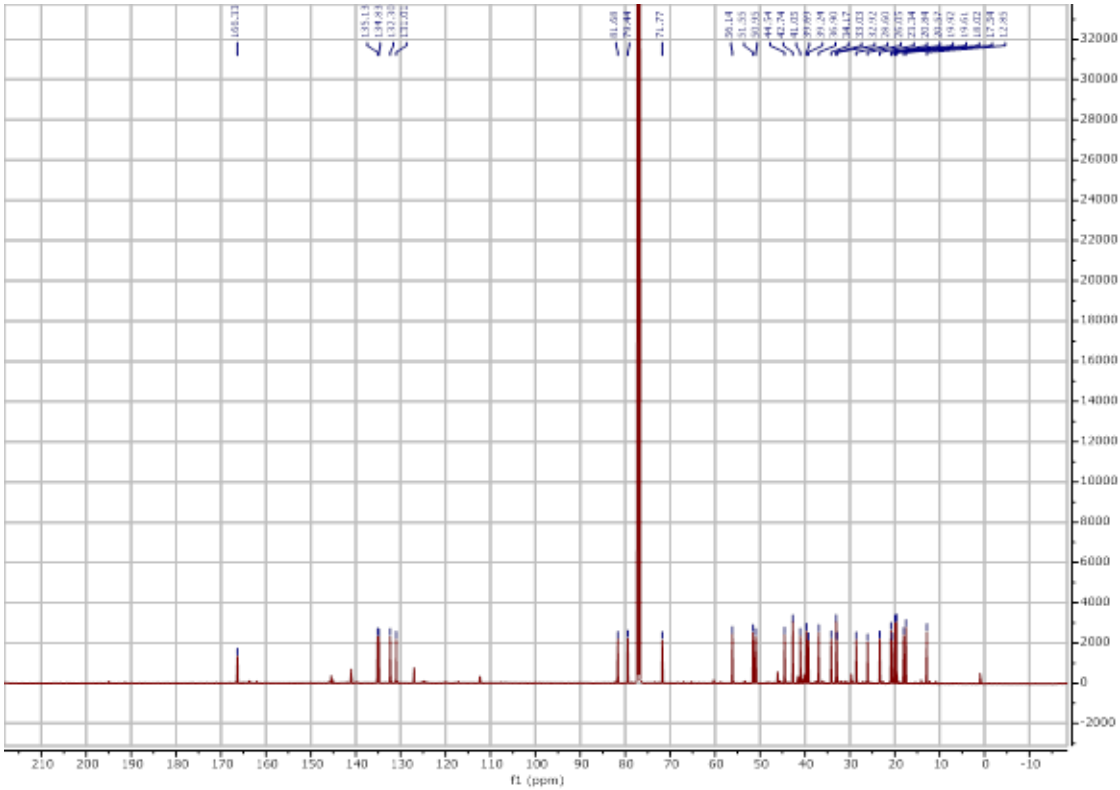

Figure S5.2 ^{13}C NMR Spectra of compound **3c**.

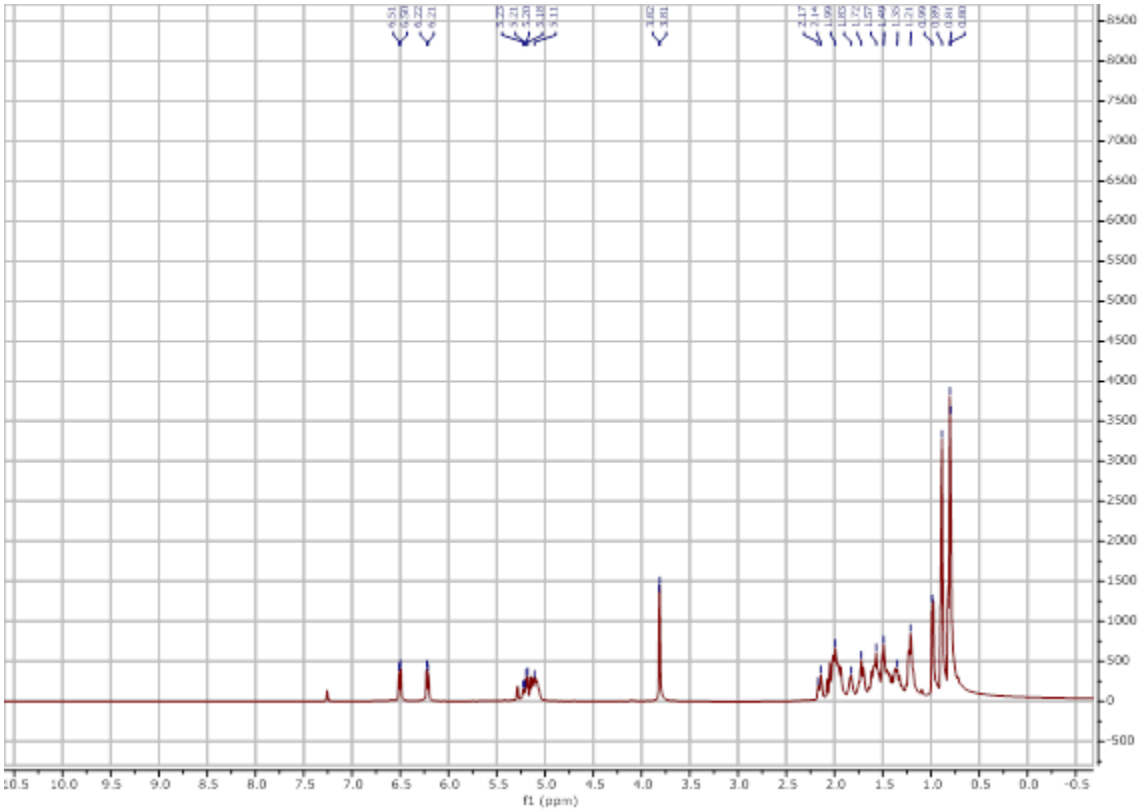

Figure S6.1 ^{1}H NMR Spectra of compound **3d**.

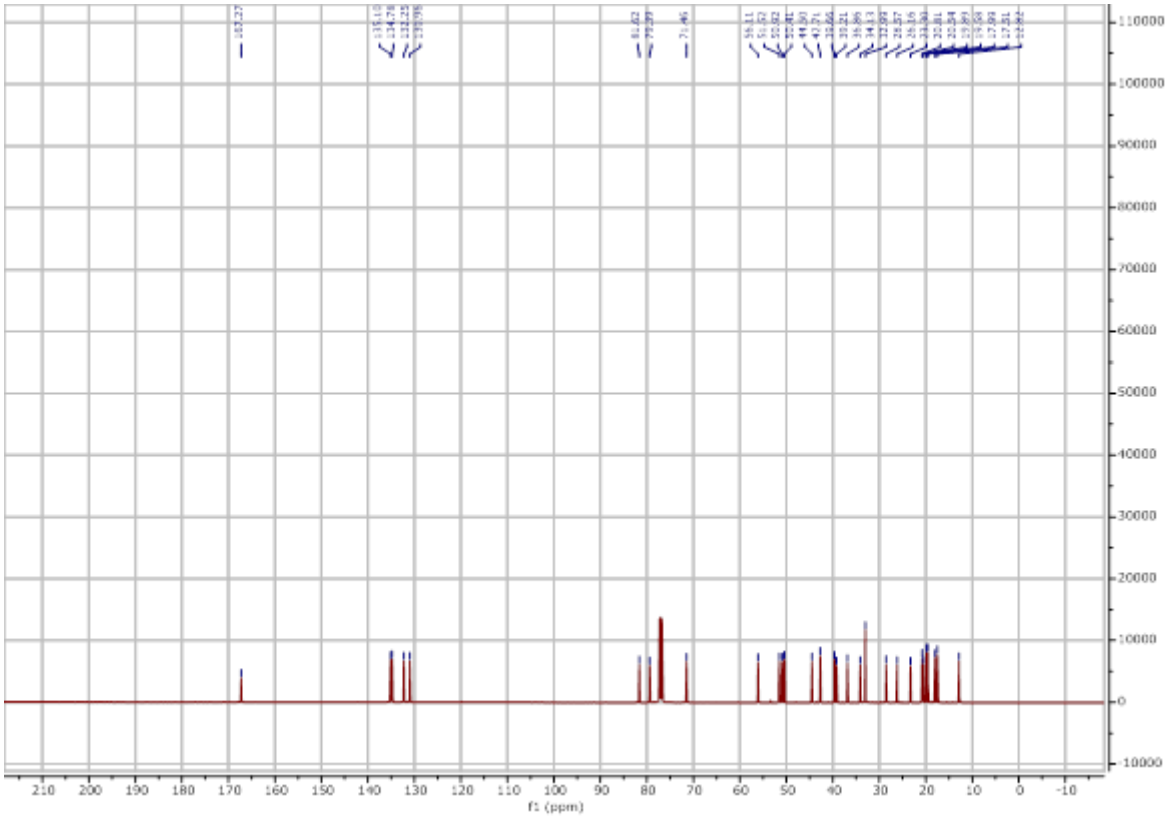

Figure S6.2 ^{13}C NMR Spectra of compound **3d**.

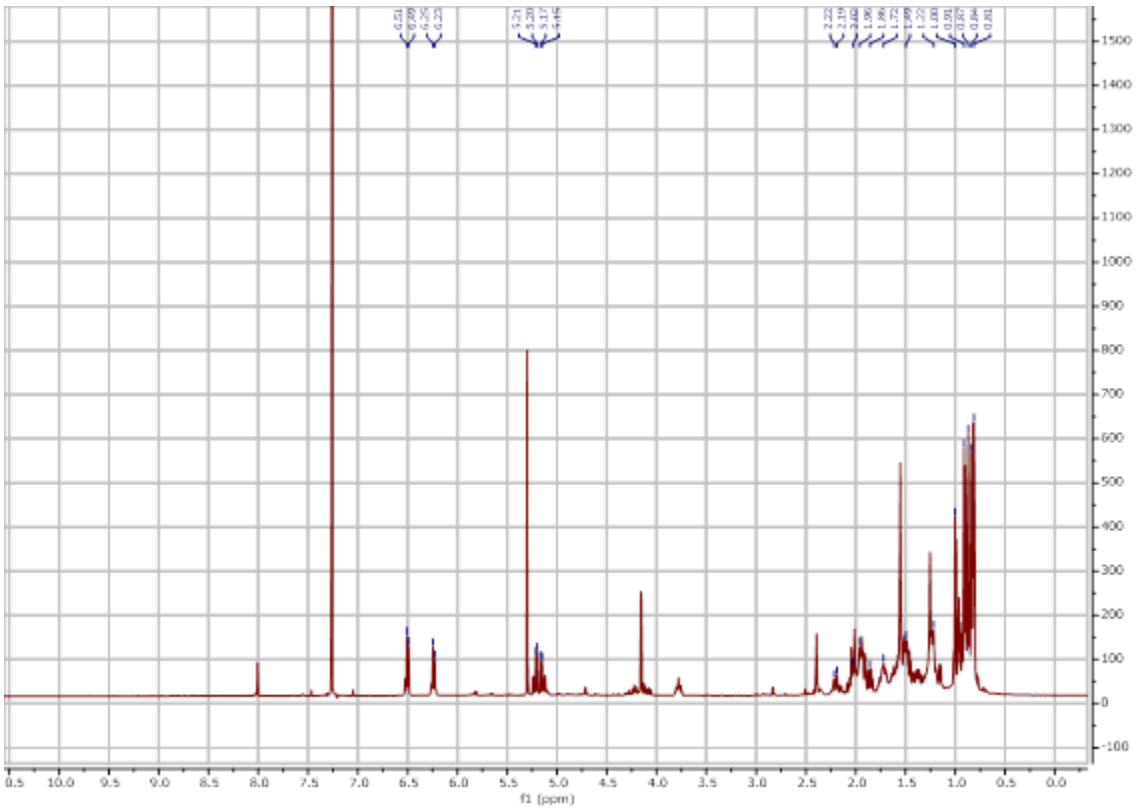

Figure S7.1 ^{1}H NMR Spectra of compound **3e**.

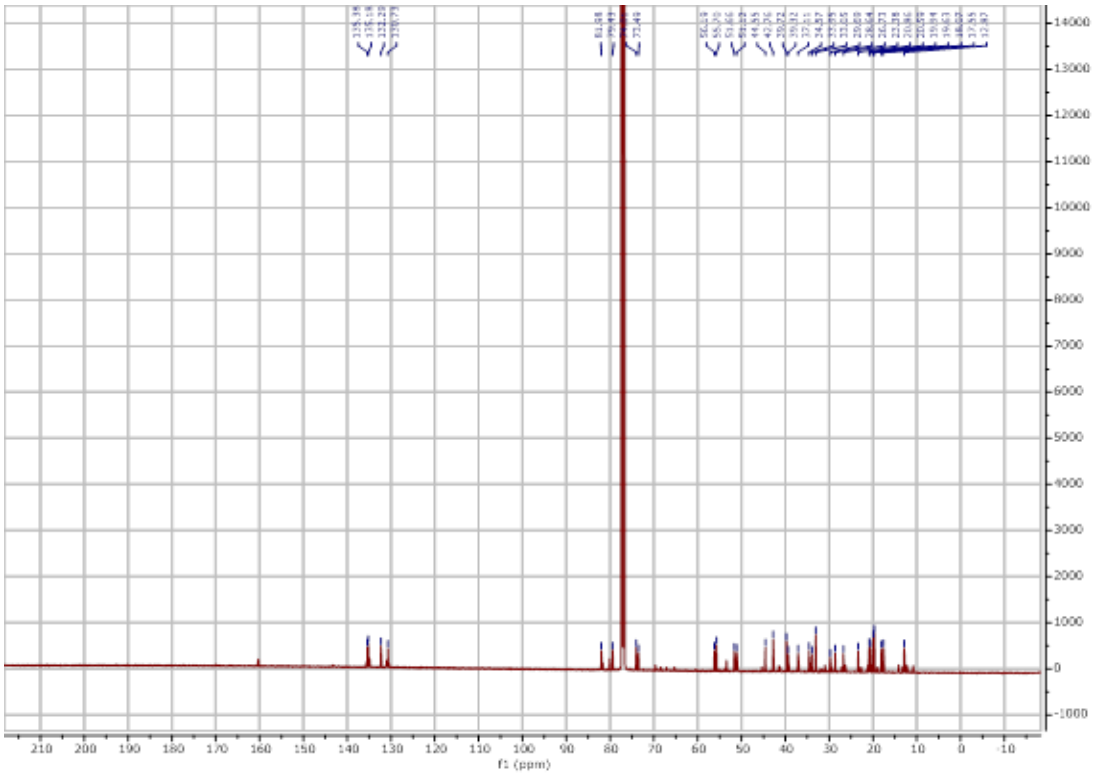

Figure S7.2 ^{13}C NMR Spectra of compound **3e**.

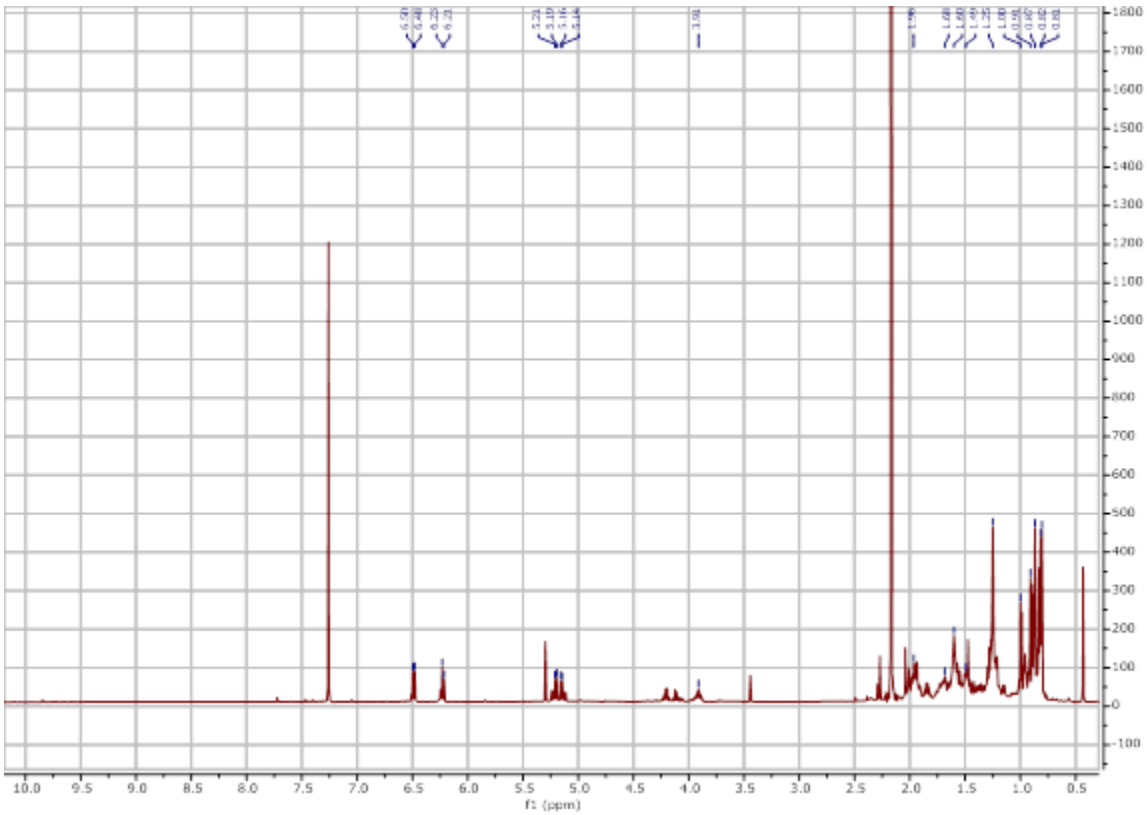

Figure S8.1 ^{1}H NMR Spectra of compound **3f**.

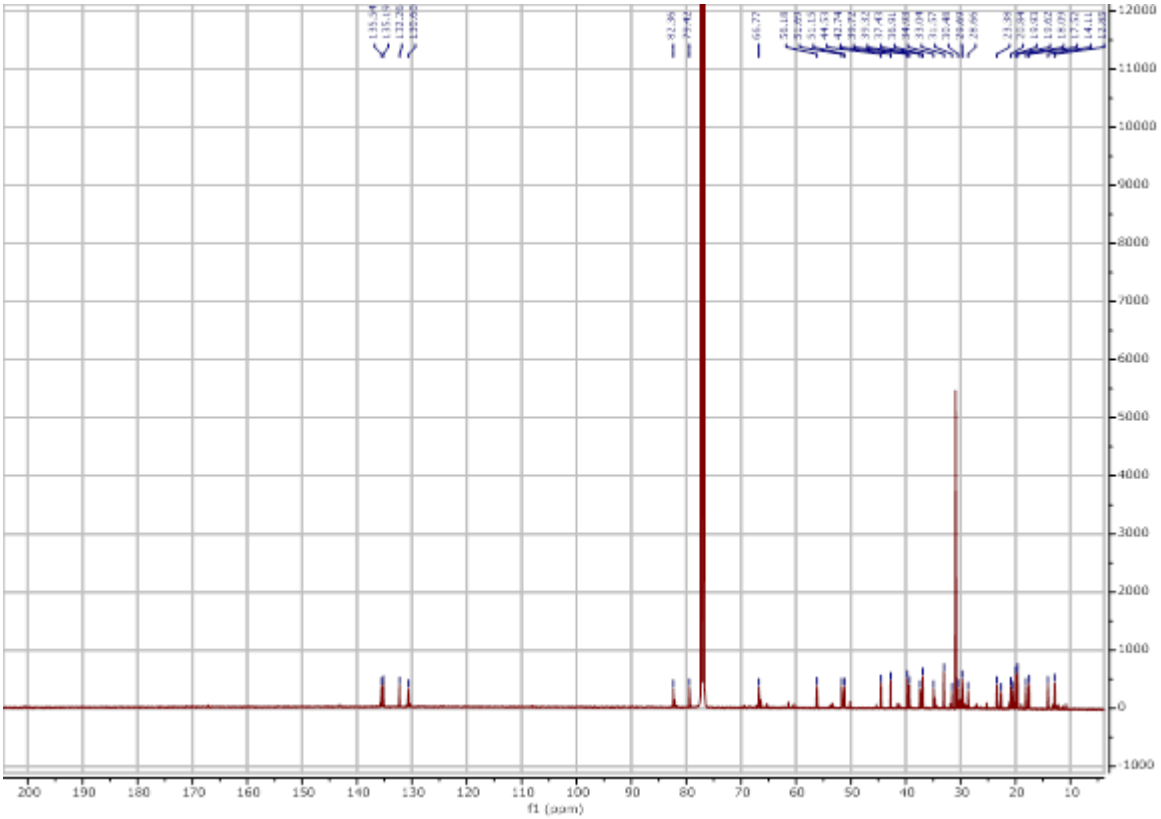

Figure S8.2 ^{13}C NMR Spectra of compound **3f**.

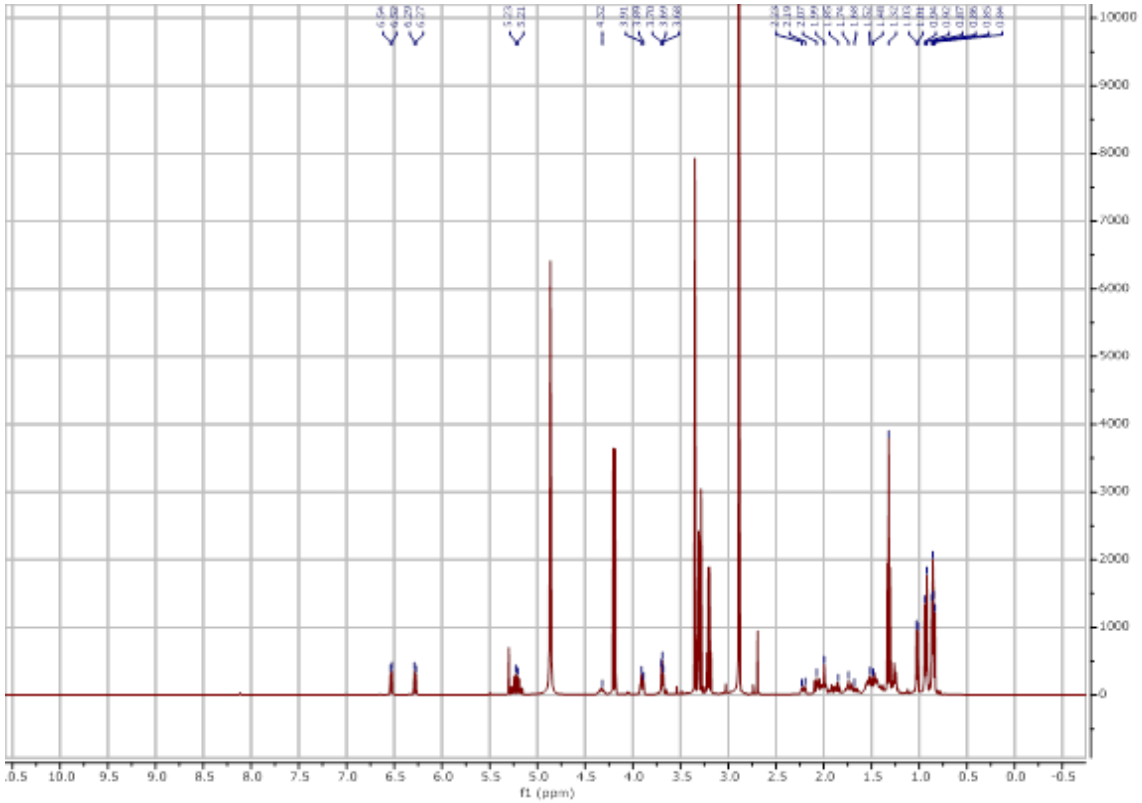

Figure S9.1 ^{1}H NMR Spectra of compound **3h**.

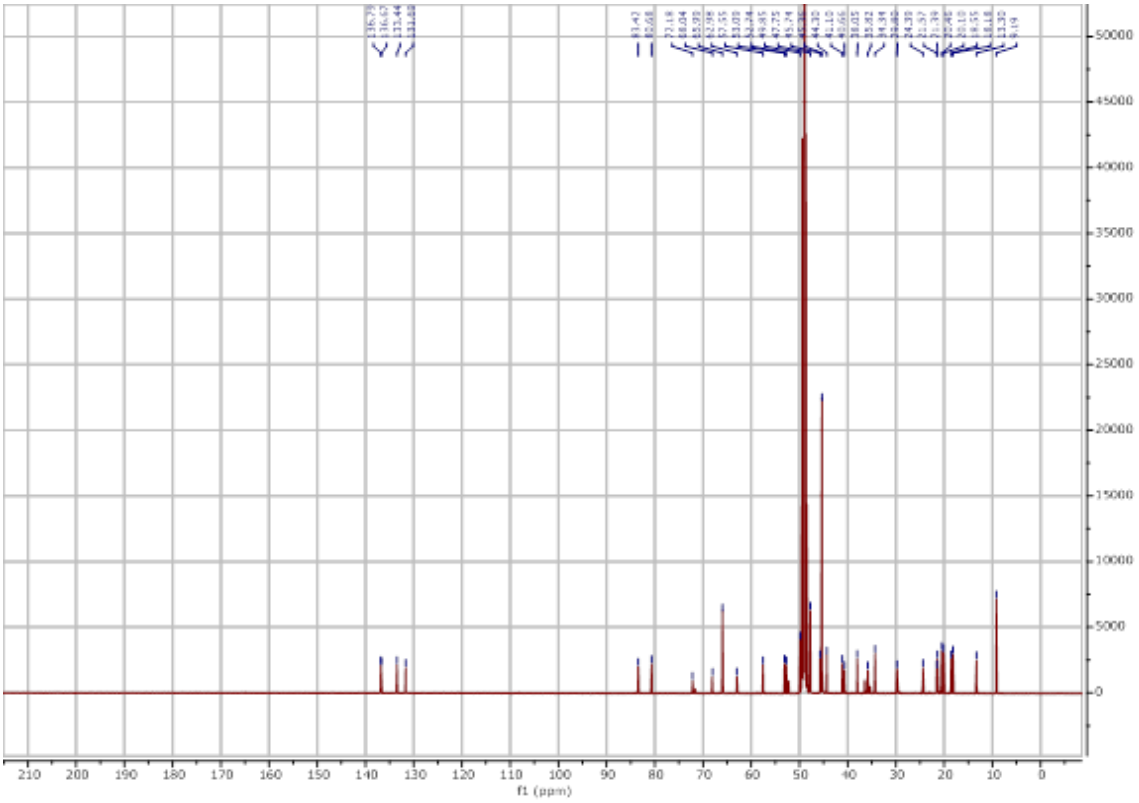

Figure S9.2 ^{13}C NMR Spectra of compound **3h**.

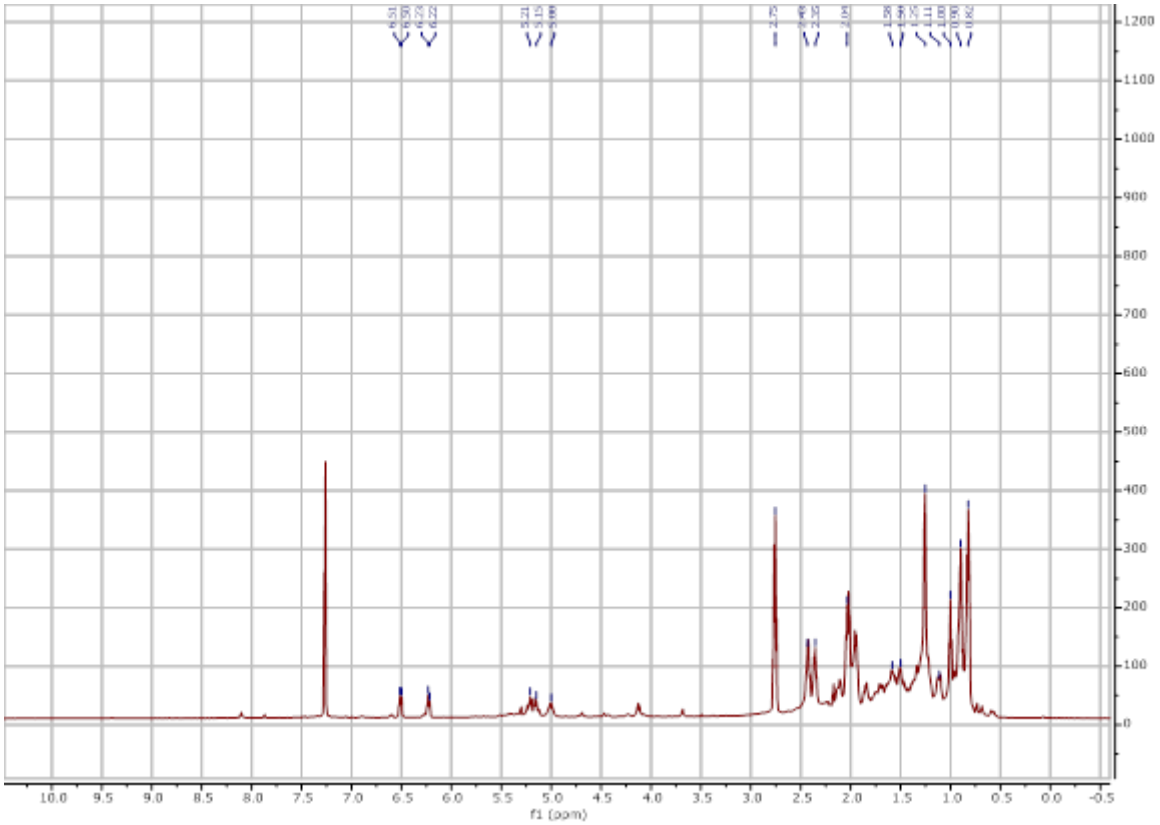

Figure S10.1 ^{1}H NMR Spectra of compound **3i**.

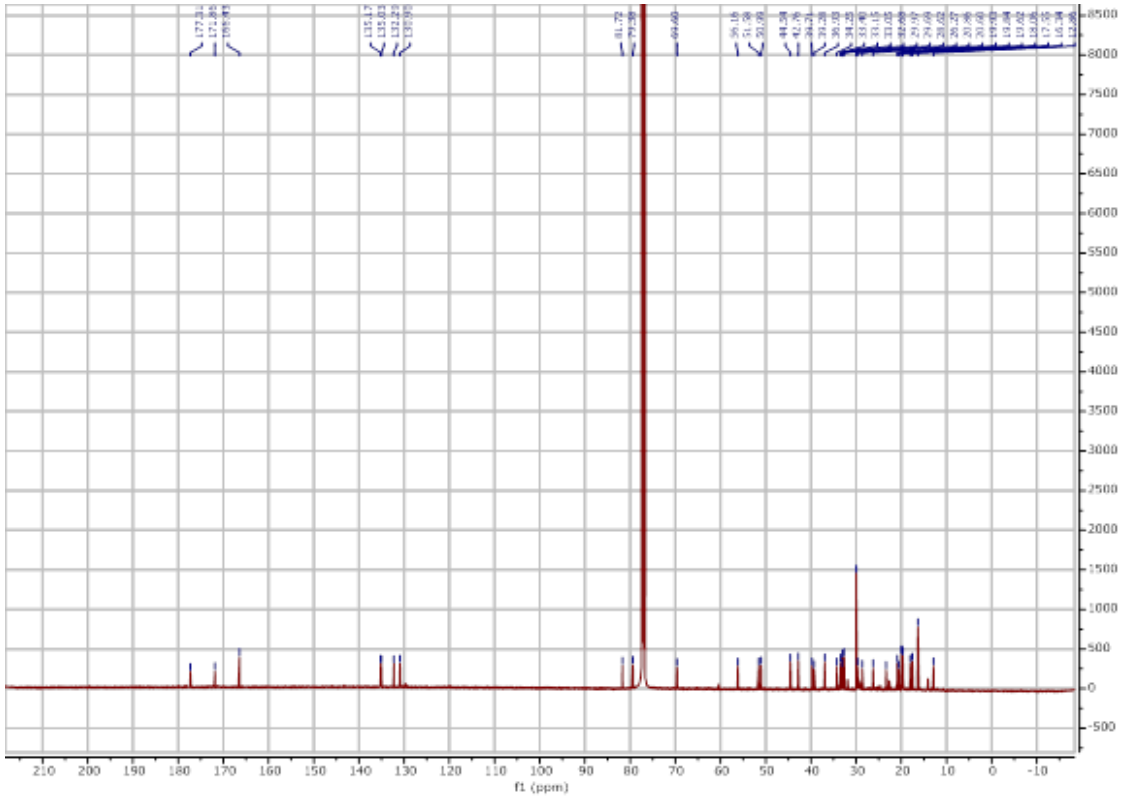

Figure S10.2 ^{13}C NMR Spectra of compound **3i**.

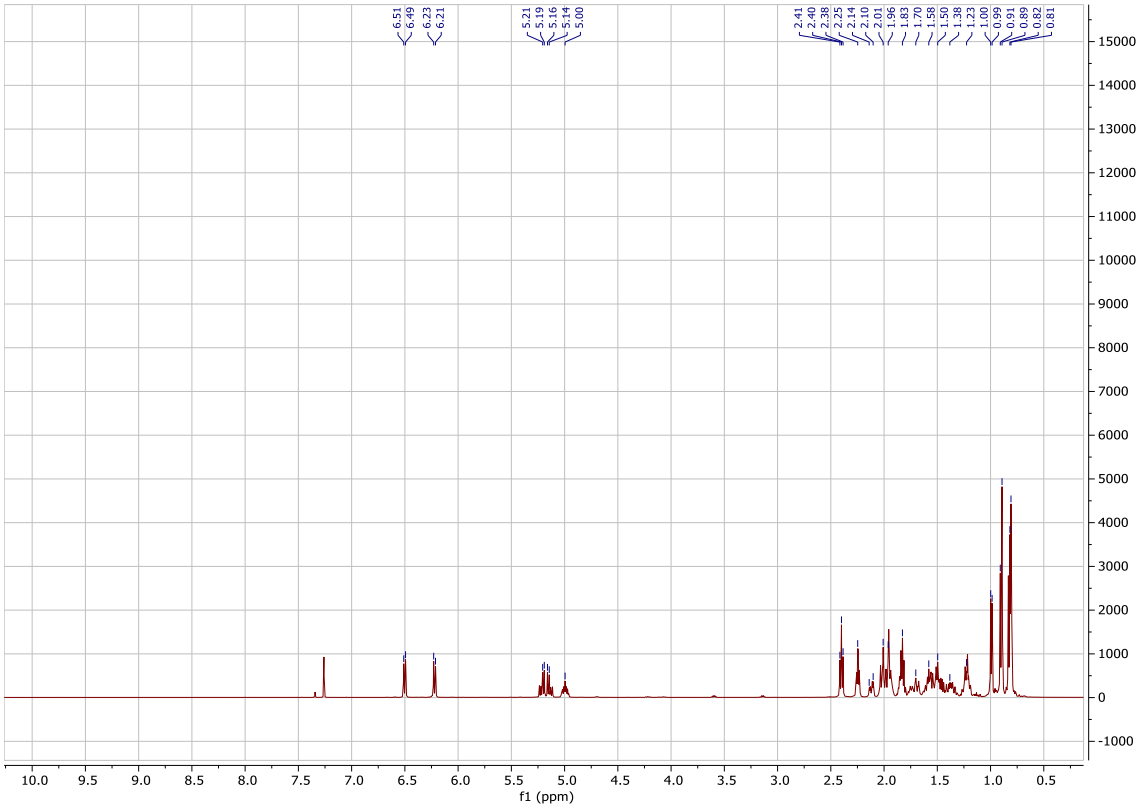

Figure S11.1 ^{1}H NMR Spectra of compound **3j**.

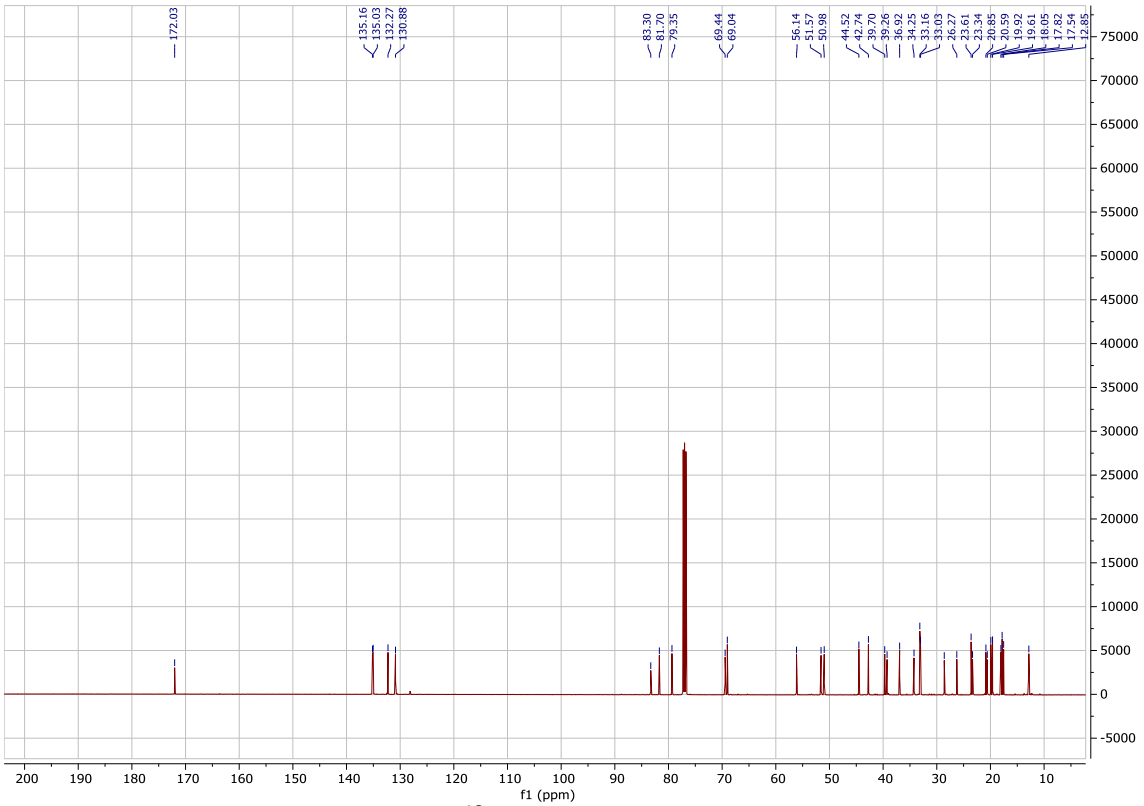

Figure S11.2 ^{13}C NMR Spectra of compound **3j**.

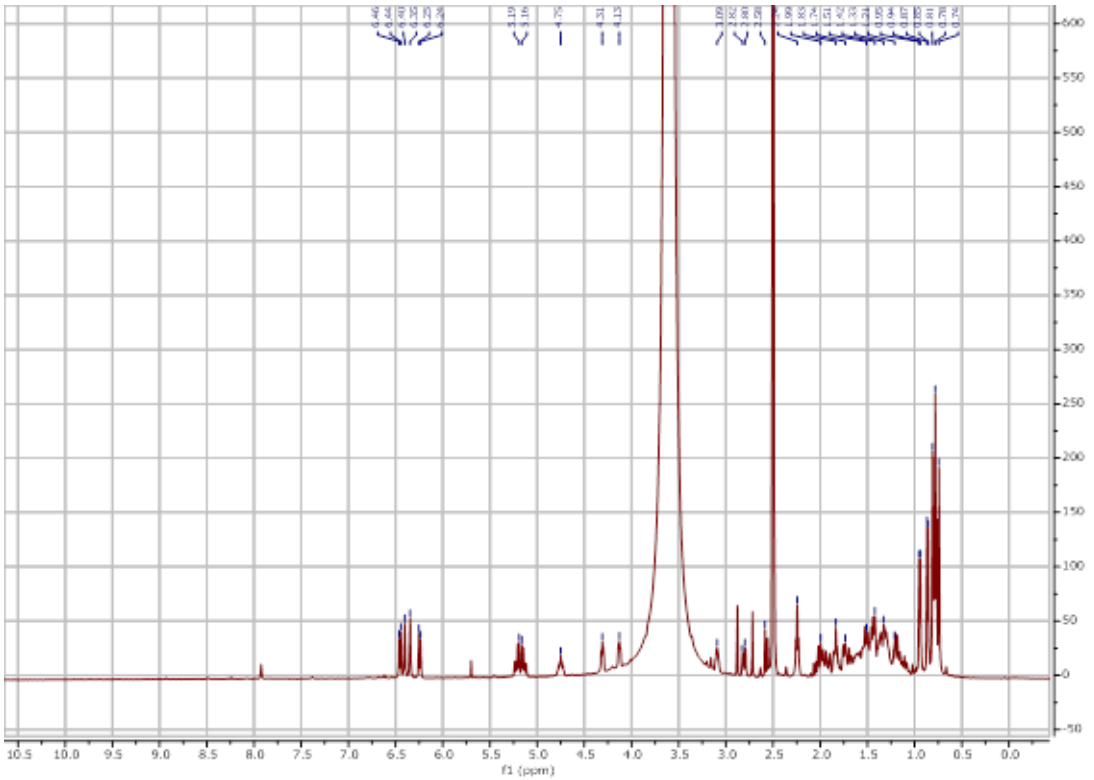

Figure S12.1 ^{1}H NMR Spectra of compound **3k**.

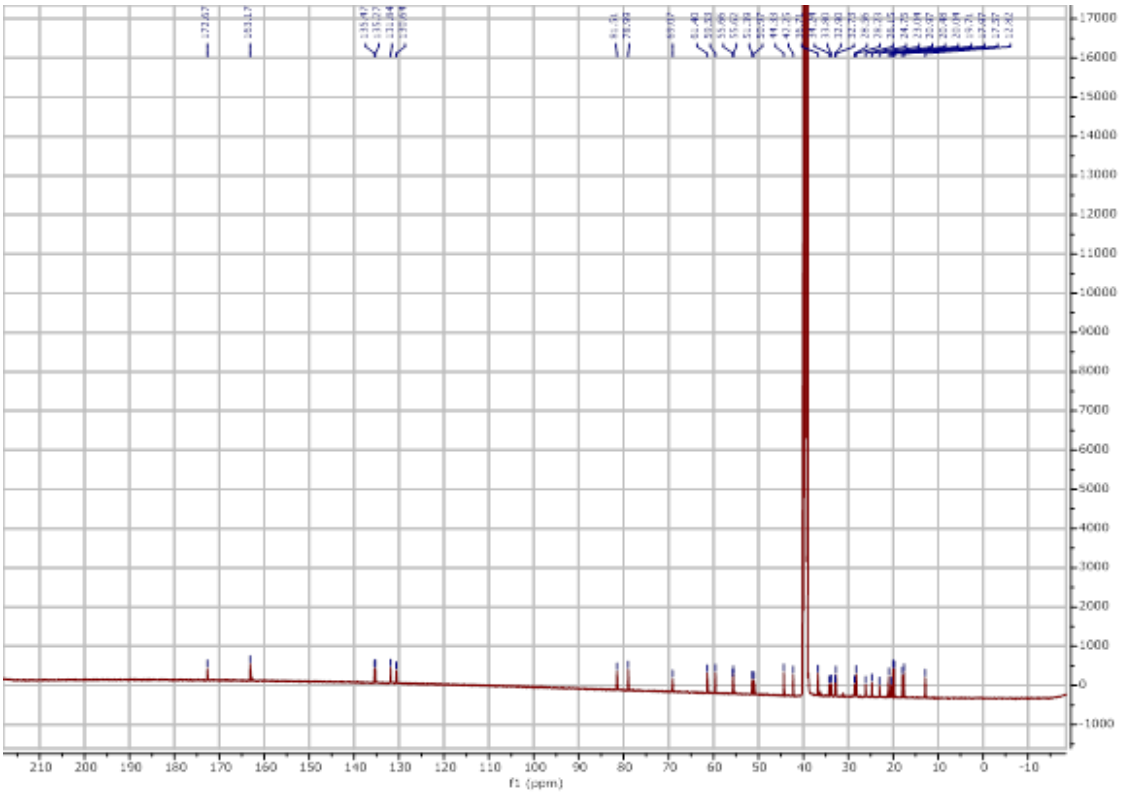

Figure S12.2 ^{13}C NMR Spectra of compound **3k**.

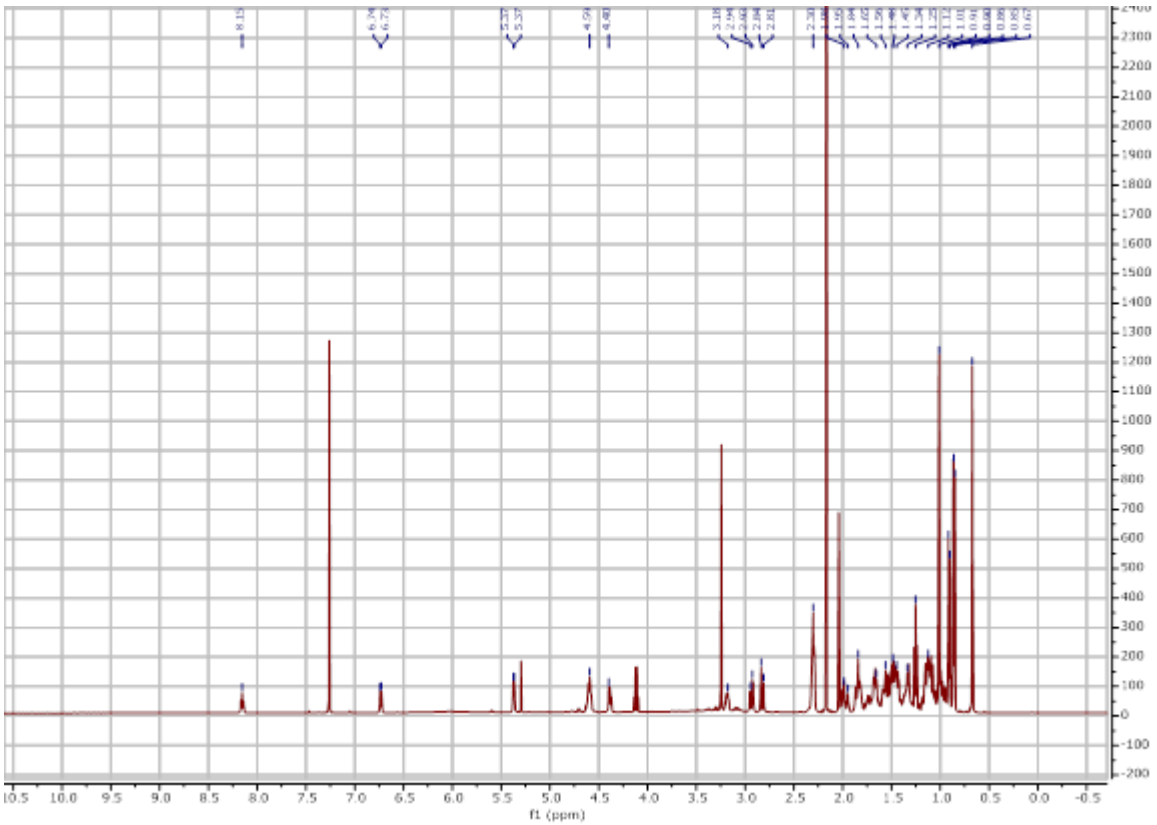

Figure S13.1 ^{1}H NMR Spectra of compound Cholesterol biotin ester.

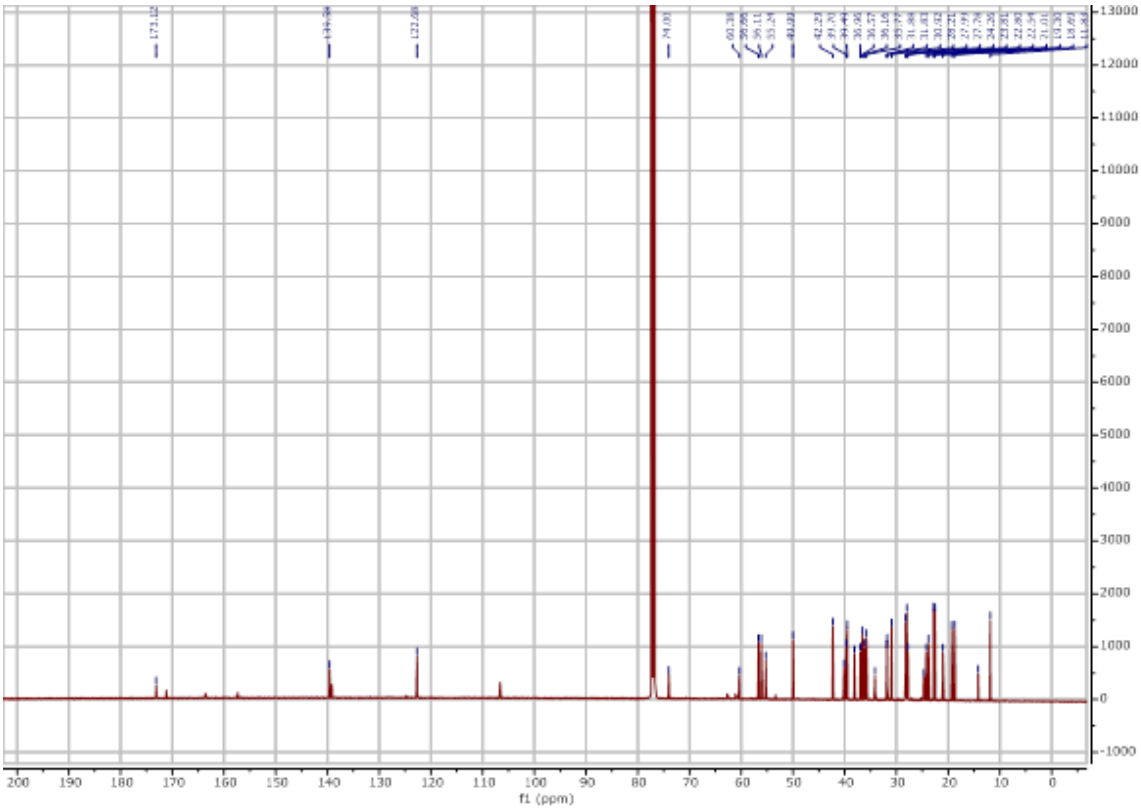

Figure S13.2 ^{13}C NMR Spectra of compound Cholesterol biotin ester.

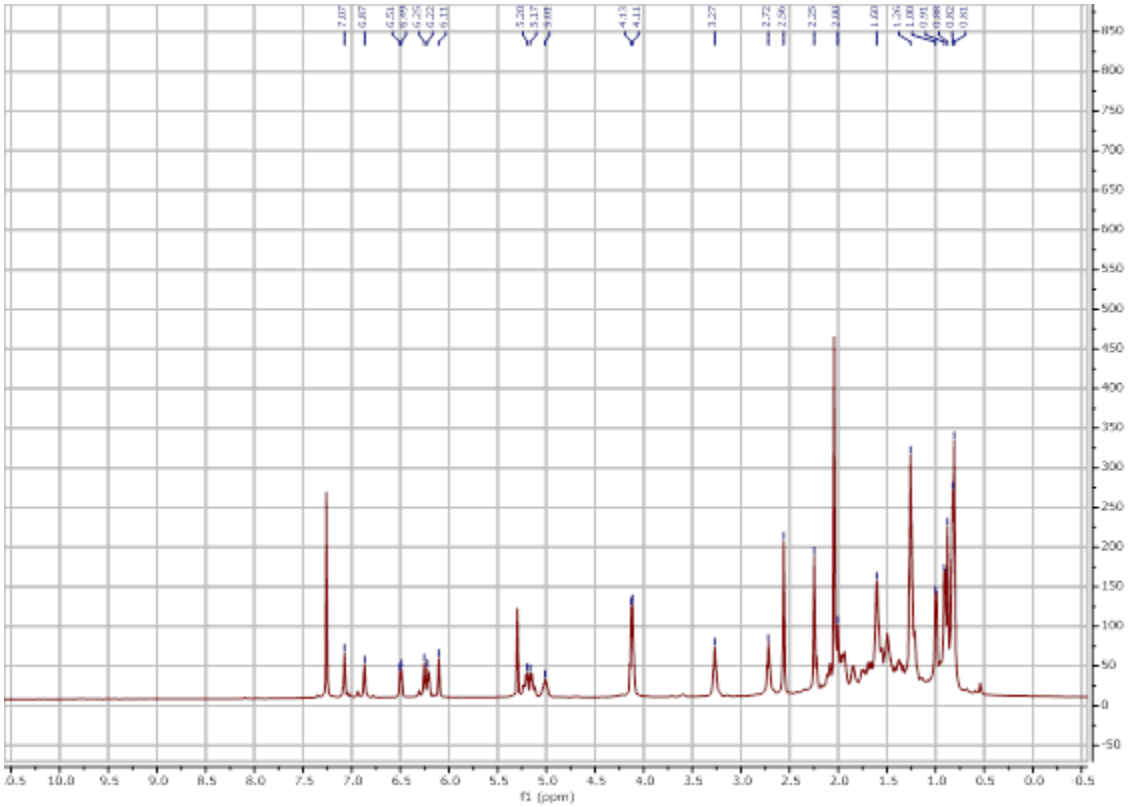

Figure S14.1 ^{1}H NMR Spectra of compound **3m**.

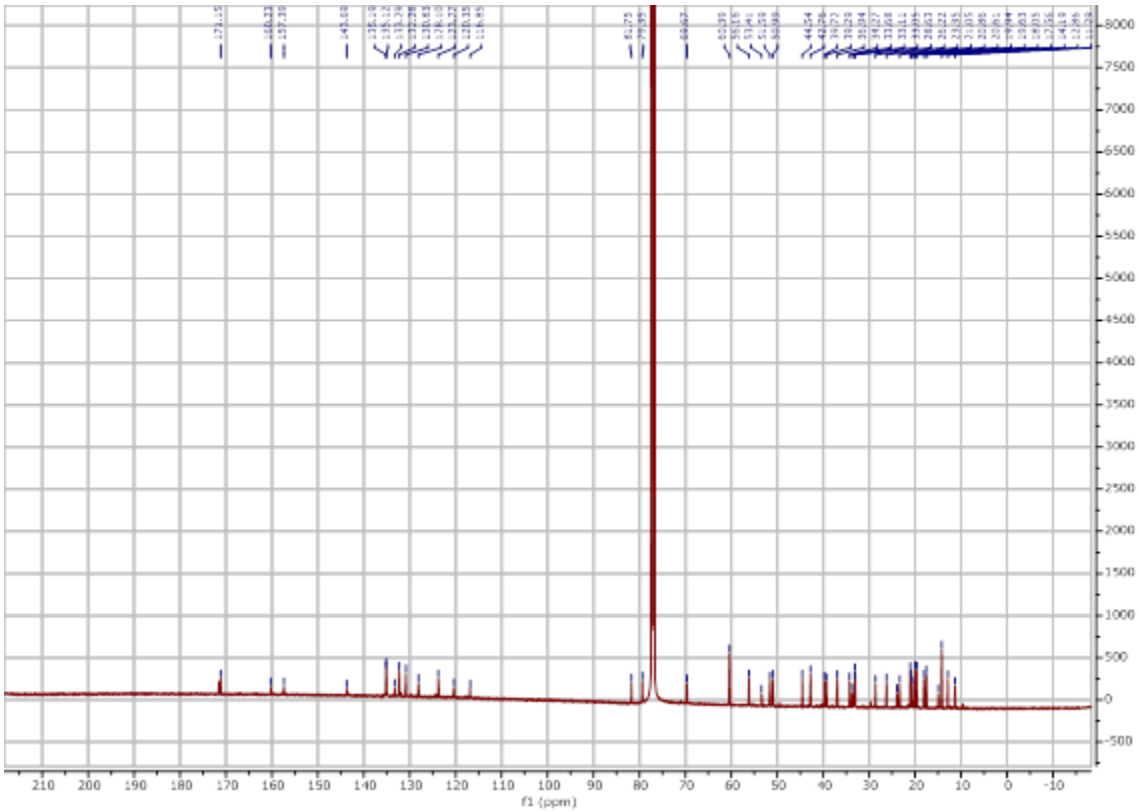

Figure S14.2 ^{13}C NMR Spectra of compound **3m**.

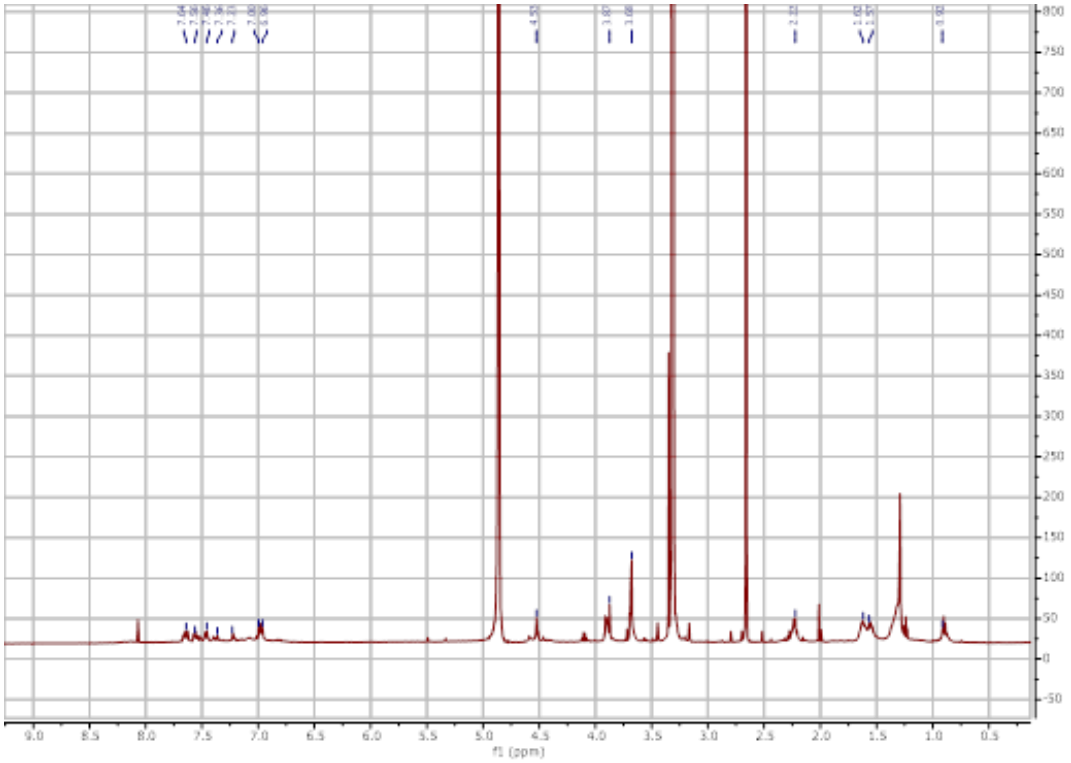

Figure S15.1 ^{1}H NMR Spectra of compound **BDY630X-glycine methyl ester control.**

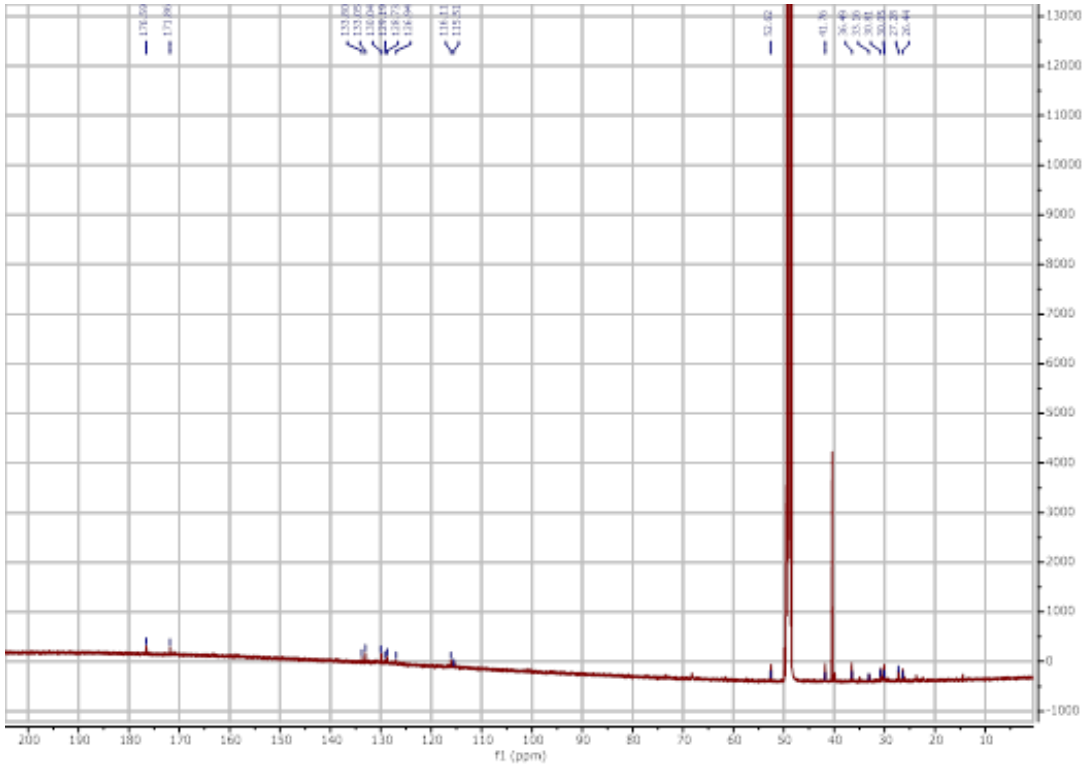

Figure S15.2 ^{13}C NMR Spectra of compound **BDY630X-glycine methyl ester control.**