Terpenoids for medicine

Fischedick, J.

Chapter 6

Cytotoxic Activity of Sesquiterpene Lactones from *Inula britannica* on Multi-Drug Resistant Human Cancer Cell Lines

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ABSTRACT

Five new sesquiterpene lactones (**1** – **5**) were isolated from *Inula britannica* collected in the wild from Serbia along with five known compounds (**6** – **10**). Sesquiterpene lactones were isolated using centrifugal partition chromatography followed by combination of flash chromatography and semi-preparative HPLC. Isolated compounds were screened for cytotoxic activity on human cancer cell line, their derived multi-drug resistant cell lines, and normal human keratinocytes. Sesquiterpene lactones showed similar cytotoxic activity towards drug sensitive and drug resistant cancer cell lines.

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Introduction

Inula britannica L. (Asteraceae) produces a variety of secondary metabolites including sesquiterpene lactones, diterpenes, triterpenes, and flavonoids (Khan et al., 2010). *Inula britannica* extracts have been reported to possess anti-inflammatory, hepatoprotective, anti-bacterial, and cytotoxic activity (Zhao et al., 2006). Sesquiterpene lactones of the germacranolide, eudesmanolide, 1,10-seco-eudesmanolide, and pseudoguaianolide groups, isolated mainly from flowers of the Chinese herb *I. britannica* var. *chinensis* are known to display cytotoxic effects against several human cancer cell lines (Zhou et al., 1993; Park and Kim, 1998; Bai et al., 2006; Qi et al., 2008). Although sesquiterpene lactones are present in *I. britannica* ecotypes growing in Europe as well, (Rybalko et al., 1968; Chugunov et al., 1971; Serkerov and Mir-Babaev, 1988) no investigation regarding the cytotoxicity of the European samples constituents has been reported so far. Therefore, we investigated the cytotoxic activity of a series of sesquiterpene lactones isolated from *I. britannica* plants collected in the wild around Belgrade, Serbia. This led to the isolation and identification of 10 sesquiterpene lactones, five of which have never been reported before. The isolated compounds were subsequently tested for their cytotoxicity against human cancer cell lines, their multi-drug resistant (MDR) counterparts, and normal human keratinocytes (HaCaT).

Materials and Methods

General Experimental Procedures

FT-IR was measured on a Perkin-Elmer FT-IR Spectrometer Paragon 1000. Optical rotations were obtained using a Propol Automatic Polarimeter. UV measurements were performed using a Shimadzu UV mini-1240. NMR spectra were recored in $CDCl_3$ or MeOD on a Bruker DMX 500 MHz NMR calibrated to residual $CDCl_3$ (7.26 ppm ^{1}H; 77.16 ppm ^{13}C) or MeOD (3.31 ppm). High resolution mass data (HRESIMS) were collected on a Thermo LC-LTQ-Orbitrap FTMS system. LC-APCI mass data (APCIMS) were collected in both positive and negative mode on an Agilent 1100 series HPLC connected to G1956 LC/MSD SL single quadropole mass spectrometer. Centrifugal partition chromatography (CPC) was carried out with a Kromaton Fast Centrifugal Partition Chromatograph with 1 L internal rotor volume and 30 mL Rheodyne injector loop. Semi-preparative HPLC (pHPLC) was performed with a Shimadzu HPLC system and a 5 mL Rheodyne manual injection loop. Normal phase (NP) separation used a Phenomenex Luna Silica (2) 100 A 5 micron 250 x 10 mm column with 10 x 10 mm silica guard cartridge while reverse phase (RP) separation used a Phenomenex Luna C18 (2) 100 A 5 micron 250 x 10 mm column. All pHPLC experiments used 5 mL/min flow rate and 10 mL fractions were collected unless otherwise noted. TLC was performed with silica gel 60 (Merck) plates using $CHCl_3$: EtOAc 1:1 and visualized with vanillin/sulfuric acid reagent. Flash chromatography used silica gel 60 (0.063- 0.2 mm, Merck). All solvents were of analytical and HPLC grade.

Plant Material

Inula britannica plant material was collected in Serbia from several natural localities: from the edge of Lipovica forest and from meadows nearby Mladenovac and Kragujevac. Species identification was confirmed by Wout Holverda at the Leiden Nationaal Herbarium Nederland and a voucher specimen was deposited in the economic botany collection under the following barcode: AsteraceaeInulabritannicaL.L 0991383J. FischedickNo. 172010.

Extraction and Isolation

Inula britannica (200 g) dried flowers were extracted with 4 L EtOH for 24 hours. Solvent removed in vacuo to yield 14 g yellow solid/syrup. Crude extract was re-dissolved in 400 mL ethyl acetate (EtOAc) and 400 mL H_2O. The H_2O layer was drained off and EtOAc rinsed 2 additional times with 250 mL H_2O. The EtOAc layer was dried over $MgSO_4$, filtered, and solvent removed in vacuo to yield 6 g yellow solid/syrup. The EtOAc extract was further fractionated with CPC. A 2 phase solvent system composed of 4: 6: 4: 6 heptane: EtOAc: MeOH: H_2O (5 L) was prepared and split into upper (2.3 L) and lower layer (2.7 L). The EtOAc extract was dissolved in 30 mL upper and lower layer (1:1). The CPC system was first filled with lower layer to act as stationary phase. Upper layer was then pumped in at flow rate of 10 mL/min and rotor rotation speed of 1000 rpm. The CPC system was considered in equilibrium when upper layer began to elute and amount of lower layer displaced recorded as void volume (220 mL). The entire sample was then injected and initial 200 mL eluent discarded after which 120 x 10 mL fractions (CPC $Fr_{\#}$) were collected (pressure 52 bar). After the 120th fraction the CPC system was rinsed with lower layer which was collected as an additional rinse fraction (Fr_R). Fractions were analyzed by TLC, combined based on profile, and solvent removed in vacuo.

CPC $Fr_{11\text{-}30}$ (318 mg) was further separated by NP pHPLC ($CHCl_3$: EtOAc, 9:1) with subsequent fractions 3-8 (110 mg) run on RP pHPLC (H_2O: acetonitrile (ACN), 8:2) to yield **1** (11.6 mg) from fractions 11-13 and an impure sesquiterpene lactone in fractions 7-9 (88.9 mg). CPC $Fr_{31\text{-}45}$ (110 mg) was separated by NP pHPLC ($CHCl_3$: EtOAc, 9:1) with subsequent fractions 1-8 (52 mg) run on RP pHPLC (H_2O: ACN, 1:1) to yield 3 additional fractions. Fractions 1-2 (27.2 mg) were combined and fractionated by RP pHPLC (H_2O: ACN, 8:2, gradient to 100% ACN) to yield **10** (8.1 mg) and **3** (2.9 mg). Fraction 3 (13.5 mg) was combined with fractions 7-9 from CPC $Fr_{11\text{-}30}$ RP pHPLC and purified with RP pHPLC (H_2O: ACN, 6:4) to yield **2** (67.8 mg). CPC $Fr_{46\text{-}65}$ (368 mg) was separated by NP pHPLC ($CHCl_3$: EtOAc, 9:1, 3 mL/min, 6 mL fractions) with **9** (127 mg) crystallized from fractions 4-16 with hexane/Et_2O while fractions 17-20 were further purified with RP pHPLC (H_2O: ACN, 1:1) to yield **3** (2.7 mg). CPC $Fr_{66\text{-}120}$ (914 mg) was separated by flash chromatography (100 g silica, $CHCl_3$ increasing ratio of acetone), with 100 mL fractions collected. Flash fractions 2-6 were combined (576 mg) and run on NP pHPLC ($CHCl_3$: EtOAc, 7:3) with subsequent fractions 7-28 further separated by NP pHPLC ($CHCl_3$: EtOAc, 9:1) to yield **8** (98 mg) as white needles (hexane/Et_2O) from fractions 12-28. CPC Fr_R (478 mg) was further separated by flash chromatography (50 g silica) using hexane with increasing ratio of acetone followed by acetone with increasing ratio of ethanol. Flash fractions 4-5 (100

mg) were purified with RP pHPLC (H_2O: ACN, 8: 2) to yield **7** (8.3 mg) from fractions 16-17 while fractions 18-23 (22.4 mg) were again separated by RP pHPLC (H_2O: ACN, gradient to 100% ACN) to yield **5** (16.8 mg) from fraction 9. Flash fractions 6-7 (118 mg) were separated by RP pHPLC (H_2O: ACN, 8: 2) from which fractions 1-3 were again separated by RP pHPLC (H_2O: ACN, 9: 1) to yield **4** (8.8 mg) from fractions 22-25 and an impure sesquiterpene lactone in the rinse fraction. The impure sesquiterpene lactone was re-purified under the same conditions to yield **6** (18.2 mg).

14-(3-Methylpentanoyl)-6-deoxybritannilactone (**1**): amorphous colorless solid; $[\alpha]^{20}_D$ +109.5 (*c* 0.15, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 212.0 (3.95) nm; IR (film) ν_{max} 3503.7, 2961.4, 2360.4, 2342.7, 1759.4, 1733.6, 1268.3 cm^{-1}; ^1H and ^{13}C NMR data, see Table1; APCIMS *m/z* 347 $[M - H_2O]^+$ (100), 363 $[M - H]^-$ (100); HRESIMS *m/z* 365.2321 $[M + H]^+$ (calcd for $C_{21}H_{33}O_5$ 365.2328).

14-(3-Methylbutanoyl)-6-deoxybritannilactone (**2**): amorphous colorless solid; $[\alpha]^{20}_D$ +112.6 (*c* 0.19, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 209 (4.06) nm; IR (film) ν_{max} 3428.1, 2959.5, 2360.3, 2343.7, 1759.6, 1734, 1267.3, 1187.8, 996 cm^{-1}; ^1H and ^{13}C NMR data, see Table1; APCIMS *m/z* 333 $[M - H_2O]^+$ (100), 349 $[M - H]^-$ (100); HRESIMS *m/z* 351.2166 (calcd for $C_{20}H_{31}O_5$ 351.2172).

14-(2-Methylpropanoyl)-6-deoxybritannilactone (**3**): amorphous colorless solid; $[\alpha]^{20}_D$ +122.9 (*c* 0.07, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 204.5 (4.29) nm; IR (film) ν_{max} 3440, 2961.5, 2360, 2343.3, 1759.1, 1734, 1268.4, 1191.7, 1157.8, 996 cm^{-1}; ^1H and ^{13}C NMR data, see Table1; APCIMS *m/z* 319 $[M - H_2O]^+$ (100), 335 $[M - H]^-$ (100); HRESIMS *m/z* 337.2011 (calcd for $C_{19}H_{29}O_5$ 337.2015).

1,3-Epi-granilin (**4**): amorphous white solid; $[\alpha]^{20}_D$ +146.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 226.5 (3.26) nm; IR (film) ν_{max} 3424, 2359.8, 2343.2, 1740, 1262, 1220, 772 cm^{-1}; ^1H NMR (MeOD, 500 MHz) δ 6.08 (1H, br s, H-13a), 5.71 (1H, br s, H-13b), 5.18 (1H, br s, H-15a), 4.67 (1H, br s, H-15b), 4.58 (1H, td, *J* = 4.8, 1.6 Hz, H-8), 4.02 (1H, dd, *J* = 11.8, 5.3 Hz, H-3), 3.42 (1H, dd, *J* = 11.9, 4.3 Hz, H-1), 3.07 (1H, m, H-7), 2.52 (1H, dd, *J* = 15.8, 1.6 Hz, H-9β), 2.09 (1H, m, H-2α), 1.80 (1H, m, H-5), 1.80 (1H, m, H-6α), 1.54 (1H, m, H-9α), 1.51 (1H, m, H-2β), 1.42 (1H, ddd, *J* = 13, 12.9 Hz, H-6β), 0.74 (3H, s, H-14); APCIMS *m/z* 265 $[M + H]^+$ (100), 263 $[M - H]^-$ (100); HRESIMS *m/z* 265.1436 $[M + H]^+$ (calcd for $C_{15}H_{21}O_4$ 265.1440).

11,13-Dihydro-inuchinenolide B (**5**): amorphous colorless solid; $[\alpha]^{20}_D$ -46.1 (*c* 0.26, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 208 (4.05) nm; IR (film) ν_{max} 3428.5, 2976.1, 2360.3, 2343.7, 1733.8, 1239.6 cm^{-1}; ^1H-NMR ($CDCl_3$, 500 MHz) δ 5.55 (1H, m, H-2β), 4.71 (1H, ddd, *J* = 10.1, 7.2, 2.5 Hz, H-8), 2.78 (1H, m, H-7), 2.76 (1H, m, H-11), 2.66 (1H, m, H-9β), 2.63 (1H, br d, H-5), 2.49 (1H, dd, *J* = 15.9, 2.5 Hz, H-9α), 2.33 (1H, dd, *J* = 13.4, 7.4 Hz, H-3β), 2.05 (3H, s, $COOCH_3$, H-2'), ~1.88 (1H, s, OH-4α), 1.77 (1H, m, H-3α), 1.77 (1H, m, H-6α), 1.68 (3H, d, *J* = 1.6 Hz, H-14), 1.33, (1H, m, H-6β), 1.26 (3H, d, *J* = 7.5 Hz, H-13β), 1.11 (3H, s, H-15); ^{13}C-NMR ($CDCl_3$, 125.8 MHz) δ 180.1 (C, $COOCH_3$, C-1'), 170.7 (C, C12), 137.6 (C, C-1), 132.4 (C, C-10), 79.4 (CH, C-8), 78.3 (C, C-4), 72.7 (CH, C-2), 52.7 (CH, C-5), 46.7 (CH_2, C-3), 41.0 (CH, C-7), 38.8

(CH, C-11), 37.4 (CH_2, C-9), 23.4 (CH_2, C-6), 23.0 (CH_3, C-15), 22.0 (CH_3, C-14), 21.2 (CH_3, $COOCH_3$, C-2'), 12.8 (CH_3, C-13); APCIMS *m/z* 307 $[M - 1]^-$ (20); HRESIMS *m/z* 309.1701 $[M + H]^+$ (calcd for $C_{17}H_{25}O_5$ 309.1702).

Pulchellin C (**6**): amorphous yellow solid; $[\alpha]^{20}_D$ +119.8 (c 0.21, MeOH); ^{1}H-NMR (MeOD, 500 MHz) δ 6.08 (1H, s, H-13a), 5.72 (1H, s, H-13b), 5.27 (1H, d, *J* = 1.2 Hz, H-15a), 4.71 (1H, d, *J* = 1.4 Hz, H-15b), 4.55 (1H, m, H-8), 3.75 (1H, br d, H-3α), 3.44 (1H, ddd, *J* = 11.6, 9.2, 4.9 Hz, H-2β), 3.11 (1H, m, H-7), 2.21 (1H, dd, *J* = 15.6, 1.3 Hz, H-9α), 1.98 (1H, br d, *J* = 12.6 Hz, H-5), 1.83 (1H, m, H-1β), 1.83 (1H, m, H-6α), 1.61 (1H, dd, *J* = 15.6, 4.6 Hz, H-9β), 1.35 (IH, m, H-6β), 1.31 (1H, m, H-1α) 0.81 (3H, s, H-14); APCIMS *m/z* 265 $[M + H]^+$ (100), 263 $[M - H]^-$ (100); HRESIMS *m/z* 265.1438 $[M + H]^+$ (calcd for $C_{15}H_{21}O_4$ 265.1440).

6-Deacetylbritanin (**7**): amorphous colorless solid; $[\alpha]^{20}_D$ +5.2 (c 0.12, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 6.25 (1H, br d, H-13a), 6.16 (1H, br d, H-13b), 4.91 (1H, ddd, *J* = 8.8, 6.6, 1.5 Hz, H-2β), 4.61 (1H, ddd, *J* = 12.1, 8.3, 4.6 Hz, H-4α), 4.28 (1H, ddd, *J* = 12.1, 9.7, 2.8 Hz, H-8), 3.76 (1H, dd, *J* = 9.8, 3.6 Hz, H-6β), ~2.88 (1H, m, OH-6α), 2.85 (1H, m, H-7), 2.35 (1H, m, H-9β), 2.05 (1H, m, H-3a), 2.04 (3H, s, $COOCH_3$, H-2'), 1.90 (1H, m, H-1), 1.84 (1H, m, H-10α), 1.82 (1H, m, H-3b), ~1.79-1.93 (1H, m, OH-4β), 1.42 (1H, m, H-9α), 0.95 (3H, d, *J* = 6.3 Hz, H-14), 0.95 (3H, s, H-15); ^{13}C-NMR ($CDCl_3$, 125.8 MHz) δ 170.6 (C, $COOCH_3$, C-1'), 170.1 (C, C-12), 139.0 (C, C-11), 123.3 (CH_2, C-13), 77.1 (CH, C-6), 76.7 (CH, C-8), 75.4 (CH, C-2), 73.8 (CH, C-4), 52.1 (CH, C-7), 51.2 (C, C-5), 51.1 (CH, C-1), 44.0 (CH_2, C-9), 36.8 (CH_2, C-3), 29.7 (CH, C-10), 21.4 (CH_3, $COOCH_3$, C-2'), 20.2 (CH_3, C-14), 17.7 (CH_3, C-15); APCIMS m/z 325 $[M + H]^+$ (100), 323 $[M - H]^-$ (95); HRESIMS m/z 325.1649 $[M + H]^+$ (calcd for $C_{17}H_{25}O_6$ 325.1651).

4H-Tomentosin (**10**): amorphous colorless solid; $[\alpha]^{20}_D$ +18.4 (*c* 0.10, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 6.26 (1H, d, *J* = 3.2 Hz, H-13a), 5.52 (1H, d, *J* = 2.8 Hz, H-13b), 5.49 (1H, dd, *J* = 9.2, 5.3 Hz, H-5), 4.64 (1H, ddd, *J* = 11.7, 8.5, 2.8 Hz, H-8), 3.78 (1H, m, H-4), 3.34 (1H, m, H-7), 2.45 (1H, m, H-6β), 2.37 (1H, m, H-10), 2.18 (1H, m, H-6α), 2.14 (1H, m, 2a), 2.00 (1H, m, H-9α), 1.99 (1H, m, H-2b), 1.90 (1H, m, H-9β), 1.57 (1H, m, H-3a), 1.46 (1H, m, H-3b), 1.33 (1H, br s, OH-4), 1.21 (3H, d, *J* = 6.2 Hz, H-15), 1.14 (3H, d, *J* = 6.9 Hz, H-14); ^{13}C-NMR ($CDCl_3$, 125.8 MHz) δ 170.4 (C, C-12), 145.7 (C, C-1), 139,2 (C, C-11), 122.2 (CH_2, C-13), 120.0 (CH, C-5), 79.8 (CH, C-8), 67.7 (CH, C-4), 42.4 (CH, C-7), 38.1 (CH_2, C-3), 36.8 (CH_2, C-9), 35.2 (CH, C-10), 33.0 (CH_2, C-2), 26.9 (CH_2, C-6), 24.0 (CH_3, C-15), 21.1 (CH_3, C-14); APCIMS *m/z* 251 $[M + H]^+$ (100), 249 $[M - H]^-$ (100); HRESIMS *m/z* 251.1642 $[M + H]^+$ (calcd for $C_{15}H_{23}O_3$ 251.1647).

Cell lines and Cell Culture

The NCI-H460, DLD1, and U87 cell lines were purchased from the American Type Culture Collection (ATCC), while COR-L23 and COR-L23/R cell lines were purchased from European Collection of Cell Cultures (ECACC). Human normal

keratinocytes – HaCaT were obtained from Cell Lines Service (CLS). NCI-H460/R cells were selected originally from NCI-H460 cells and cultured in a medium containing 100 nM doxorubicin (Pešić et al., 2006). DLD1-TxR and U87-TxR cells were selected from DLD1 and U87 cells, respectively, and cultured in a medium containing 300 nM paclitaxel (Podolski-Renić et al., 2011). All cell lines were sub-cultured at 72 h intervals using 0.25% trypsin/EDTA and seeded into a fresh medium at the following densities: 8,000 cells/cm^2 for NCI-H460, DLD1, DLD1-TxR, COR-L23 and COR-L23/R, 16,000 cells/cm^2 for U87 and NCI-H460/R, and 32,000 cells/cm^2 for U87-TxR and HaCaT.

Cytotoxicity assays

Cells grown in 25 cm^2 tissue flasks were trypsinized, seeded into flat-bottomed 96-well tissue culture plates, and incubated overnight. Treatment with all compounds (10 μM) lasted 72 h. The cellular proteins were stained with Sulforhodamine B assay (SRB), following a slightly modified protocol (Skehan et al., 1990). To further assess the cytotoxic effects of the most potent sesquiterpene lactones in NCI-H460 and NCI-H460/R cells, beside SRB assay, the MTT assay based on the reduction of 3-(4,5-dimethyl-2-thizolyl)-2,5-diphenyl-2H-tetrazolium bromide into formazan dye by active mitochondria of living cells was applied as well. The cells were incubated with indicated compounds for 72 h. Afterwards, 100 μl of MTT solution (1 mg /ml) was added to each well and plates were incubated at 37°C for 4 h. Formazan product was dissolved in 200 μl of DMSO. The absorbance of obtained dye after SRB or MTT assay was measured at 540 nm using an automatic microplate reader (LKB 5060-006 Micro Plate Reader, Vienna, Austria).

Appendix

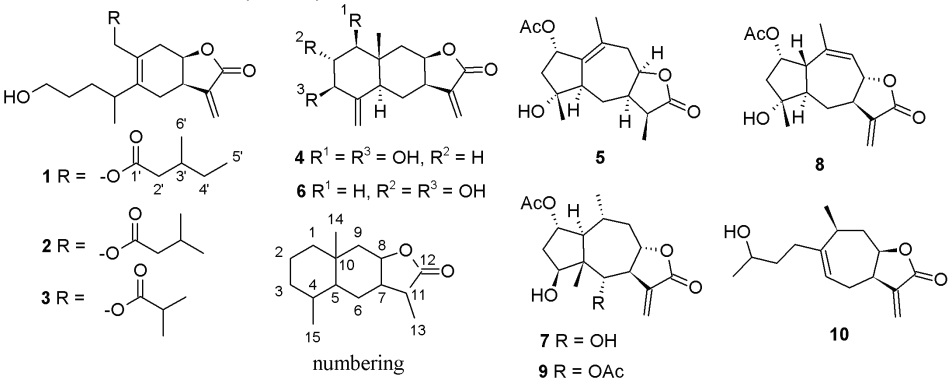
Detailed LC-MS conditions, NMR parameters, ^{1}H-NMR data for compounds **6** and **7**, and NMR spectra of all isolated compounds are available in the chapter 6 appendix.

Results and Discussion

An EtOH extract from dried *I. britannica* flowers was partitioned between EtOAc and H_2O. The EtOAc fraction was further purified with CPC followed by combination of flash chromatography and pHPLC to yield 10 sesquiterpene lactones. Three of these compounds were new 1,10-seco-eudesmanolides assigned the trivial names; 14-(3-methylpentanoyl)-6-deoxybritannilactone (**1**), 14-(3-methylbutanoyl)-6-deoxybritannilactone (**2**), and 14-(2-methylpropanoyl)-6-deoxybritannilactone (**3**). In addition, a new stereoisomer of granillin, 1,3-epi-granillin (**4**), and a new pseudoguaianolide, 11,13-dihydro-inuchinenolide B (**5**), were isolated. Five known sesquiterpene lactone compounds; pulchellin C (**6**) (Serkerov and Mir-Babaev, 1988; Adekenov et al., 1990), 6-deacetylbritannin (**7**) (Dzhazin and Adekenov, 1996; Yang et al., 2010), gaillardin (**8**) (Kupchan et al., 1965; Kupchan et al., 1966; Ito and Iida, 1981), britannin (**9**) (Adekenov et al., 1990; Rybalko et al., 1968), and 4H-tomentosin

(**10**) (Bohlmann et al., 1978; Kim et al., 2004) were also isolated, with **10** being reported in *I. britannica* for the first time.

Chemical structures (1 – 10)



Compounds **1-3** were isolated as amorphous colorless solids. Complete ^{1}H and ^{13}C-NMR assignments are shown in table 1. Carbon atom multiplicity was confirmed with ^{13}C-APT and HSQC. Complete HMBC correlations are shown in table 2. Similarity of ^{1}H and ^{13}C-NMR with literature (Zhou et al., 1993; Bai et al., 2006; Qi et al., 2008) suggested that **1-3** contained a 1,10-seco-eudesmanolide skeleton. A mass of 364 was suggested by the APCIMS spectrum of **1**. The molecular formula $C_{21}H_{32}O_5$ was established for **1** by HRESIMS (obsd 365.2321, calcd 365.2328, $[M + H]^+$). The IR suggested the presence of a hydroxyl group (3507.7 cm^{-1}), a γ unsaturated lactone (1759.4 cm^{-1}), and an ester (1733.6 cm^{-1}). The terminal methylene functionality of the α-methylene-γ-lactone was confirmed by the pair of 1H doublets appearing at δ_H 5.64 and 6.27, both attached to δ_C 122.0 (C-13). Furthermore the carbonyl carbon at δ_C 170.5 (C-12) and the quaternary carbon at δ_C 140.1 (C-11) correlated in HMBC to H-13. Correlations in COSY between H-13a and H-13b with δ_H 3.23 (H-7) were observed. The rest of the spin system connectivity was easily deduced by COSY showing correlations between H-7 with δ_H 4.90 (H-8), 2.25 (H-6), and 2.09 (H-6) as well as correlations in COSY between H-8 with δ_H 2.43 (H-9) and 2.62 (H-9). HSQC was used to unambiguously assign proton containing carbons on the bicyclic ring and HMBC confirmed connectivity of the entire spin system. The dd splitting pattern of protons at position 6 and 9 indicated that carbons at C-5 and C-10 were quaternary. Through analysis of HMBC spectra 2 alkene region quaternary carbons at δ_C 139.6 and δ_C 129.2 were assigned as C-5 and C-10 respectively (C-5→ 2H-6, 2H-9; C-10→ 2H-6, H-8, 2H-9). An additional HMBC correlation from C-5 to the methyl protons at δ_H 0.97 (CH_3-15) confirmed connectivity of side chain. Connectivity of C-5 to C4, C-15 to C4, C-4 to C-3, C-3 to C-2, and C-2 to C-1 was established through analysis of COSY, HSQC spectra, and confirmed by HMBC. The chemical shift of protons at position 1 (δ_H 3.98) and carbon (δ_C 64.0) confirmed the presence of hydroxyl functionality at position 1. Alkyl ester substitution connected to C-14 was suggested by the IR, remaining $C_6H_{11}O_2$, and the deshielded protons and carbon at C-14 (δ_C 61.4; δ_H 4.13, 4.19). Connectivity

from C-1' (δ_C 173.7) to C-2' (δ_C 41.6, CH_2) and C-3' (δ_C 32.1, CH) was established by HMBC. The methine at H-3' (δ_H 1.86) COSY spectrum showed correlations to H-2a' (δ_H 2.29), H-2b' (δ_H 2.09), CH_3-6' (δ_H 0.92), H-4a' (δ_H 1.34), and H-4b' (δ_H 1.22). The remaining methyl group was assigned to 5' (δ_H 0.88) and confirmed with COSY. The ester substituent was thus identified as a 3-methylpentanoyl group. The *cis* and axial relative configuration of protons H-7 and H-8 was confirmed by correlation in NOESY spectrum (Figure 1). Correlations were observed between H-7 and both H-6 as well as between H-8 and both H-9 (not shown in figure 1) and thus could not be used to establish their relative configuration. However due to the *cis* configuration of H-7 and H-8 as well as a cross peak between H-13b and H-6 (δ_H 2.09) confirmed β configuration. A NOESY correlation from H-6β (δ_H 2.09) to H-9β (δ_H 2.62) confirmed β configuration of H-9β. A NOESY correlation between H-6 (δ_H 2.25) and H-9 (δ_H 2.43) was also observed and confirmed α configuration of these protons. Compound **1** was therefore identified as 14-(3-methylpentanoyl)-1-hydroxy-1,10-seco-5(10),11(13)-eudesadien-12,8β-olide.

The same 1,10-seco-eudesmanolide skeleton as **1** was recognized in compounds **2** and **3** by ^{1}H-NMR, COSY, ^{13}C-APT, HSQC and HMBC spectra. A mass of 350 was suggested by APCIMS of **2**. The molecular formula $C_{20}H_{30}O_5$ was established for **2** by HRESIMS (obsd 351.2166, calcd 351.2172, $[M + H]^+$). The difference between **1** and **2** was therefore due to a different functional group at the C-14 position. Alkyl ester substitution was again suggested from IR (1734 cm^{-1}) and remaining $C_5H_9O_2$. Connectivity from the carbonyl C-1' (δ_C 177.5) to 2'-H (δ_H 2.17) and 3'-H (δ_H 2.08) was determined by HMBC and 2' correlated in COSY with 3'. The 2 overlapping methyl groups (δ_H 0.94) showed correlations in COSY with 3' and were assigned at the 4' and 5' positions. The substituent was therefore established as a 3-methylbutanoyl group. The relative configuration was the same as **1**, confirmed by NOESY. Therefore compound **2** was identified as 14-(3-methylbutanoyl)-1-hydroxy-1,10-seco-5(10),11(13)-eudesadien-12,8β-olide.

The mass of compound **3** was suggested to be 336 based on APCIMS. Confirmation of the molecular formula as $C_{19}H_{28}O_5$ was established by HRESIMS (obsd 337.2011, calcd 337.2015, $[M + H]^+$). Alkyl ester substitution at C-14 was suggested by IR (1734 cm^{-1}) and remaining $C_4H_7O_2$. In HMBC correlations between carbonyl C-1', 2'-H (δ_H 2.53) and 2 overlapping methyl groups (δ_H 1.16). Connectivity between 2'-H and the methyl groups at 3' and 4' was confirmed by coupling constant (J = 7.0 Hz) and COSY. The substituent was therefore established as a 2-methylpropanoyl group. NOESY established that the relative configuration for **3** was the same as in **1** and **2**. Compound **3** was thus identified as 14-(2-methylpropanoyl)-1-hydroxy-1,10-seco-5(10),11(13)-eudesadien-12,8β-olide.

Table 1. ^{1}H and ^{13}C-NMR data for compounds **1** – **3** ($CDCl_3$)

	1		**2**		**3**	
position	δ_C type	δ_H (J in Hz)	δ_C type	δ_H (J in Hz)	δ_C type	δ_H (J in Hz)
1	64.0, CH_2	3.98, m	64.0, CH_2	3.98, m	64.1, CH_2	3.97, m
2	27.0, CH_2	1.32, m 1.42, m	27.0, CH_2	1.31, m 1.41, m	27.0, CH_2	1.32, m 1.41, m
3	30.8, CH_2	1.16, m 1.31, m	30.7, CH_2	1.15, m 1.29, m	30.7, CH_2	1.17, m 1.30, m
4	33.6, CH	2.76, m	33.5, CH	2.76, m	33.6, CH	2.77, m
5	139.6, C		139.4, C		139.6, C	
6α	29.0, CH_2	2.25, dd (15.0, 6.6)	28.9, CH_2	2.24, dd (15.0, 6.7)	29.0, CH_2	2.25, dd (15.0, 7.1)
6β		2.09, m		2.08, m		2.09, dd (15.0, 4.5)
7	37.5, CH	3.23, m	37.5, CH	3.23, m	37.5, CH	3.24, m
8	77.1, CH	4.90, m	77.1, CH	4.90, m	77.1, CH	4.91, m
9α	31.8, CH_2	2.43, dd (15.3, 4.4)	31.8, CH_2	2.42, dd (15.4, 4.3)	31.8, CH_2	2.43, dd (15.0, 4.4)
9β		2.62, dd (15.3, 4.6)		2.61, dd (15.3, 4.6)		2.64, dd (15.0, 4.5)
10	129.2, C		129.2, C		129.2, C	
11	140.1, C		140.1, C		140.1, C	
12	170.5, C		170.5, C		170.5, C	
13b	122.0, CH_2	5.64, d (2.3)	122.9, CH_2	5.64, d (2.3)	122.0, CH_2	5.65, d (2.3)
13a		6.27, d (2.7)		6.27, d (2.7)		6.28, d (2.7)
14	61.4, CH_2	4.13, d (11.9) 4.19, d (11.9)	61.3, CH_2	4.13, d (11.9) 4.19, d (11.9)	61.5, CH_2	4.14, d (12) 4.19, d (12)
15	19.5, CH_3	0.97, d (6.8)	19.5, CH_3	0.97, d (6.8)	19.5, CH_3	0.98, d (6.8)
1’	173.7, C		173.4, C		177.5, C	
2’	41.6, CH_2	2.09, m 2.29, dd (14.7, 6.1)	43.5, CH_2	2.17, d (7.1)	34.16, CH	2.53, septet (7)
3’	32.1, CH	1.86, m	25.8, CH	2.08, m	19.2, CH_3	1.16, d (7)
4’	29.8, CH_2	1.22, m 1.34, m	22.5, CH_3	0.94, d (6.6)	19.2, CH_3	1.16, d (7)
5’	11.4, CH_3	0.88, t (7.5)	22.5, CH_3	0.94, d (6.6)		
6’	19.4, CH_3	0.92, d (6.7)				
OH		~1.51, br s		~1.50, br s		~1.43, m

Figure 1. NOESY correlations of **1** – **3**.

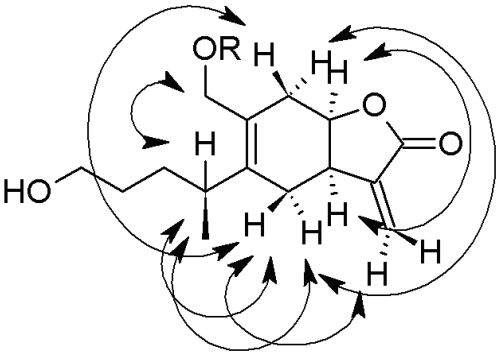


Table 2. HMBC correlations **1** - **3**

position	**1** (C→ H#)	**2** (C→ H#)	**3** (C→ H#)
1		2, 3	2, 3
2	1, 3	1, 3	1, 3
3	1, 15	1, 2, 4, 15	1, 15
4	6β, 15	2, 3, 6, 15	6β, 15
5	6, 9, 15	3, 6, 9, 15	6, 9, 14, 15
6	8, 13a	8, 13	8, 13a
7	6, 9, 13	6, 9, 13	6, 9, 13
8	6, 9	6, 9	6, 9
9	6, 14	4	14
10	6, 8, 9, 14	6, 8, 9	6, 8, 9, 14
11	6, 13a	13	13a
12	8, 13	8, 13	8, 13
13			
14	9	9	9
15		3	
1’	2’, 3’	2’, 3’	2’, 3’, 4’
2’	4’, 6’	3’, 4’, 5’	3’, 4’
3’	2’, 5’	2’, 4’, 5’	2’, 4’
4’	2’, 5’	2’, 3’, 5’	2’, 3’
5’	4’	2’, 3’, 4’	
6’	2’, 4’		

A mass of 264 was suggested from the APCIMS spectrum of **4** and HRESIMS (obsd 265.1436, calcd 265.1440 $[M + H]^+$) confirmed $C_{15}H_{20}O_4$ as the molecular formula. Compound **4** had very similar ^{1}H-NMR and IR spectra as that of granilin (Nikonova and Nikonov, 1972; Maruyama and Shibata, 1975; Vichnewski et al., 1976). However differences were noted in the δ and *J* values for protons at position 1, 2, and 3 suggesting a different relative configuration of the hydroxyl groups. The spin system connecting 2H-13 to H-7, H-7 to 2H-6 and H-8, H-8 to 2H-9, H-6 (δ_H 1.42) to H-5, and H-5 to 2H-15 was confirmed by COSY. Protons at δ_H 3.42 and δ_H 4.02 were assigned to H-1 and H-3 respectively due to deshielding from hydroxyl groups and for H-3 a correlation in COSY with methylene protons 2H-15. Connectivity between H-1 to 2H-2 (δ_H 2.09; δ_H 1.51) to H-3 was confirmed by COSY. In the NOESY spectrum a correlation between H-7 and H-8 was observed confirming a *cis* and axial configuration (Figure 4). Further correlations in NOESY between H-7 and H-6 (δ_H 1.80) as well as H-8 and H-9 (δ_H 1.54) confirmed α configuration for these protons. Both H-6β (δ_H 1.42) and H-9β (δ_H 2.52) correlated in NOESY with the methyl group at position 14 (δ_H 0.74) confirming β configuration. The α configuration of H-1 was confirmed by a correlation in NOESY with H-9α. Further correlations from H-1 to H-2α (δ_H 2.09) and H-2α to H-3 confirmed the hydroxyl groups at 1 and 3 as β oriented. Finally a correlation from H-2β (δ_H 1.51) to the β oriented methyl group at C-14 was also observed in the NOESY spectrum. Compound **4** was therefore identified as 1β,3β-dihydroxy-4(15),11(13)-eudesmadien-12,8β-olide.

Figure 2. NOESY correlations of **4**.

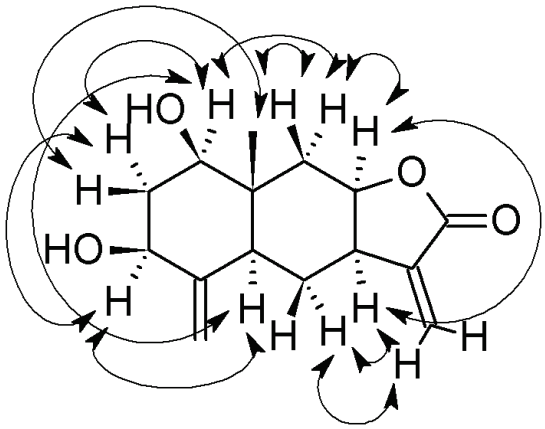
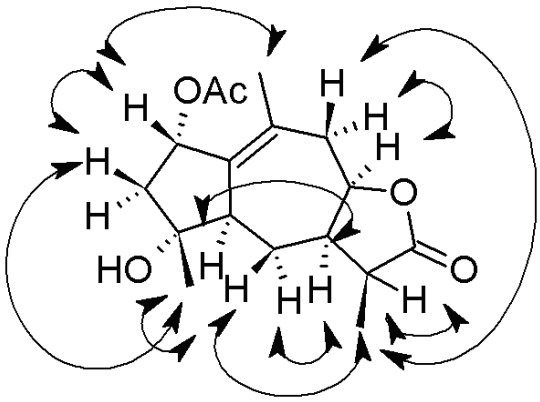


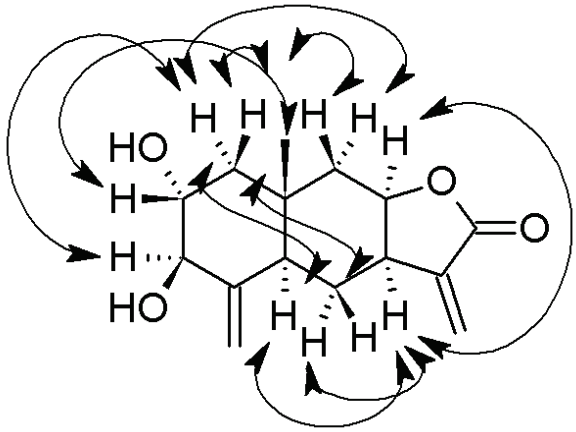
Figure 3. NOESY correlations of **5**.



The molecular formula of compound **5** was established as $C_{17}H_{24}O_5$ by HRESIMS (obsd 365.2321, calcd 365.2328 $[M + H]^+$). The ^1H and ^{13}C-NMR spectra of **5** resembled inuchinenolide B, previously reported from *I. Britannica* (Ito and Iida, 1981), however the methylene H-13 doublets were lacking and an additional methyl group (δ_H 1.26) was present. The connectivity of the spin system H-2 (δ_H 5.55) and 2H-3 (δ_H 2.33; 1.77) was confirmed with COSY. Correlations in COSY from H-8 (δ_H 4.71) to 2H-9 (δ_H 2.66; 2.49), H-8 to H-7 (δ_H 2.78), H-7 to 2H-6 (δ_H 1.77; 1.33), and H-6 (δ_H 1.33) to H-5 (δ_H 2.63) were observed. The methyl groups at positions 14 (δ_H 1.63), 15 (δ_H 1.11), and 2' (δ_H 2.05) were assigned based on typical δ_H from neighboring functional groups and similarity with inuchinenolide B (Ito and Iida, 1981). The remaining methine proton and methyl group were assigned to H-11 (δ_H 2.76) and 3H-13 (δ_H 1.26) respectively with correlation in COSY confirming connectivity. Carbon resonances containing protons were unambiguously assigned with ^{13}C-APT and HSQC.

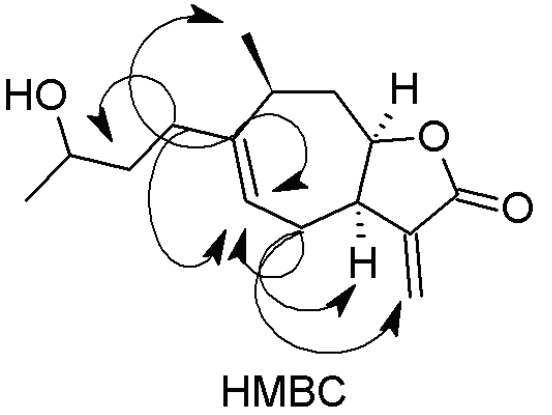
Remaining quaternary carbons were assigned based on similarity to inuchinenolide B (Ito and Iida, 1981). This data confirmed that **5** was the 11,13-dihydro version of inuchinenolide B. A correlation in NOESY between H-7 and H-8 was observed confirming axial and *cis* configuration (Figure 3). Correlations in NOESY from H-7 to H-6 (δ_H 1.77) and H-5 as well as H-8 to H-9 (δ_H 2.49) and H-5 confirmed α configuration of these protons. Both H-6β and H-9β correlated with 3H-13 in NOESY confirming the methyl group was in β orientation. Correlations in NOESY from H-6β to 3H-15 confirmed the methyl group was β orientated and hydroxyl group at position 4 α orientated. The methyl group at 15 also correlated with H-3 (δ_H 2.33) and H-2 confirming their β configuration. Therefore compound **5** was identified as 2α-acetoxy-4α-hydroxy-1(10)-guaiadien-12,8β-olide.

Figure 4. NOESY correlations of **6**.

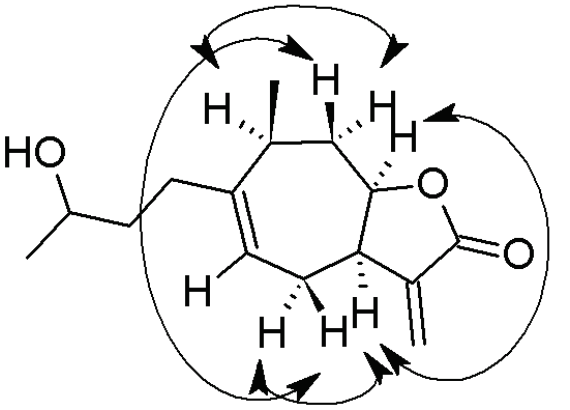


Previously literature concerning **6** did not complete all proton assignments (Serkerov and Mir-Babaev, 1988; Adekenov et al., 1990). By measuring **6** in MeOD with ^{1}H-NMR, COSY, and NOESY all protons and their relative configuration was completed (see experimental) (Figure 4). For **7** it was noted that no ^{13}C-NMR data was available (Dzhazin and Adekenov, 1996). Using ^{13}C-APT and HSQC carbon resonances for **7** were assigned. Close inspection of previous NMR data regarding **10** (Bohlmann et al., 1978; Kim et al., 2004) compared with ^{1}H-NMR, COSY, HMBC, and NOESY spectra obtained revealed incorrect assignments of 2H-2 and 2H-6 (Figure 5). Furthermore no complete ^{13}C-NMR assignments were found for **10**. Therefore the corrected proton assignments, relative configuration based on NOESY, and ^{13}C-NMR assignments are reported for **10** (see experimental).

Figure 5. HMBC (C → H) and NOESY correlations of **10**.



HMBC



NOESY

MDR cancer cell lines with P-gp over-expression (NCI-H460/R, DLD1-TxR and U87-TxR) have been developed from their sensitive counterparts (NCI-H460, DLD1 and U87, respectively) by continuous exposure to increasing concentrations of chemotherapeutic drugs (Pešić et al., 2006; Podolski-Renić et al., 2011). COR-L23/R cell line is an MDR cancer cell line with MRP1 over-expression originating from corresponding sensitive cancer cell line (COR-L23/R). P-gp and MRP1 are ABC-type transporters (ATP-dependent drug efflux pumps) for xenobiotic compounds with broad substrate specificity. Isolated compounds were screened for their activity against non-small cell lung carcinoma (NCI-H460 and COR-L23), colorectal adenocarcinoma (DLD1), glioblastoma (U87), and their respective MDR cancer cell lines. Initially, 10 μM of each compound was tested by Sulforhodamine B assay (SRB) (Table 3).

Compound **2** was not assayed due to low purity (86%), as determined by HPLC at 230 nm. All other compounds were > 90% pure (data not shown). Compounds that achieved more than 50% inhibition (**7-10**) on the majority of cell lines after 10 μM application were selected for further testing. To compare their effects (the IC50 values) toward sensitive (NCI-H460) and MDR (NCI-H460/R) cancer cell lines as well as non-cancer cell line (HaCaT), SRB and MTT assays were applied (Table 4). Doxorubicin, a known anti-cancer drug, was used as a positive control. The results obtained by MTT and SRB assays were similar. Doxorubicin had approximately 100 times weaker activity against NCI-H460/R compared to corresponding sensitive NCI-H460 and 3 to 4 times stronger activity against NCI-H460 compared to non-cancer cell line – HaCaT. Contrary, no selectivity towards NCI-H460, NCI-H460/R or HaCaT was observed for **7-10,** suggesting that these compounds are generally cytotoxic. In addition, this data suggests that sesquiterpene lactones exhibit cytotoxic effects regardless of the presence of P-gp/MRP1.

Table 3. The percentage of cell growth inhibition induced by 10 μM (SRB assay)

Cell Lines	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
NCI-H460	25±9	35±5	n	20±5	13±9	32±6	71±15	83±13	81±6
NCI-H460/R	52±11	5±2	n	n	no	9±5	47±3	90±9	86±1
DLD1	14±6	12±5	n	n	26±9	25±5	86±4	78±2	87±9
DLD1-TxR	28±3	40±13	n	n	52±5	53±6	87±10	80±7	86±7
U87	24±6	20±4	10±4	25±10	26±6	67±4	74±6	63±6	66±2
U87-TxR	36±5	26±2	33±2	21±5	17±4	54±5	68±1	71±5	73±8
COR-L23	44±8	17±7	n	9±1	58±7	66±10	78±10	74±13	78±6
COR-L23/R	48±8	15±7	n	9±5	5±3	41±4	59±1	72±3	67±12

n = no inhibition.

Table 4. The IC50 values (μM) of **7**-**10** in NCI-H460, NCI-H460/R and HaCaT

	NCI-H460*		NCI-H460/R**		HaCaT***	
compounds	MTT	SRB	MTT	SRB	MTT	SRB
7	15.0±0.1	10.6±0.6	20.2±0.5	17.0±0.2	9.3±0.1	16.5±3.3
8	3.8±0.1	3.6±0.2	15.1±0.4	12.1±0.3	4.1±0.1	5.8±0.2
9	4.7±0.1	1.9±0.2	3.3±0.3	7.3±0.1	8.3±0.1	9.5±0.8
10	8.2±0.1	5.7±0.5	4.5±0.1	14.3±0.2	8.0±0.1	19.4±0.1
dox	0.036±0.001	0.037±0.003	4.42±0.17	2.04±0.05	0.146±0.01	0.09±0.01

*NCI-H460 were seeded at 1000 cells/well, **NCI-H460/R were seeded at 2000 cells/well, ***HaCaT were seeded at 4000 cells/well

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Appendix Chapter 6

LC-MS

LC-MS with an Agilent 1100 series HPLC with a G1379A degasser, a G1312A binary pump, a G1367A WPALS (automated liquid sampler), a G1315B DAD detector, and a G1956 LC/MSD SL single quadropole mass spectrometer equipped with an atmospheric pressure chemical ionization probe (APCI) was used for initial mass determination. A 150 x 4.6 mm Luna 5 micron C18 (2) 100A column equipped with a guard column containing C18 4 x 3 mm cartridges was used for separation (Phenomenex Inc, Torrance, California, USA). Gradient elution with a flow of 0.5 mL/min consisted initially of 80% H_2O and 20% ACN which increased to 100% ACN in 20 min and remained at 100% ACN for 5 min. The DAD detector was set at 210 and 254 nm with a UV spectrum scan from 190-390 nm. Mass spectra were acquired in both positive and negative mode with a mass range of 50-500 amu and a fragmentor voltage of 150. The APCI spray chamber gas temperature was set to 350 °C, a vaporizer temperature of 325 °C, a drying gas flow rate of 5 L/min, and a nebulizer pressure of 40 psig. The VCap was set to 4000 V for positive scans and 3000 V for negative scans while the corona current was 5 µA for positive scans and 15 µA for negative scans.

LC-HRESIMS

High resolution mass spectrometry was performed on an LC-LTQ-Orbitrap FTMS system, (Thermo Scientific, Waltham, Massachusetts, USA) with an Accela HPLC, an Accela photodiode array detector, connected to a LTQ/Orbitrap hybrid mass spectrometer equipped with an electrospray (ESI) source. Chromatographic separation used a Phenomenex Luna C18(2) analytical column (150 x 2.0 mm, 3 µm particle size). Both H_2O and acetonitrile contained 0.1 % v/v formic acid, were run at a flow rate of 0.19 mL/min, and a column temperature of 40 °C. A linear gradient from 5 to 75% acetonitrile in 45 min was applied, which was followed by 15 min of washing and equilibration. FTMS full scans (*m/z* 100−1200) were recorded with a resolution of 60.000, whereas for MS^n scans a resolution of 15.000 was used. The FTMS was externally calibrated in negative mode using sodium formate clusters in the range *m/z* 150-1200 and automatic tuning was performed on *m/z* 384.93.

Typical NMR parameters

^{1}H-NMR: Solvent $CDCl_3$, Temperature 298.0, Pulse Sequence zg30, Experiment 1D, Number of Scans 128, Receiver Gain 181, Relaxation Delay 1.0000, Pulse Width 7.3000, Acquisition Time 3.1719, Spectrometer Frequency 500.13, Spectral Width 10330.6, Lowest Frequency -2090.8, Nucleus 1H, Acquired Size 32768, Spectral Size 65536; ^{1}H-^{1}H COSY: Parameter Value (f2, f1), Solvent $CDCl_3$, Temperature 298.0, Pulse Sequence cosygpqf, Experiment COSY, Number of Scans 4, Receiver Gain 256, Relaxation Delay 1.4869, Pulse Width 7.3000, Acquisition Time 0.2048, Spectrometer Frequency (500.13, 500.13), Spectral Width (5000.0, 5000.0),

Lowest Frequency (-263.4, -263.2), Nucleus (1H, 1H), Acquired Size (1024, 512), Spectral Size (1024, 1024); ^{13}C-APT: Solvent $CDCl_3$, Temperature 298.0, Pulse Sequence jmod, Experiment JMOD, Number of Scans 10240, Receiver Gain 23170, Relaxation Delay 2.0000, Pulse Width 9.5000, Acquisition Time 1.0912, Spectrometer Frequency 125.77, Spectral Width 30030.0, Lowest Frequency -2421.9, Nucleus 13C, Acquired Size 32768, Spectral Size 65536; HSQC: Parameter Value (f2, f1), Solvent $CDCl_3$, Temperature 298.1, Pulse Sequence hsqcetgp, Experiment HSQC, Number of Scans 4, Receiver Gain 20642, Relaxation Delay 1.5000, Pulse Width 7.3000, Acquisition Time 0.4096, Spectrometer Frequency (500.13, 125.77), Spectral Width (5000.0, 20833.3), Lowest Frequency (-312.0, -1044.5), Nucleus (1H, 13C), Acquired Size (2048, 512), Spectral Size (2048, 2048); HMBC: Parameter Value (f2, f1), Solvent $CDCl_3$, Temperature 298.0, Pulse Sequence hmbcgplpndqf, Experiment HMBC, Number of Scans 4, Receiver Gain 23170, Relaxation Delay 1.5000, Pulse Width 7.3000, Acquisition Time 0.2925, Spectrometer Frequency (500.13, 125.77), Spectral Width (7002.8, 26411.8), Lowest Frequency (-1315.3, -642.0), Nucleus (1H, 13C), Acquired Size (2048, 512), Spectral Size (2048, 2048); NOESY: Parameter Value (f2, f1), Solvent $CDCl_3$, Temperature 298.0, Pulse Sequence noesygpphpp, Experiment NOESY, Number of Scans 4, Receiver Gain 1626, Relaxation Delay 1.5000, Pulse Width 7.3000, Acquisition Time 0.2048, Spectrometer Frequency (500.13, 500.13), Spectral Width (5000.0, 5000.0), Lowest Frequency (-163.0, -163.5), Nucleus (1H, 1H), Acquired Size (1024, 400), Spectral Size (1024, 1024).

Gaillardin (**8**): white needles; $[\alpha]^{20}_{D}$ -2.1 (*c* 0.09, $CHCl_3$); APCIMS *m/z* 307 $[M + H]^+$ (35), 305 $[M - H]^-$ (5); HRESIMS *m/z* 307.1543 $[M + H]^+$ (calcd for $C_{17}H_{23}O_5$ 307.15455).

Britannin (**9**): clear crystals; $[\alpha]^{20}_{D}$ -15.1 (*c* 0.11, $CHCl_3$); APCIMS *m/z* 367 $[M + H]^+$ (100), 365 $[M - H]^-$ (100); HRESIMS *m/z* 367.1755 $[M + H]^+$ (calcd for $C_{19}H_{27}O_7$ 367.1757).

Table A1. ^{1}H-NMR and COSY data for compounds **8** and **9**

	8			**9**	
^{1}H	δ_H (J in Hz)	COSY	^{1}H	δ_H (J in Hz)	COSY
			1α	1.94, m*	2β
1β	2.46, br d	2β, 5, 9, 14			
2β	5.34, m	1, 3α,β	2β	4.9, br dd	1, 3α
3α	2.03, d (15.4)	3β	3α	2.07, m	2, 3β, 4
3β	1.93, dd (15.4, 4)	2β, 3α	3β	1.83, m	3α, 4
			4α	4.12, ddd (11.5, 8.2, 3.5)	3α,β, OH
5α	2.17, td (12.2, 3)	1, 6α,β			
6α	2.56, m	5, 6β, 7			
6β	1.41, m	5, 6α, 7	6β	5.06, d (8.3)	7
7	2.62, m	6α,β 8, 13a,b	7	3.03, m	6, 8, 13a,b
8	4.5, br d	7, 9, 14	8	4.53, m	7, 9α,β
9	5.94, br s	1, 8, 14	9α	2.45, m	8, 9β, 10
			9β	1.44, dt	8, 9α, 10
			10β	1.91, m*	9α,β, 14
13a	6.23, d (3.3)	7	13a	6.17, d (3.5)	7
13b	5.55, d (3.1)	7	13b	5.37, d (3.2)	7
14	1.83, br s		14	0.99, d (5.9)	10
15	1.26, s		15	1.02, s	
Ac	2.11, s		Ac	2.05, s	
			Ac	2.25, s	
			OH	2.38, d (3.5)	4

*Overlap

Figure A1. ^{1}H-NMR of 14-(3-methylpentanoyl)-6-deoxybritannilactone (**1**) ($CDCl_3$)

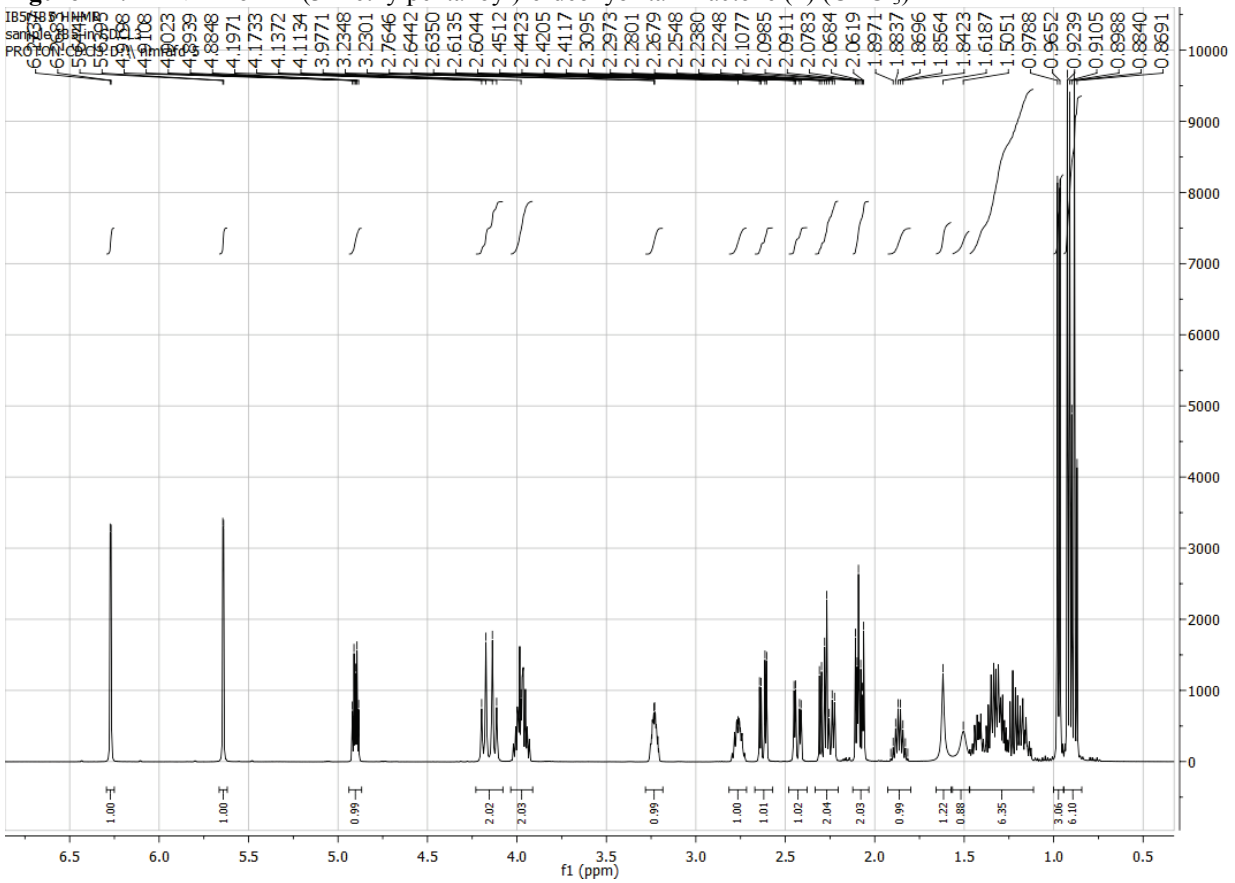

Figure A2. ^{1}H-NMR of 14-(3-methylbutanoyl)-6-deoxybritannilactone (**2**) ($CDCl_3$)

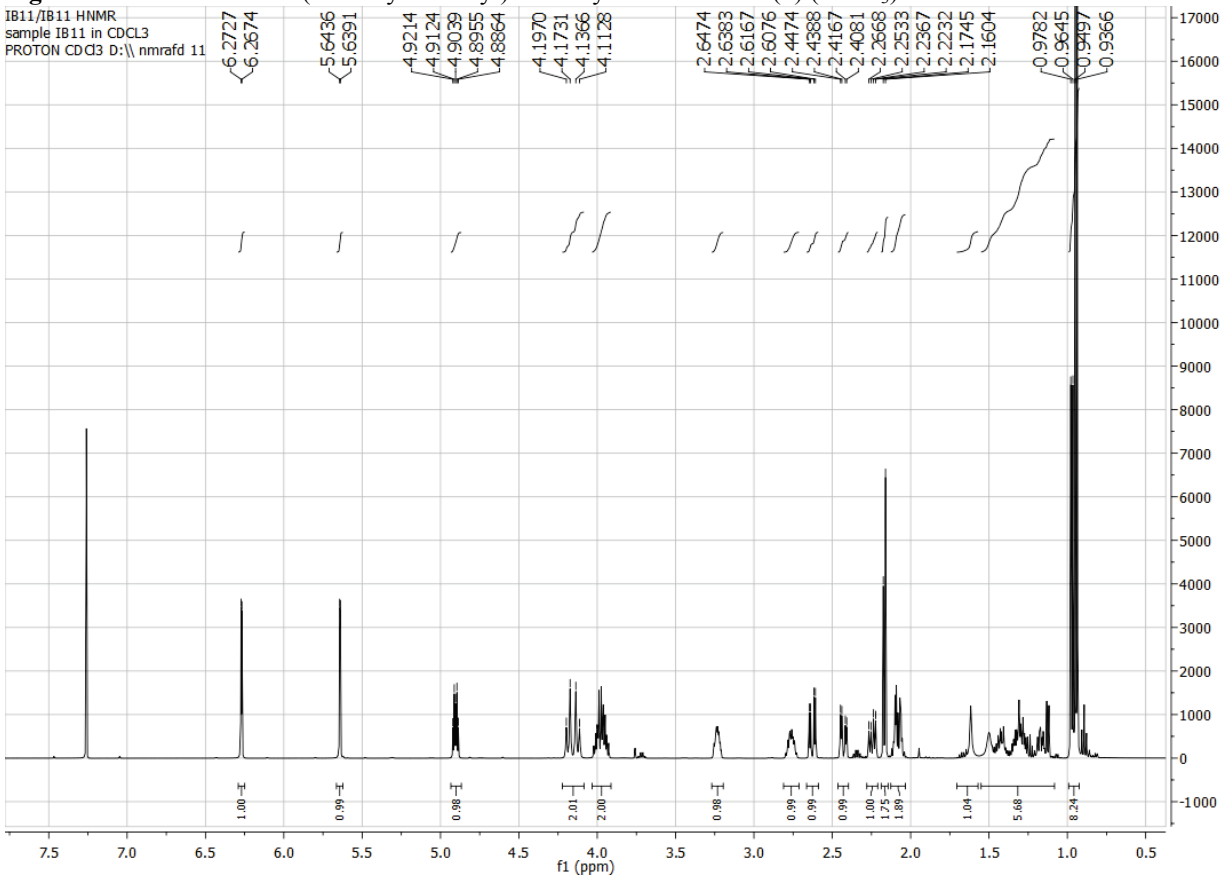

Figure A3. ^{1}H-NMR of 14-(2-methylpropanoyl)-6-deoxybritannilactone (**3**) ($CDCl_3$)

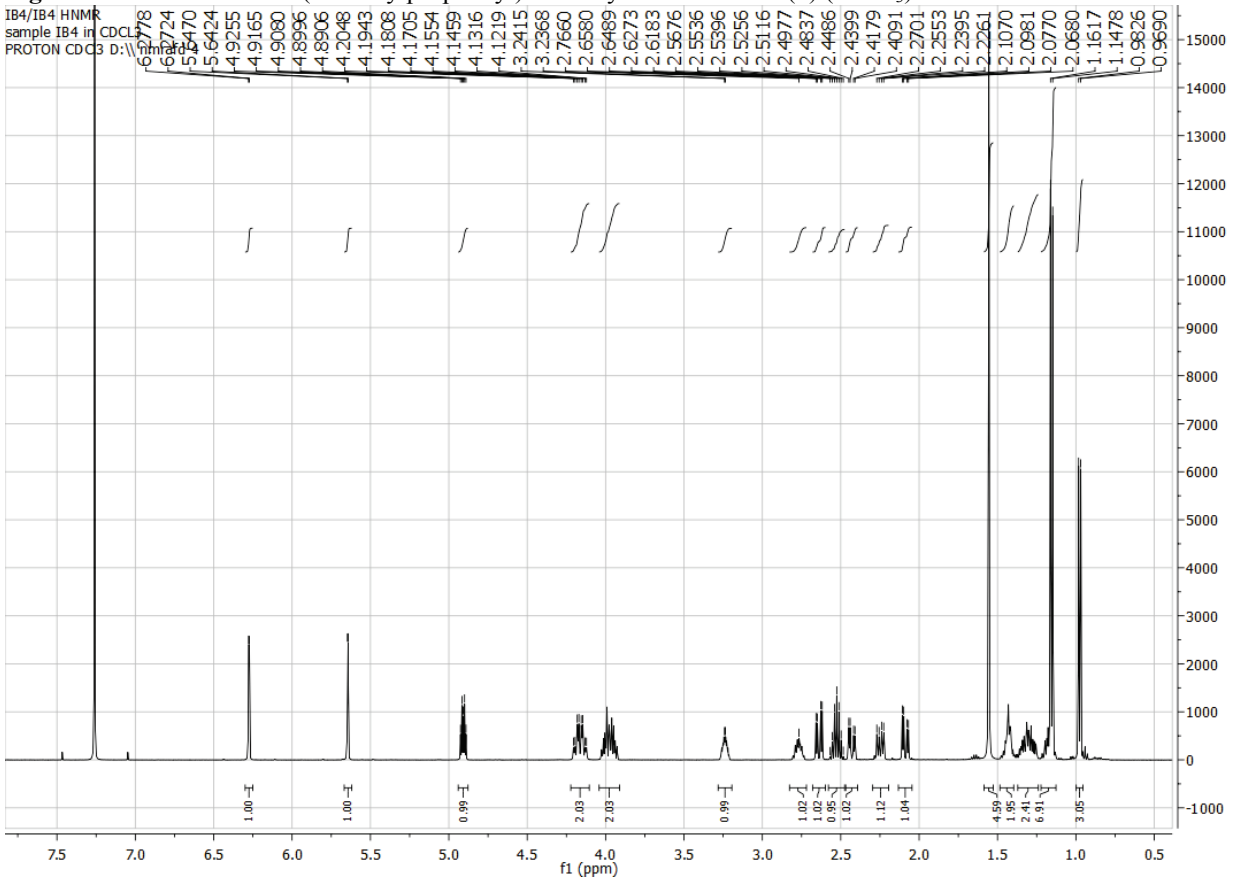

Figure A4. [1]H-NMR of 1,3-Epi-Granilin (**4**) (MeOD)

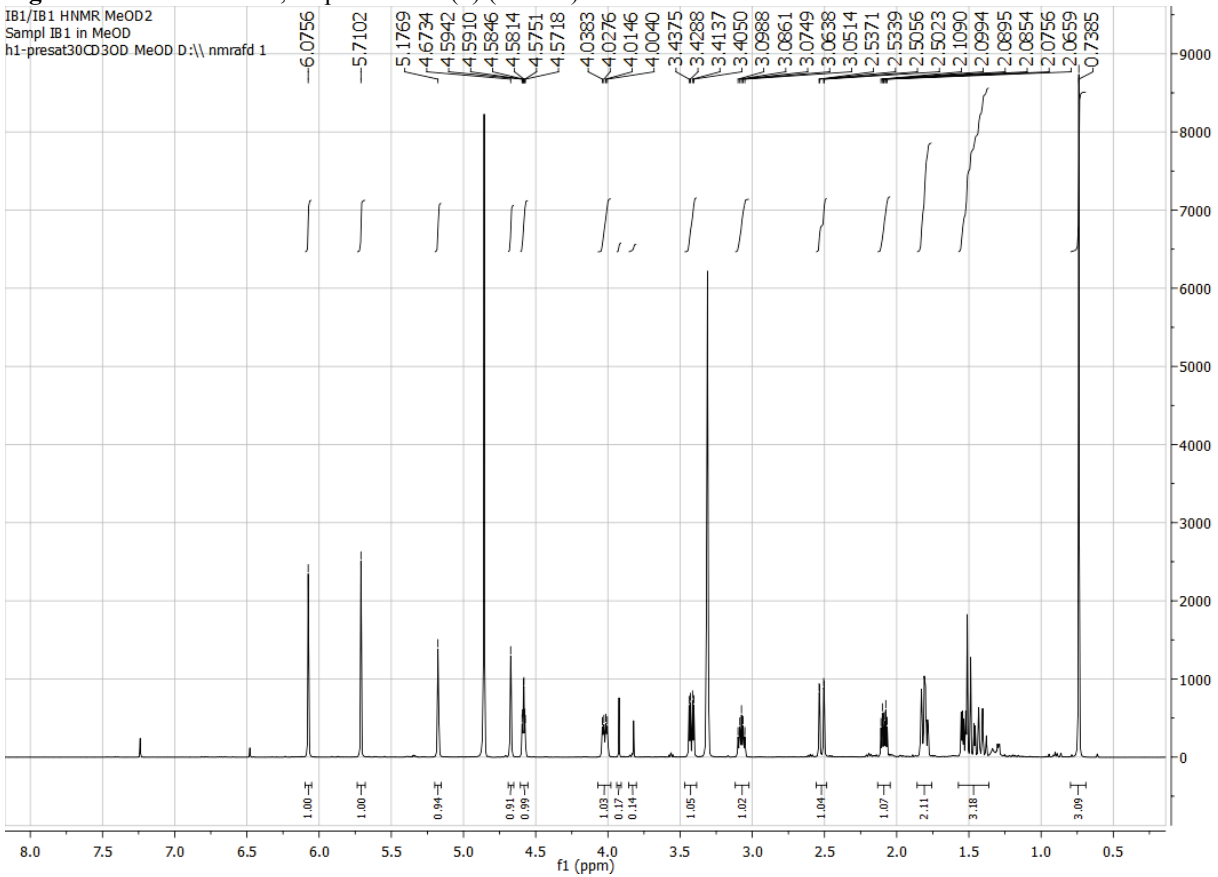

Figure A5. ^{1}H-NMR of 11,13-Dihydro-inuchinenolide B (**5**) ($CDCl_3$)

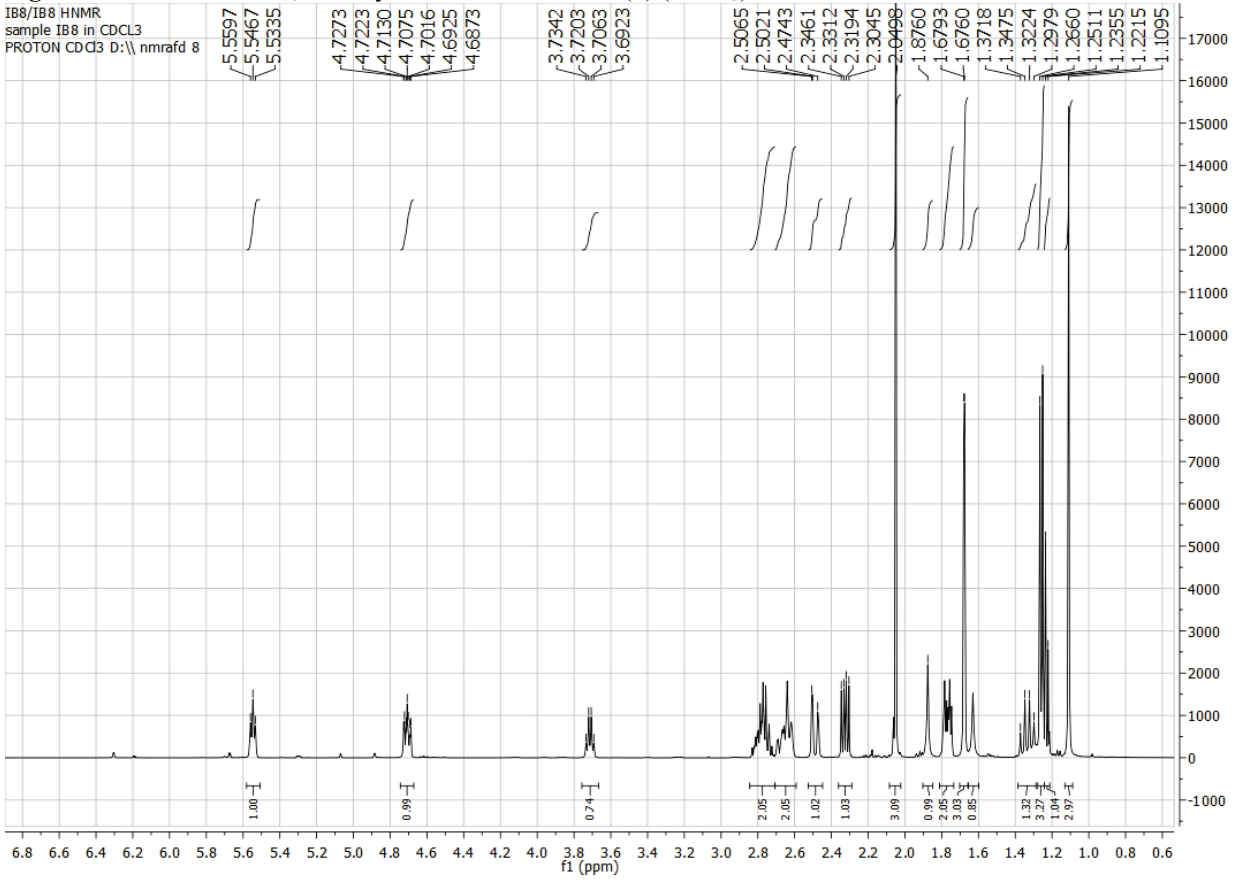

Figure A6. ^{1}H-NMR of Pulchellin C (**6**) (MeOD)

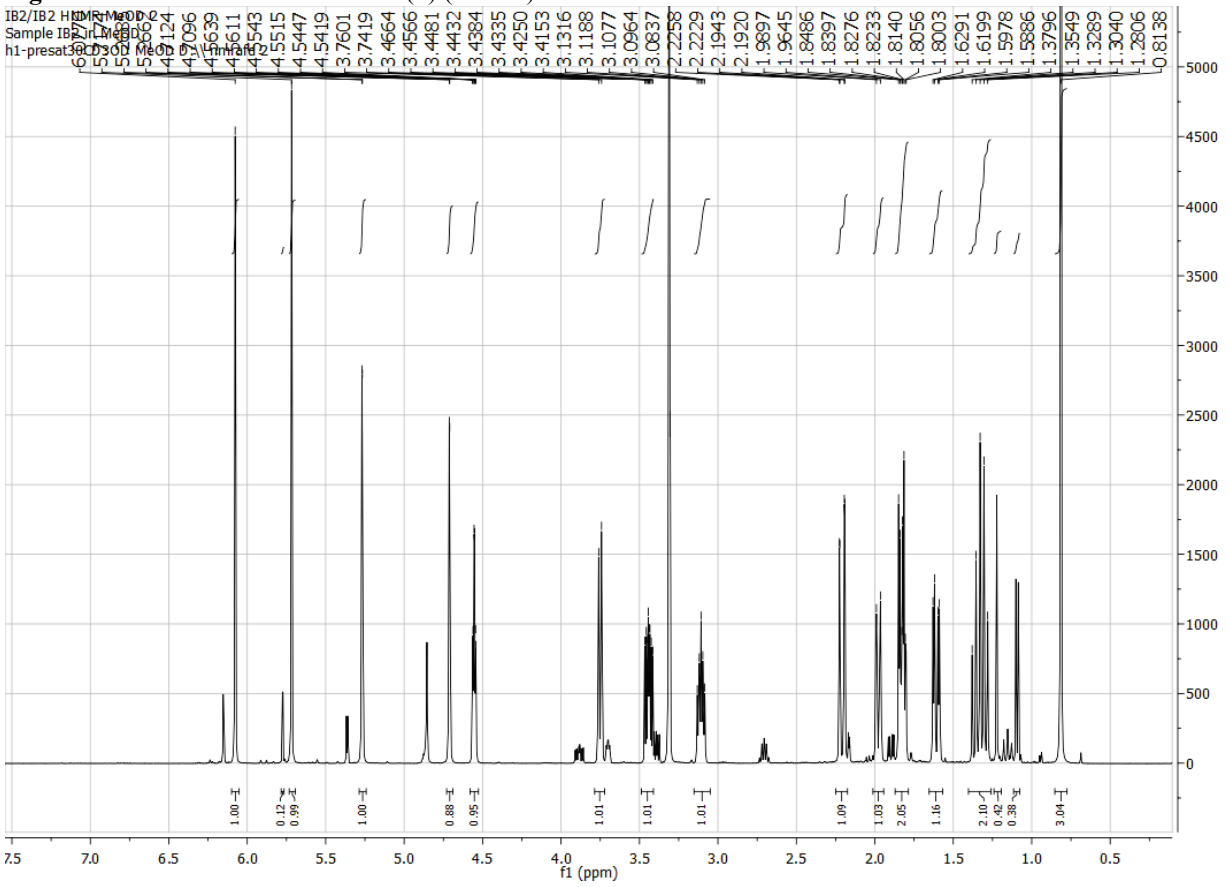

Figure A7. ^{1}H-NMR of 6-Deacetylbritanin (**7**) ($CDCl_3$)

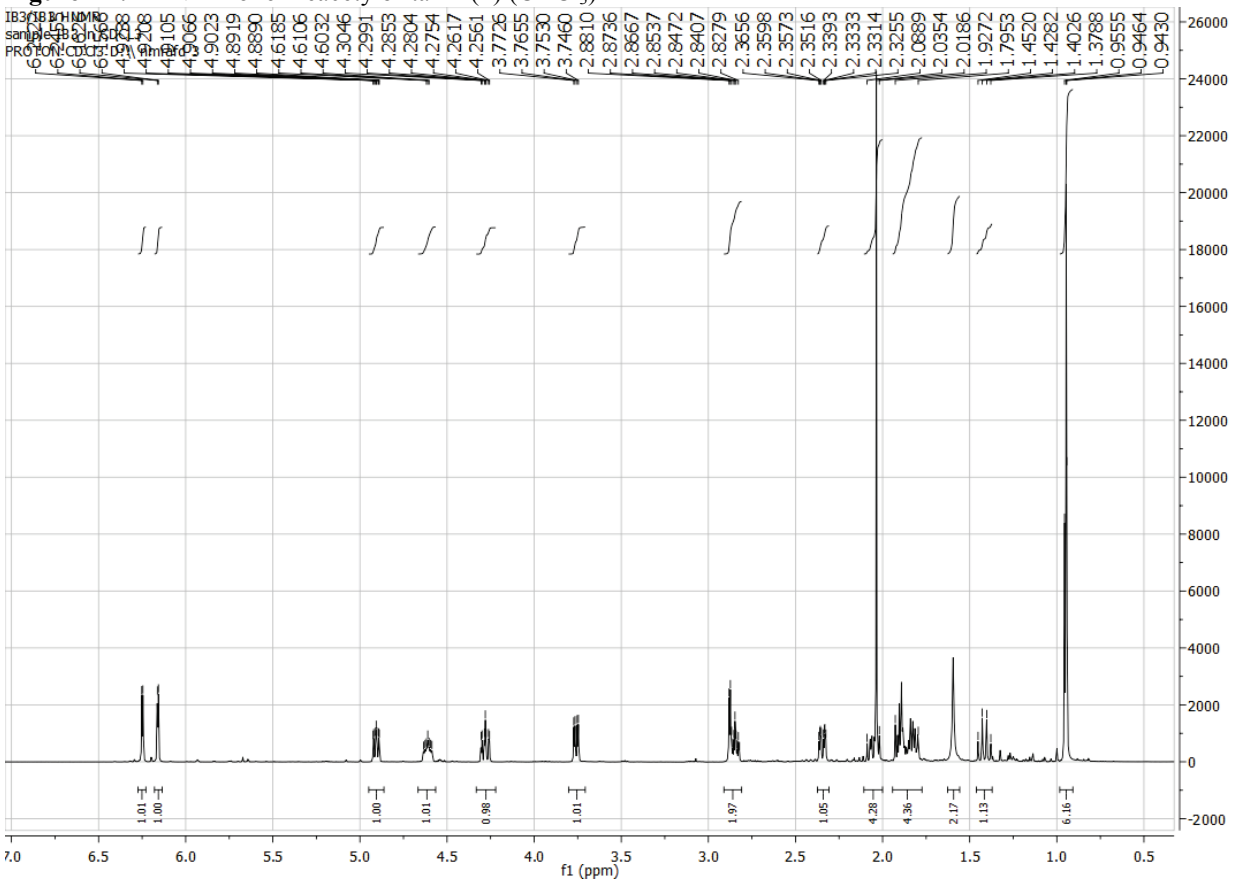

Figure A8. ^{1}H-NMR of Gaillardin (**8**) ($CDCl_3$)

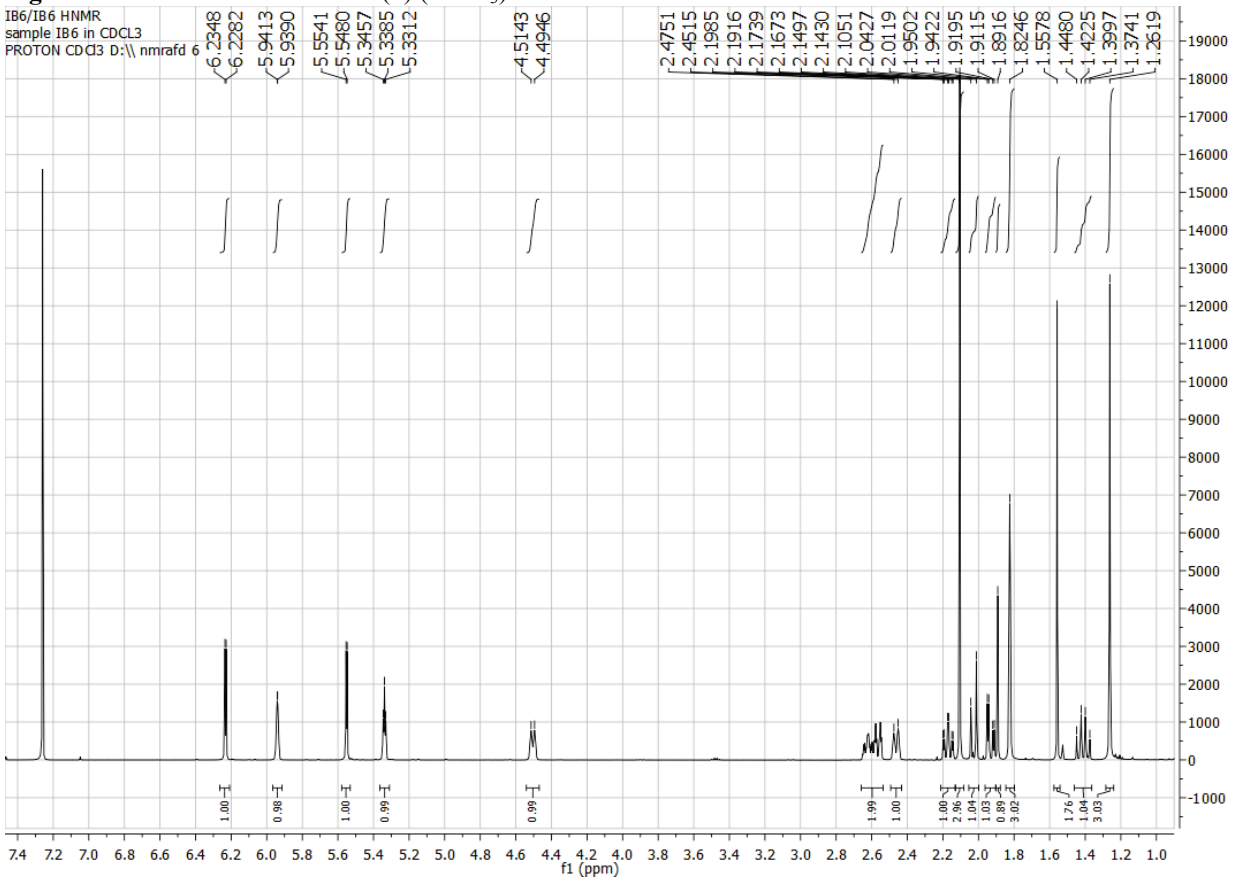

Figure A9. ^{1}H-NMR of Britannin (**9**) ($CDCl_3$)

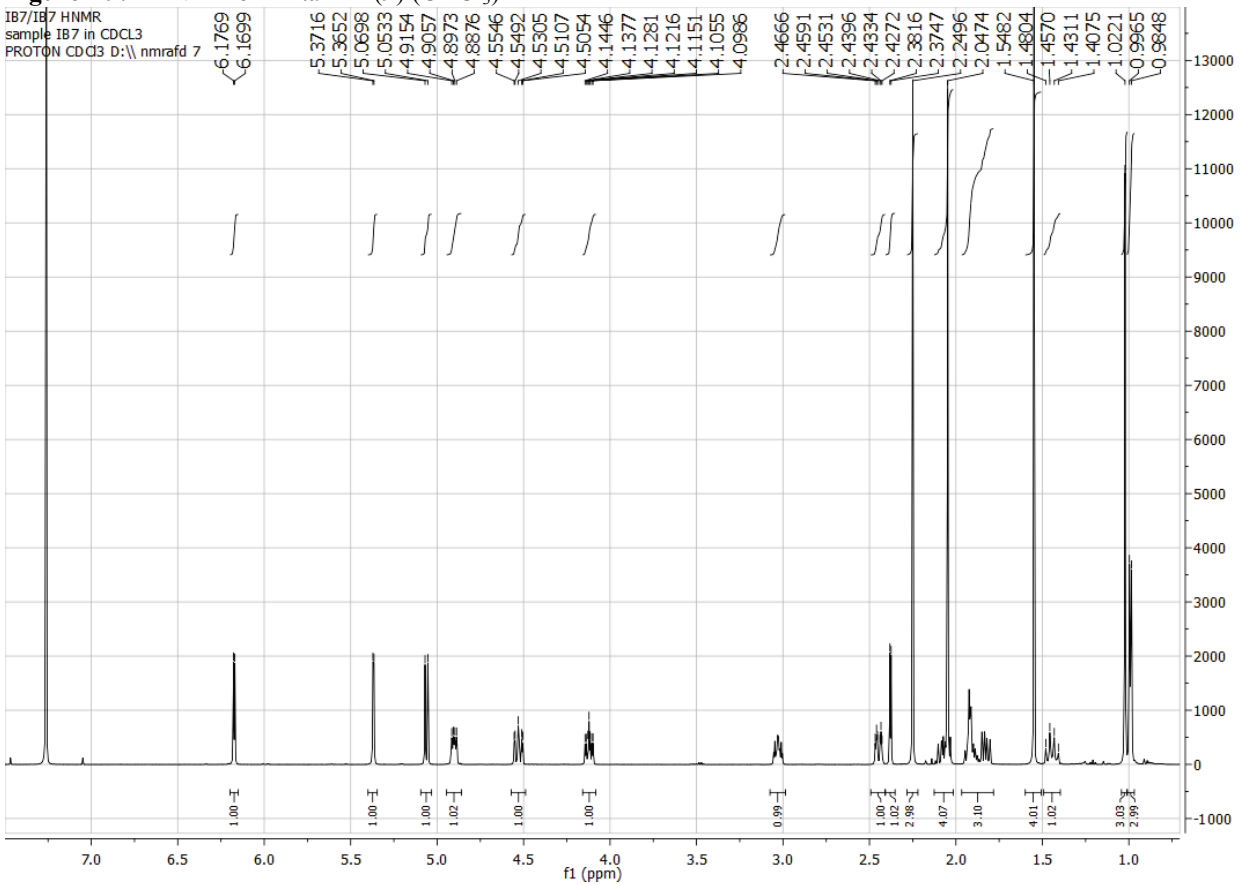

Figure A10. ^{1}H-NMR of 4H-Tomentosin (**10**) ($CDCl_3$)