

## ***Supporting Information for***

### **The Alternative Medicine Pawpaw and Its Acetogenin Constituents Suppress Tumor Angiogenesis via the HIF-1/VEGF Pathway**

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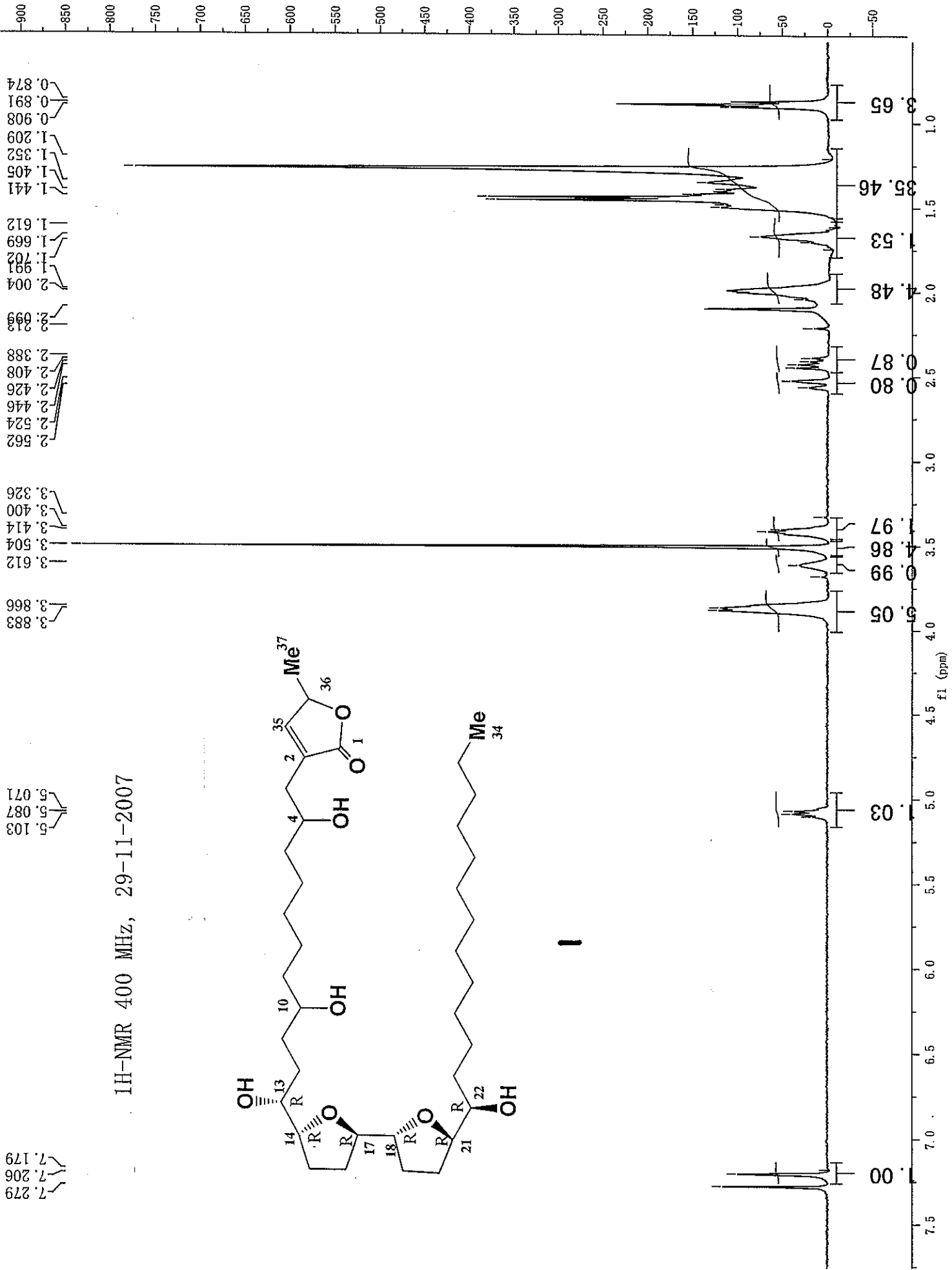
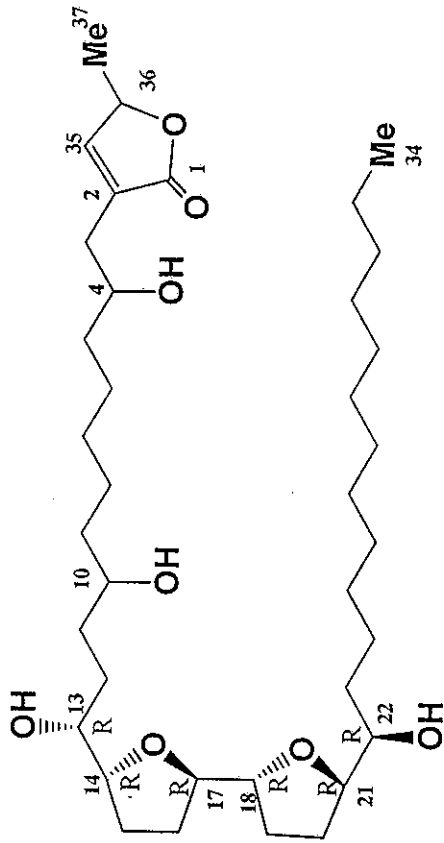
<sup>‡</sup>Department of Biology

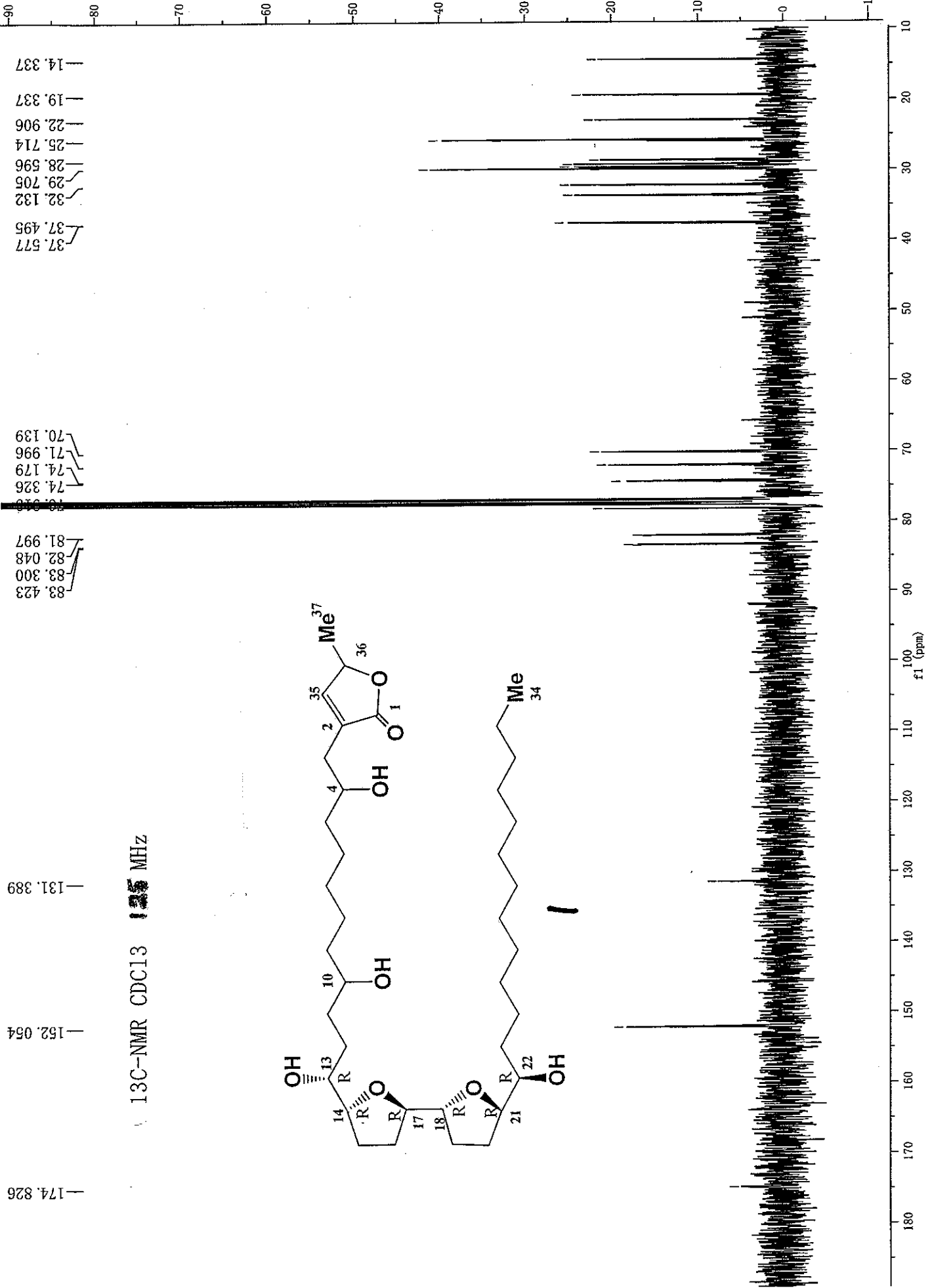
<sup>§</sup>Research Institute of Pharmaceutical Sciences

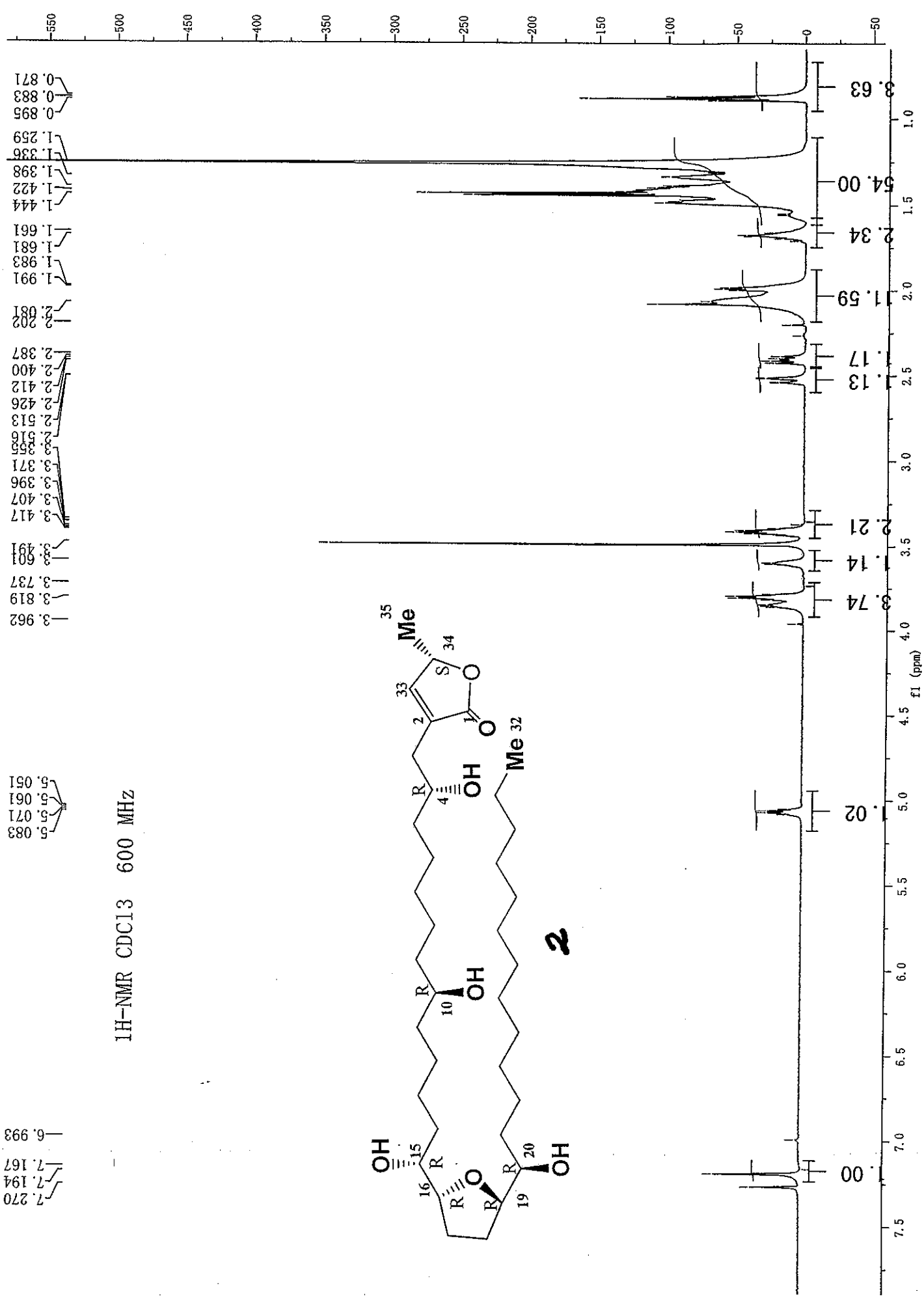
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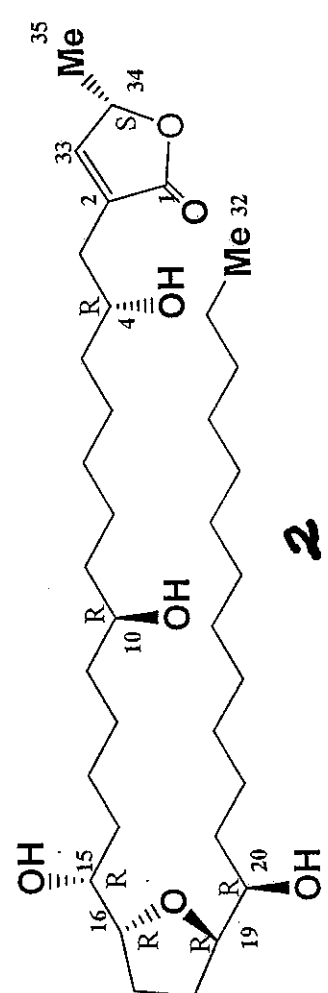
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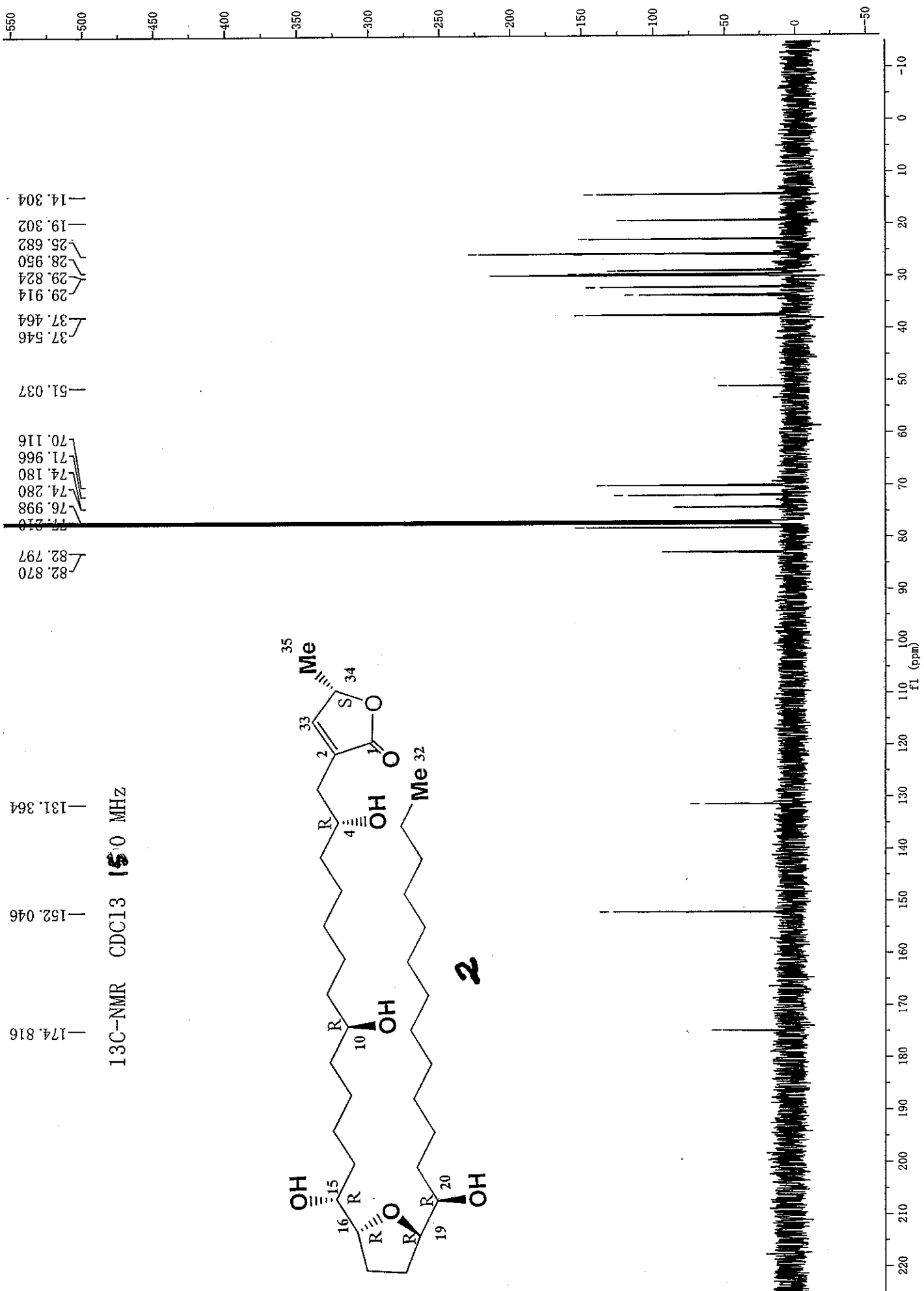


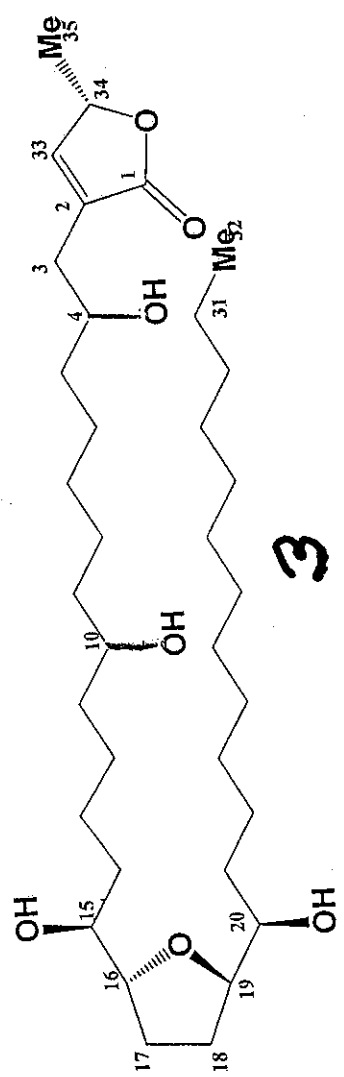
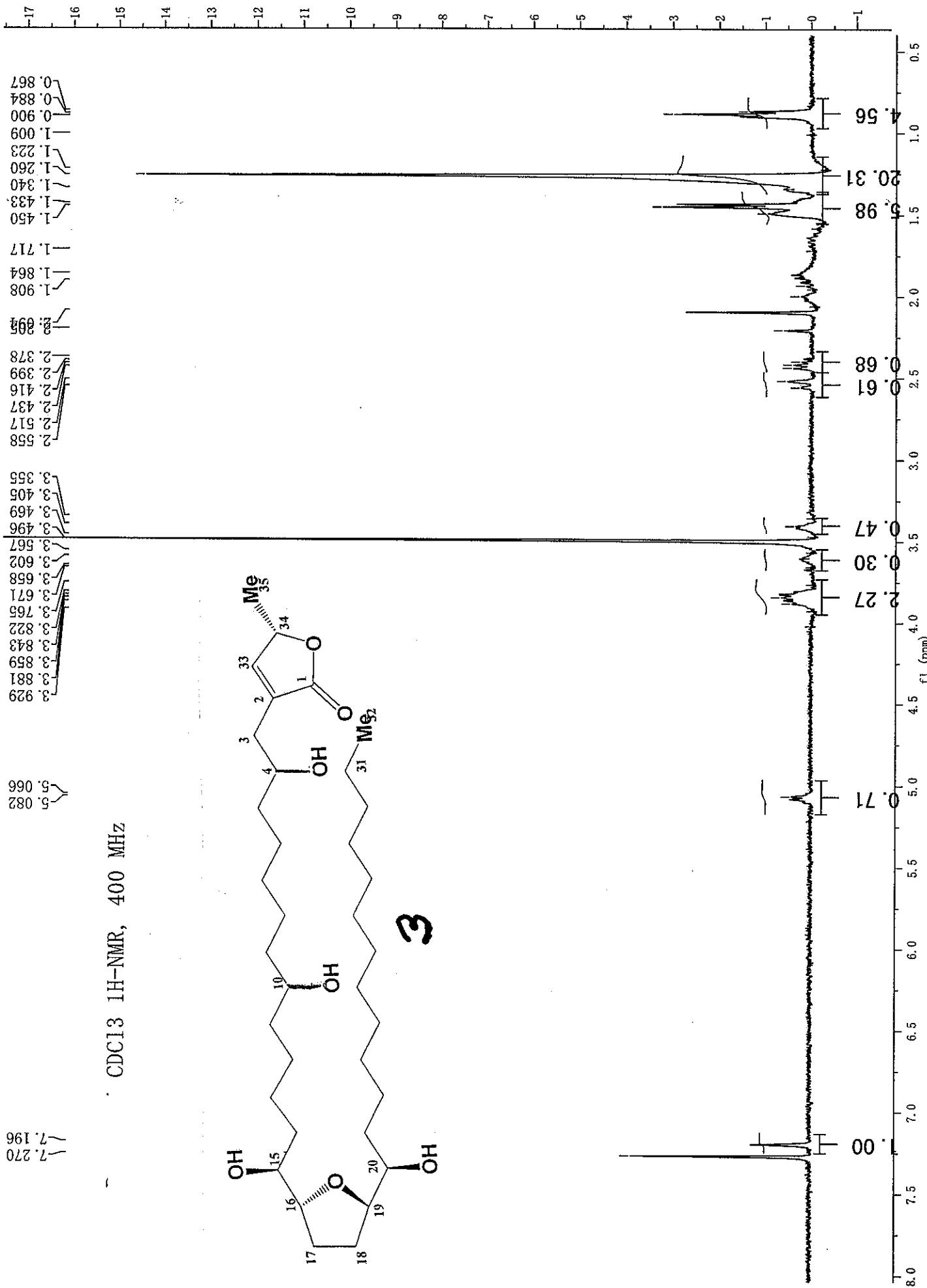


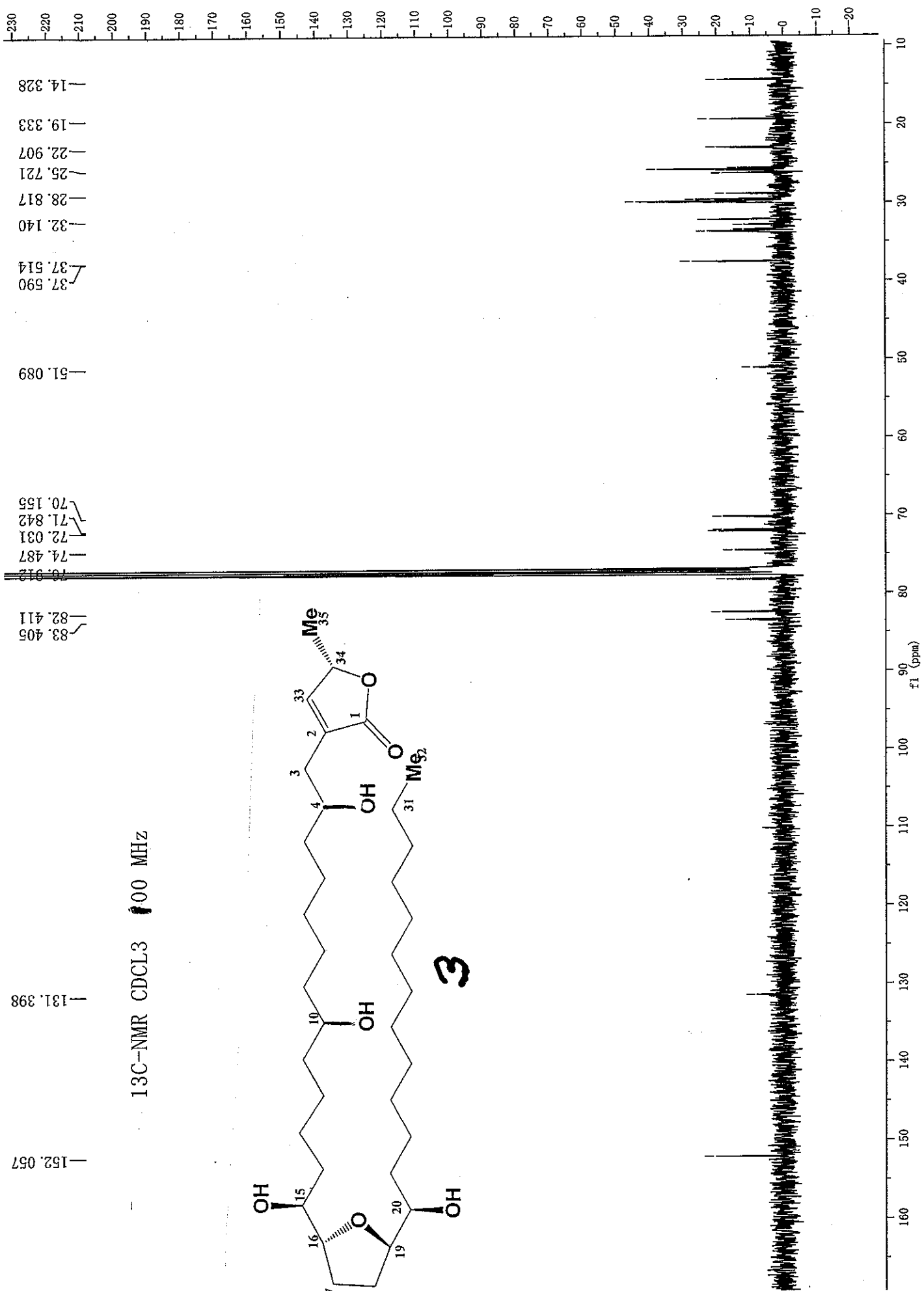


1H-NMR CDCl3 600 MHz











### Bioassay-Guided Isolation of Compounds 1 – 3

The crude extract (3.6 g) was separated by HP-20SS column chromatography that was eluted with step gradients of MeOH/H<sub>2</sub>O (1:1), MeOH, EtOAc, CH<sub>2</sub>Cl<sub>2</sub>, and hexanes. The active fraction (94% inhibition at 0.1 µg/mL) was subjected to Sephadex LH-20 column separation eluted with CHCl<sub>3</sub>/MeOH (1:1). The active subfraction (92% inhibition at 0.02 µg/mL) was further separated by Sephadex LH-20 CC eluted with petroleum ether/CHCl<sub>3</sub>/MeOH (2:1:1). Final purification by HPLC (Luna<sup>®</sup> 5µ, ODS-3 100 Å, 250 x 10.0 mm, isocratic 65% CH<sub>3</sub>CN in water, 4.0 mL per min) afforded three compounds: **1** (1.8 mg, 0.05% yield, *t<sub>R</sub>* 29 min), **2** (0.7 mg, 0.02% yield, *t<sub>R</sub>* 33 min) and **3** (3.4 mg, 0.09% yield, *t<sub>R</sub>* 40 min).

### <sup>1</sup>H NMR, <sup>13</sup>C NMR, Optical Rotation and MS Data of Compounds 1 – 3

**10-Hydroxyglaucanetin (1):** White powder;  $[\alpha]_D^{24} +14.5$  (*c* 0.038, MeOH); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz): δ 7.18 (1H, br s, H-35), 5.09 (1H, d, *J* = 6.8 Hz, H-36), 3.87 (5H, m, H-4, 14, 17, 18, 21), 3.61 (1H, br s, H-10), 3.40 (2H, m, H-13, 21), 2.54 (1H, br d, *J* = 15.4 Hz, H-5a), 2.42 (1H, dd, *J* = 15.4, 8.0 Hz, H-5b), 1.70-2.00 (8H, m, H-15, 16, 19, 20), 1.45 (3H, d, *J* = 6.8 Hz, H-37), 0.89 (3H, t, *J* = 6.8 Hz, H-34); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz): δ 174.8 (C, C-1), 152.1 (CH, C-35), 131.4 (C, C-2), 83.4 (CH, C-21), 83.3 (CH, C-14), 82.1 (CH, C-18), 82.0 (CH, C-17), 78.2 (CH, C-36), 74.3 (CH, C-13 or 22), 74.2 (CH, C-13 or 22), 72.0 (CH, C-10), 70.1 (CH, C-4), 19.3 (CH<sub>3</sub>, C-37), 14.3 (CH<sub>3</sub>, C-34); LRESIMS *m/z* 639 [M+H]<sup>+</sup> and 657 [M+H<sub>2</sub>O+H]<sup>+</sup>.

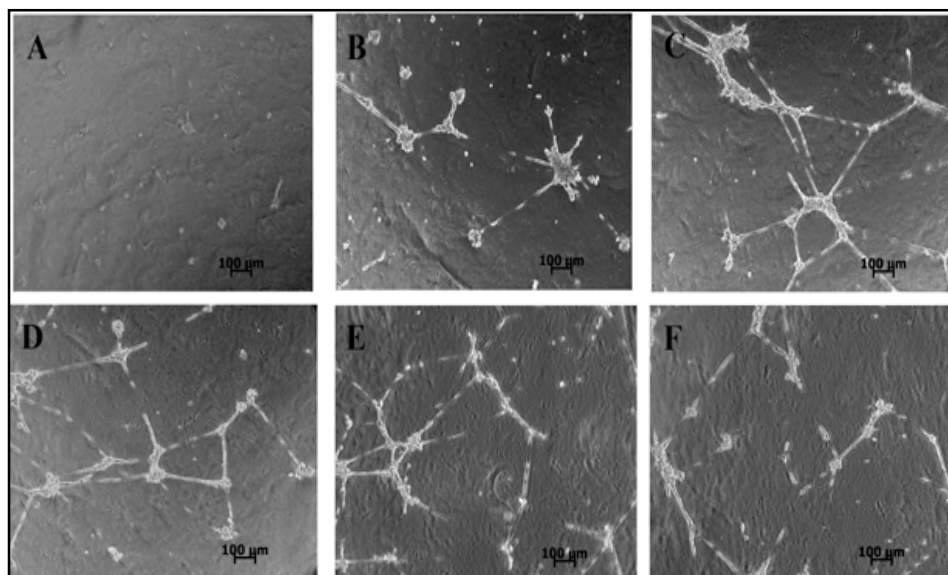
**Annonacin (2):** White powder;  $[\alpha]_D^{24} +3.46$  (*c* 0.15, MeOH); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz): δ 7.17 (1H, br s, H-33), 5.06 (1H, d, *J* = 6.6 Hz, H-34), 3.86 (1H, m, H-4), 3.78 (2H, m, H-16, 19), 3.60 (1H, br d, *J* = 3.0 Hz H-10), 3.40 (2H, dt, *J* = 11.4, 5.4 Hz, H-15, 20), 2.53 (1H, dd, *J* = 15.0, 1.2 Hz, H-5a), 2.41 (1H, dd, *J* = 15.0, 8.4 Hz, H-5b), 1.99 (2H, m, H-17b, 18a), 1.68 (2H, m, H-17a, 18b), 1.41 (3H, d, *J* = 6.6 Hz, H-35), 0.88 (3H, t, *J* = 7.2 Hz, H-32); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz): δ 152.1 (CH, C-33), 131.4 (C, C-2), 82.9 (CH, C-16 or 19), 82.8 (CH, C-16 or 19), 78.2 (CH, C-34), 174.8 (C, C-1), 74.3 (CH, C-15 or 20), 74.2 (CH, C-15 or 20), 72.0 (CH, C-10), 70.1 (CH, C-4), 37.6 (CH<sub>2</sub>, C-5), 19.3 (CH<sub>3</sub>, C-35), 14.3 (CH<sub>3</sub>, C-32); LRESIMS *m/z* 619 [M+Na]<sup>+</sup> and 1215 [2M+Na]<sup>+</sup>.

**Annonacin A (3):** White powder;  $[\alpha]_D^{24} +18.66$  (*c* 0.033, CH<sub>2</sub>Cl<sub>2</sub>); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz): δ 7.20 (1H, br s, H-33), 5.07 (1H, d, *J* = 6.8 Hz, H-34), 3.84 (3H, m, H-4, 16, 19), 3.57 (1H, m, H-10), 3.40 (1H, dt, *J* = 11.4, 5.4 Hz, H-20), 3.36 (1H, m, H-15), 2.54 (1H, dd, *J* = 16.4, 8.4 Hz, H-5a), 2.41 (1H, br d, *J* = 16.4 Hz, H-5b), 2.00 (2H, m, H-17b, 18a), 1.72 (2H, m, H-17a, 18b), 1.44 (3H, d, *J* = 6.8 Hz, H-35), 0.88 (3H, t, *J* = 6.8 Hz, H-32); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz): δ 174.8 (C, C-1), 152.1 (CH, C-33), 131.4 (C, C-2), 83.4 (CH, C-19), 82.4 (CH, C-16), 78.2 (CH, C-34), 74.4 (CH, C-15), 72.0 (CH, C-10), 71.8 (CH, C-20), 70.2 (CH, C-4), 37.6 (CH<sub>2</sub>, C-5), 19.3 (CH<sub>3</sub>, C-35), 14.3 (CH<sub>3</sub>, C-32); LRESIMS *m/z* 619 [M+Na]<sup>+</sup>.

### Detailed Procedure for Immunofluorescence Analysis for HIF-1 $\alpha$ Protein

T47D cells ( $1.5 \times 10^5$  cells/well) were seeded onto 4-well chamber slides (Lab-Tek II, Nunc) in DMEM/F12 medium supplemented with 10% FBS. After the cells attached (overnight at 37 °C), half of the conditioned media was replaced with serum-free media containing compound. Thirty minutes later, the cells were exposed to specified oxygen condition for 4 h, fixed with a freshly prepared solution of 3.7% formaldehyde (Sigma) for 10 min, and permeabilized with 0.1% Triton X-100 in 1x PBS (pH 7.4, Fisher Scientific) for 5 min. After blocking with 1% BSA (fraction V,  $\geq 96\%$ , Sigma) in 1x PBS for 1 h, the cells were incubated with a monoclonal anti-HIF-1 $\alpha$  antibody (BD Biosciences, 1:200 dilution in 1x PBS containing 0.1% BSA) at 4°C overnight. After three washes with 1x PBS containing 0.1% Tween 20, the cells were incubated with Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, 1:250 dilution) for 1 h at room temperature. The slides were mounted with Vectashield® mounting medium with DAPI (Vector Laboratories). The samples were visualized with the Plan-Apochromat 63x oil immersion objective and the images acquired using a LSM 510 META laser scanning confocal microscope system (Zeiss). Images were processed with Adobe Photoshop CS3.

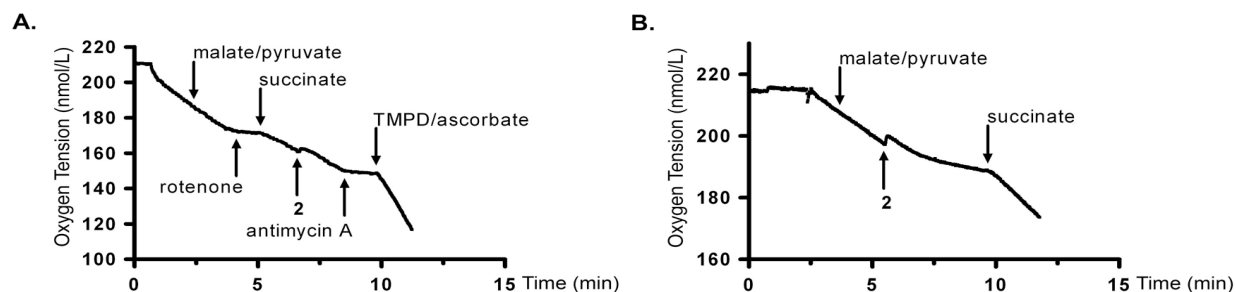
Effects of Compounds 1 and 2, and CE on Normoxic Tumor Angiogenesis *in vitro*



**Figure S1. Effects of compounds 1 and 2, and pawpaw crude extract on the ability of normoxic T47D cells to induce angiogenesis *in vitro***

The effects of normoxic T47D cell conditioned media on angiogenesis *in vitro* were evaluated in a HUVEC tube formation assay. Representative pictures are shown and each panel includes a 100  $\mu\text{m}$  scale bar. The conditions are: (A) basal media (negative control); (B) basal media supplemented with 20 ng recombinant human VEGF protein (R & D, positive control); (C) conditioned media (CM) sample from normoxic T47D cells; (D) CM sample from normoxic T47D cells treated with **1** (0.1  $\mu\text{M}$ ); (E) CM sample from normoxic T47D cells treated with **2** (0.1  $\mu\text{M}$ ); and (F) CM sample from normoxic T47D cells treated with pawpaw crude extract (0.1  $\mu\text{g/mL}$ ).

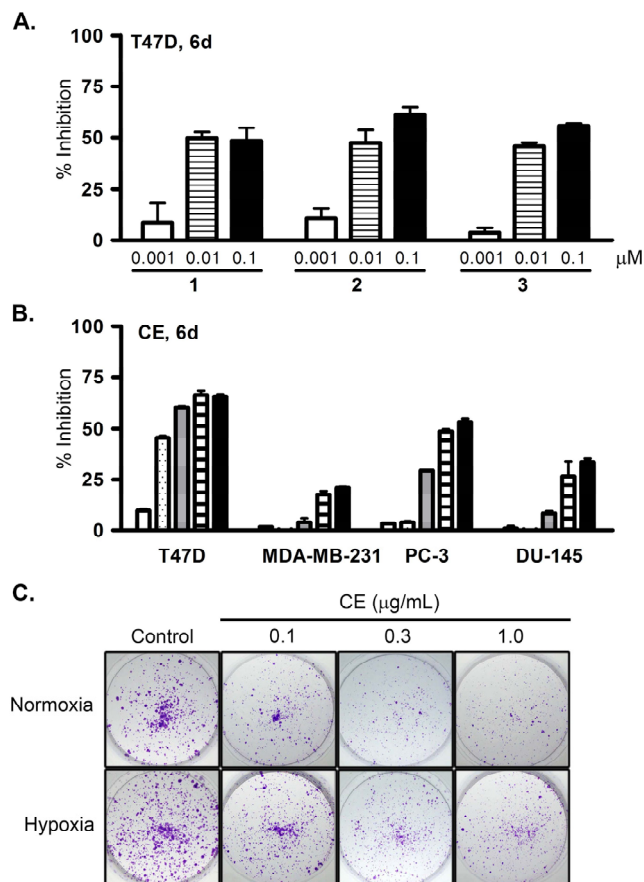
## Annonaicin (2) Inhibits Mitochondrial Respiration at Complex I in T47D Cells



**Figure S2. Compound 2 (annonaicin) inhibits oxygen consumption in T47D cells by suppressing mitochondrial respiration at complex I**

In T47D cells, compound 2 (0.3  $\mu$ M) does not affect mitochondrial respiration at complex II, III, or IV (A). Substrates and inhibitors were added in a sequential manner to T47D cells ( $5 \times 10^6$ , 30  $^{\circ}$ C) at the specified time point. Compound 2 suppresses mitochondrial respiration by inhibiting complex I (B).

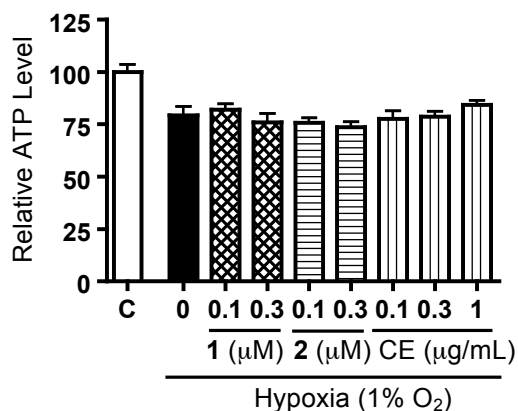
## Acetogenins and Pawpaw Crude Extract Suppress Cell Proliferation/Viability in a Cell Line-Dependent Manner



### Figure S3. Cell line-dependent effects of compounds 1 – 3 and pawpaw crude extract on proliferation/viability and colony formation

(A) Effects of compounds 1 – 3 on the proliferation/viability of T47D cells under normoxic conditions (95% air, 6 d) were quantified using the sulphorhodamine B method. The conditioned media were replaced with extract/compound in fresh media after three days. Absorbance at 490 nm (background at 690 nm) was measured using a BioTek Synergy HT microplate reader, and presented as % inhibition of the untreated control. Data shown are mean  $\pm$  SD ( $n = 3$ ). (B) Concentration-response results of pawpaw crude extract (CE) on T47D, MDA-MB-231, PC-3, and DU-145 cell proliferation/viability after 6 day exposure were presented as % inhibition of the control [0.01  $\mu\text{g/mL}$  (open bar), 0.03  $\mu\text{g/mL}$  (dotted bar), 0.1  $\mu\text{g/mL}$  (gray bar), 0.3  $\mu\text{g/mL}$  (striped bar), and 1  $\mu\text{g/mL}$  (black bar)]. Data are mean  $\pm$  SD ( $n = 3$ ). (C) Representative images from a T47D cell-based clonogenic survival assay. T47D cells were seeded at the density of 1,000 cells/well into 6-well plates (Cellstar) and incubated at 37  $^{\circ}\text{C}$  for 4 h to allow the cells to adhere. The cells were exposed to normoxic or hypoxic conditions (95% air or 1%  $\text{O}_2$ , respectively) in the presence of pawpaw crude extract for 24 h. The conditioned media were replaced with fresh DMEM/F12 media containing FBS (10%) and P/S. The cells were incubated for an additional 14 days with a medium change every 5 days, fixed with MeOH, stained with crystal violet (1 mg/mL in 20% EtOH), and the images acquired with a Kodak digital camera..

Effects of **1**, **2**, and Pawpaw Crude Extract on the Levels of ATP in T47D Cells under Experimental Conditions



**Figure S4. Compounds **1**, **2** and pawpaw crude extract does not inhibit ATP levels under hypoxia**

T47D cells were exposed to hypoxic conditions (1% O<sub>2</sub>, 37 °C, 16 h) in the presence of compounds **1** and **2**, and pawpaw crude extract (CE) at the specified concentrations. The cells were lysed and the levels of ATP determined using a commercial kit (ATPlite™, PerkinElmer). Luminescence was measured on a BioTek Synergy HT microplate reader. Data are mean ± SD of the normoxic control "C" (n = 3). There is no statistically significant difference between the hypoxic control and the treated samples ( $p \geq 0.05$ ).