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## APPENDICES



## Appendix A

### 1. YPD media (Difco™)

#### Broth formula

Formula per Liter

Yeast Extract .....	10.0 g
Peptone .....	20.0 g
Dextrose.....	20.0 g

#### Agar formula

Formula per Liter

Yeast Extract .....	10.0 g
Peptone .....	20.0 g
Dextrose.....	20.0 g
Agar .....	15.0 g

#### Media preparation

1. Suspend the powder in 1 L of purified water: Difco™ YPD Agar – 65 g;  
Difco™ YPD Broth – 50 g. Mix thoroughly.
2. Heat the agar medium with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Autoclave the agar and broth media at 121°C for 15 minutes.

### 2. Synthetic complete media lacking uracil

#### Agar formula for repressed system

Formula per Liter

Yeast nitrogen base (Difco Laboratories).....	6.7 g
Amino acid mixtures lacking uracil (Sigma).....	1.92 g
Bactro™ agar (Difco Laboratories).....	20.0 g
Glucose (Difco Laboratories).....	20.0 g

## The composition of amino acid mixtures lacking uracil (Sigma)

Component	Product No.	Amount per liter of medium (mg)
Adenine hemisulfate	A 9126	18
Alanine	A 7627	76
Arginine hydrochloride	A 5131	76
Asparagine monohydrate	A 8381	76
Aspartic acid	A 9256	76
Cysteine hydrochloride monohydrate	C 7880	76
Glutamic acid monosodium salt	G 1626	76
Glutamine	G 3126	76
Glycine	G 7126	76
Histidine	H 8000	76
Myo-Inositol	I 5125	76
Isoleucine	I 2752	76
Leucine	L 8000	380
Lysine monohydrochloride	L 5626	76
Methionine	M 9625	76
p-Aminobenzoic acid potassium salt	A 0254	8
Phenylalanine	P 2126	76
Proline	P 0380	76
Serine	S 4500	76
Threonine	T 8625	76
Tryptophan	T 0254	76
Tyrosine disodium salt	T 2269	76
Valine	V 0500	76

**Media preparation**

1. Suspend the powder in 1 L of purified water: 6.7 g of yeast nitrogen base and 1.92 g of amino acid mixtures lacking uracil. Mix thoroughly to completely dissolve the powder.
2. Adjusted media to pH 7.2 by 1N NaOH
3. Add 20 g of glucose and 20.0 g of Bactro™ agar
4. Autoclave the agar and broth media at 121°C for 15 minutes.

**Agar formula for induced system**

## Formula per Liter

Yeast nitrogen base (Difco Laboratories).....	6.7 g
Amino acid mixtures lacking uracil (Sigma).....	1.92 g
Bactro™ agar (Difco Laboratories).....	20.0 g
Galactose (Difco Laboratories) .....	20.0 g

**Media preparation**

1. Suspend the powder in of purified water to 900 ml: 6.7 g of yeast nitrogen base and 1.92 g of amino acid mixtures lacking uracil. Mix thoroughly to completely dissolve the powder.
2. Adjusted media to pH 7.2 by 1N NaOH
3. Add 20.0 g of Bactro™ agar
4. Autoclave the agar and broth media at 121°C for 15 minutes.
5. Add 100 ml of 20% galactose (20 g galactose/100 ml purified water), filter-sterilized through a 0.45 µm filter.

**Table A1** The number of yeast colony forming unit.

Serial dilutions of yeast cultures	Yeast colony forming unit per plate			
	Plate 1	Plate 2	Plate 3	Average
1 (O.D. <sub>600</sub> = 0.3)	> 300	> 300	> 300	> 300
10 <sup>-1</sup>	> 300	> 300	> 300	> 300
10 <sup>-2</sup>	> 300	> 300	> 300	> 300
10 <sup>-3</sup>	> 300	> 300	> 300	> 300
10 <sup>-4</sup>	46	68	52	58.6
10 <sup>-5</sup>	7	10	5	7.3
10 <sup>-6</sup>	0	0	0	0

## APPENDIX B

### Bioassay laboratory protocol

#### 1. Cytotoxic against primate cell line (Vero)

Method:	Green fluorescent protein (GFP) detection
Positive control:	Ellipticine
Negative control:	0.5% DMSO
Maximum final test concentration:	50 µg/ml

The GFP-expressing Vero cell line was generated in-house by stably transfecting the African green monkey kidney cell line (Vero, ATCC CCL-81), with pEGFP-N1 plasmid (Clontech). The cell line is maintained in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, at 37°C in a humidified incubator with 5% CO<sub>2</sub>.

The assay is carried out by adding 45 µl of cell suspension at 3.3x10<sup>4</sup> cells/ml to each well of 384-well plates containing 5 µl of test compounds previously diluted in 0.5% DMSO, and then incubating for 4 days in 37°C incubator with 5% CO<sub>2</sub>. Fluorescence signals are measured by using SpectraMax M5 microplate reader (Molecular Devices, USA) in the bottom reading mode with excitation and emission wavelengths of 485 and 535 nm. Fluorescence signal at day 4 is subtracted with background fluorescence at day 0. The percentage of cytotoxic is calculated by the following equation, where FU<sub>T</sub> and FU<sub>C</sub> represent the fluorescence units of cells treated with test compound and untreated cells, respectively.

$$\% \text{ cytotoxicity} = [1 - (FU_T / FU_C)] \times 100$$

IC<sub>50</sub> values are derived from dose-response curves, using 6 concentrations of 2-fold serially diluted samples, by the SOFTMax Pro software (Molecular device). Ellipticine and 0.5% DMSO are used as a positive and a negative control, respectively.

## 2. Cancer cell growth inhibition

Method:	Resazurin microplate assay (REMA)
Positive control:	Ellipticine and doxorubicin
Negative control:	0.5% DMSO
Maximum final test concentration:	50 µg/ml

Three cancerous human-cell lines are available for this assay:

- 1) KB cell line (epidermoid carcinoma of oral cavity, ATCC CCL-17)
- 2) MCF-7 cell line (breast adenocarcinoma, ATCC HTB-22)
- 3) NCI-H187 (small cell lung carcinoma, ATCC CRL-5804)

This assay is performed using the method described by Brien *et al.* (2000). In brief, cells at a logarithmic growth phase are harvested and diluted to  $7 \times 10^4$  cell/ml for KB and  $9 \times 10^4$  cells/ml for MCF-7 and NCI-H187, in fresh medium. Successively, 5 µl of test sample diluted in 5% DMSO, and 45 µl of cell suspension are added to 384-well plates, incubated at 37°C in 5% CO<sub>2</sub> incubator. After the incubation period (3 days for KB and MCF-7, and 5 days for NCI-H187), 12.5 µl of 62.5 µg/ml resazurin solution is added to each well, and the plates are then incubated at 37°C for 4 hours. Fluorescence signal is measured using SpectraMax M5 multi-detection microplate reader (Molecular Devices, USA) at the excitation and emission wavelengths of 530 nm and 590 nm. Percent inhibition of cell growth is calculated by the following equation, where  $FU_T$  and  $FU_C$  represent the mean fluorescent unit from treated and untreated conditions, respectively.

$$\% \text{Inhibition} = [1 - (FU_T / FU_C)] \times 100$$

Dose response curves are plotted from 6 concentrations of 2-fold serially diluted test compounds and the sample concentrations that inhibit cell growth by 50% ( $IC_{50}$ ) can be derived using the SOFTMax Pro software (Molecular Devices, USA).

## Appendix C

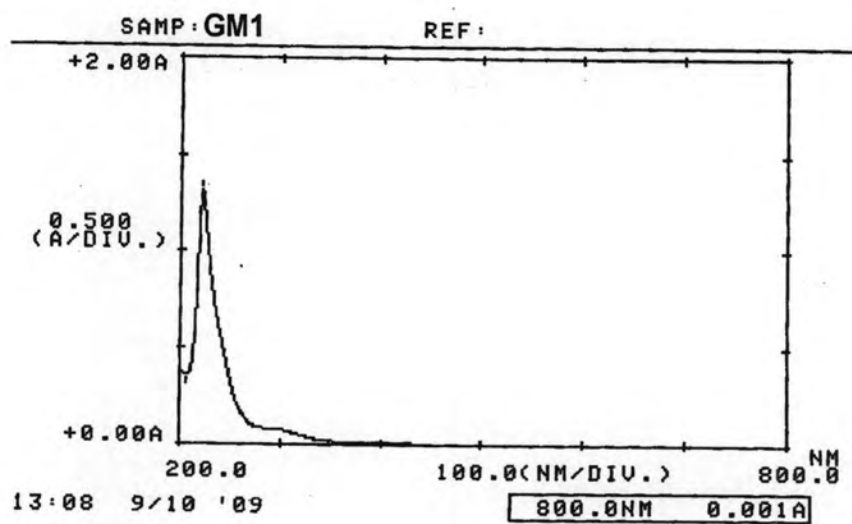


Figure C1 UV spectrum of compound GM1.

Scientific and Technological Research Equipment Centre  
Chulalongkorn University

GM1

Fourier Transform Infrared Spectrometer, PerkinElmer (Spectrum One)

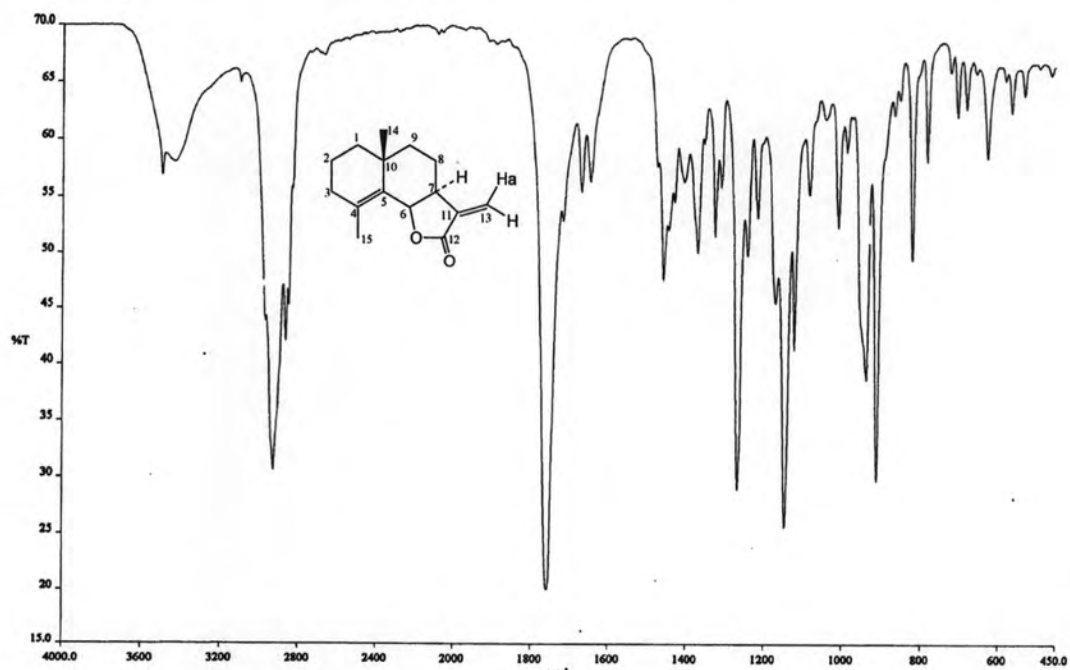


Figure C2 IR spectrum of compound GM1.

C:\xcalibur\data\EI\data\GM1  
 DIP:Probe\_EI:temp30-350,rate20/min,hold30sec  
 G3A #29 RT: 0.35 AV: 1 NL: 1.36E6  
 T: + c Full ms [ 50.00-900.00]

22/7/2552 12:36:33

GM1

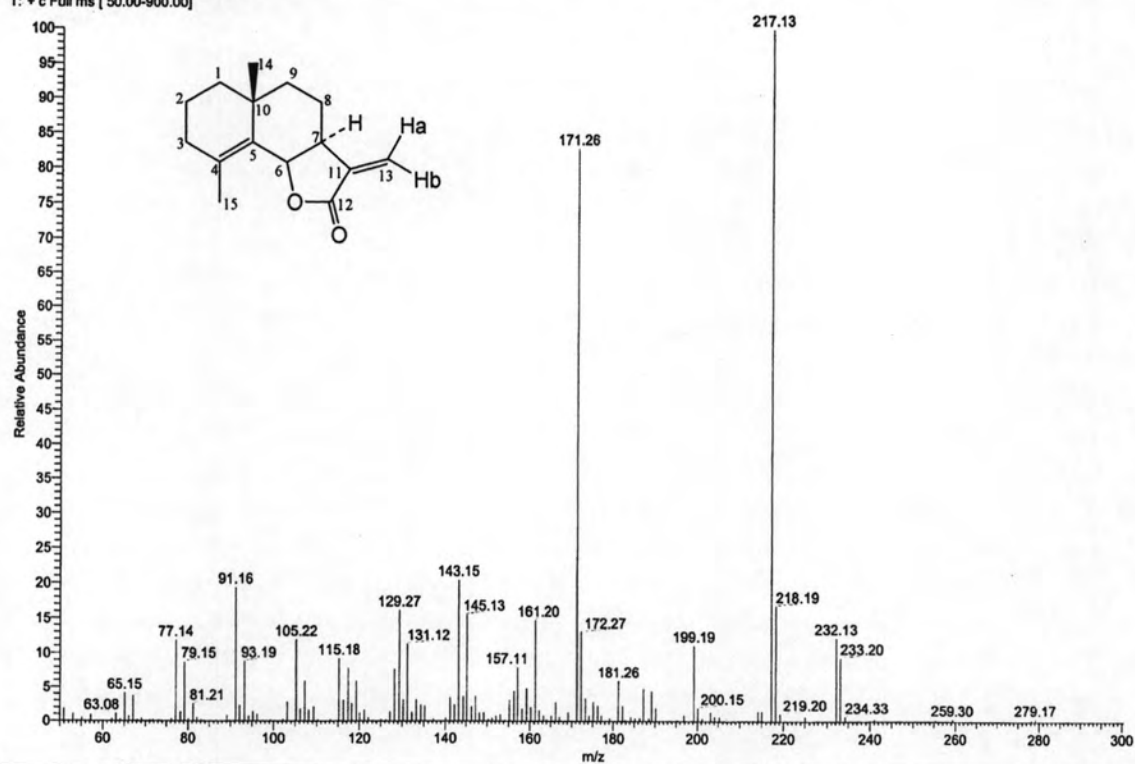


Figure C3 Mass spectrum of compound GM1.

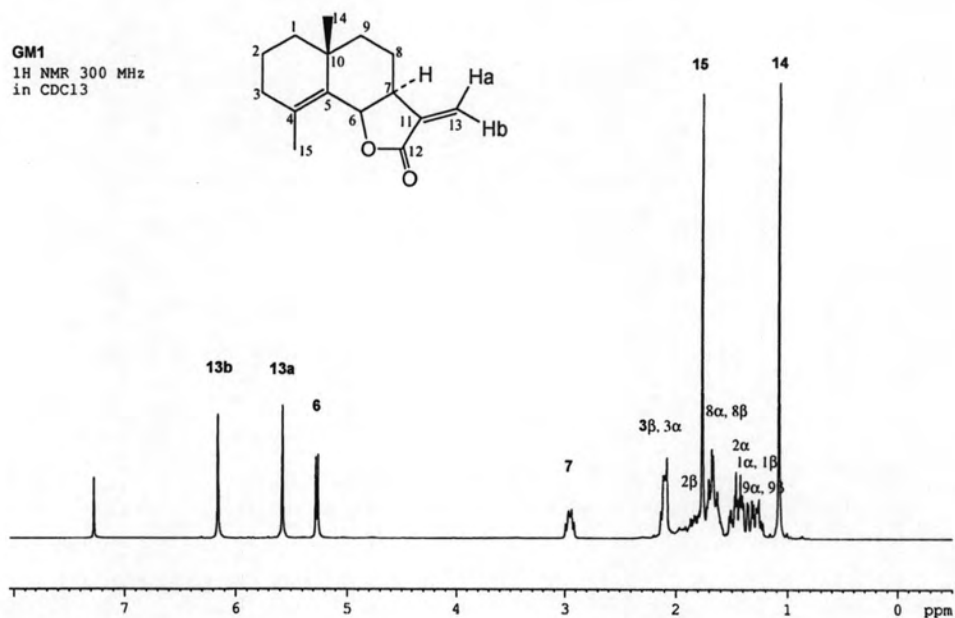


Figure C4 <sup>1</sup>H-NMR (300 MHz) spectrum of compound GM1 (CDCl<sub>3</sub>).



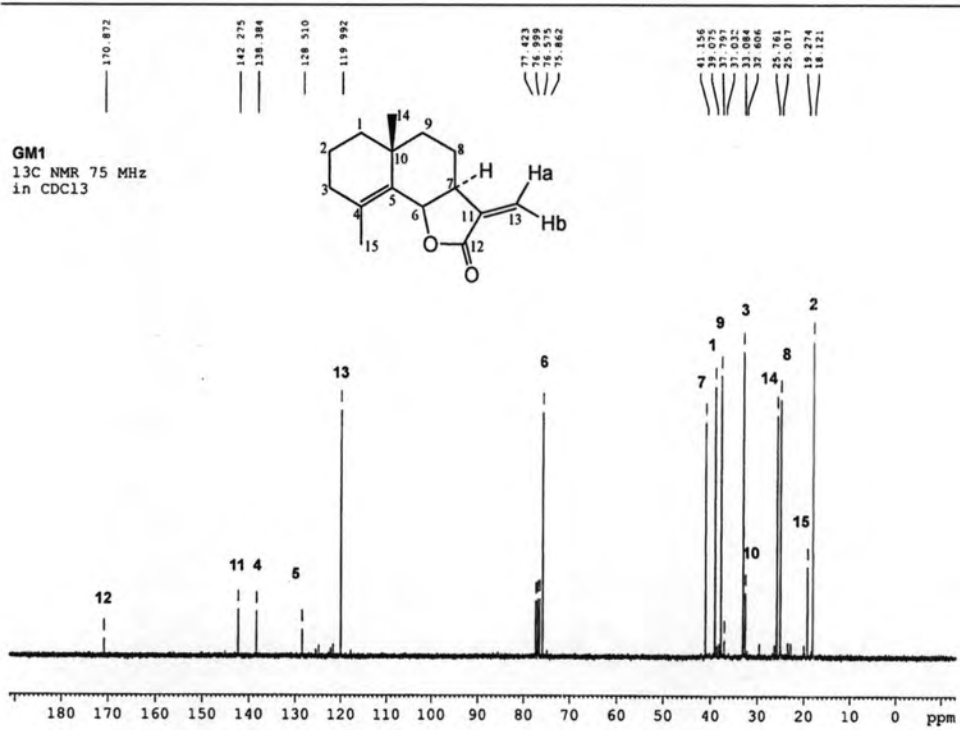


Figure C5  $^{13}\text{C}$ -NMR (75 MHz) spectrum of compound GM1 ( $\text{CDCl}_3$ ).

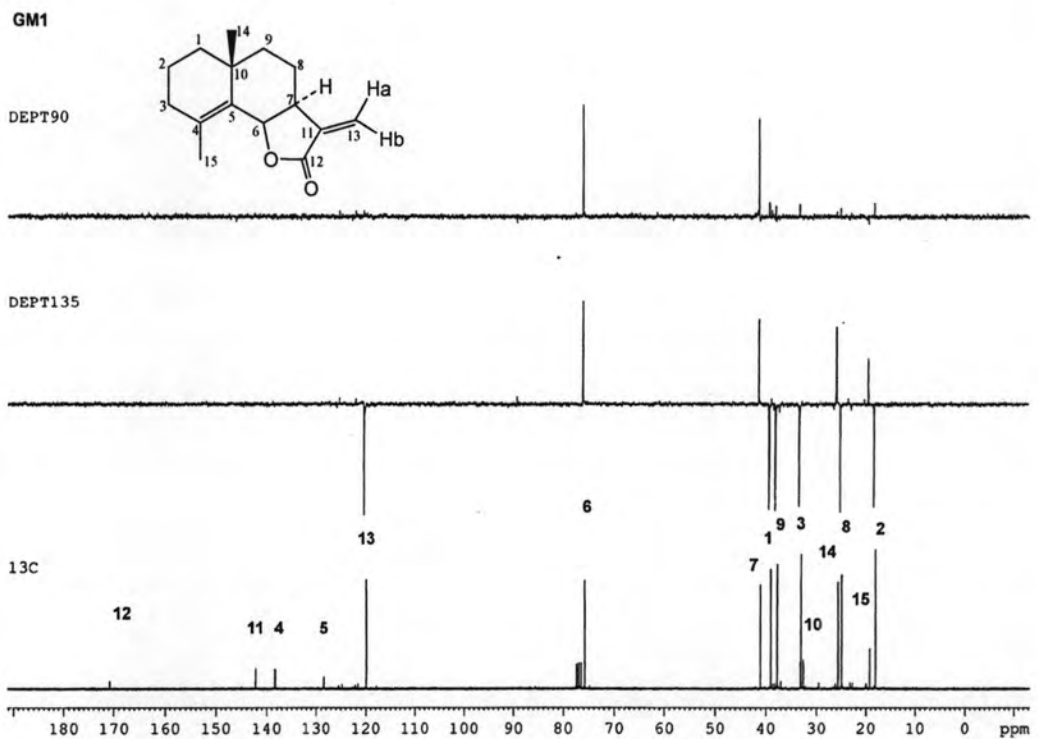


Figure C6 DEPT spectrum of compound GM1 ( $\text{CDCl}_3$ ).

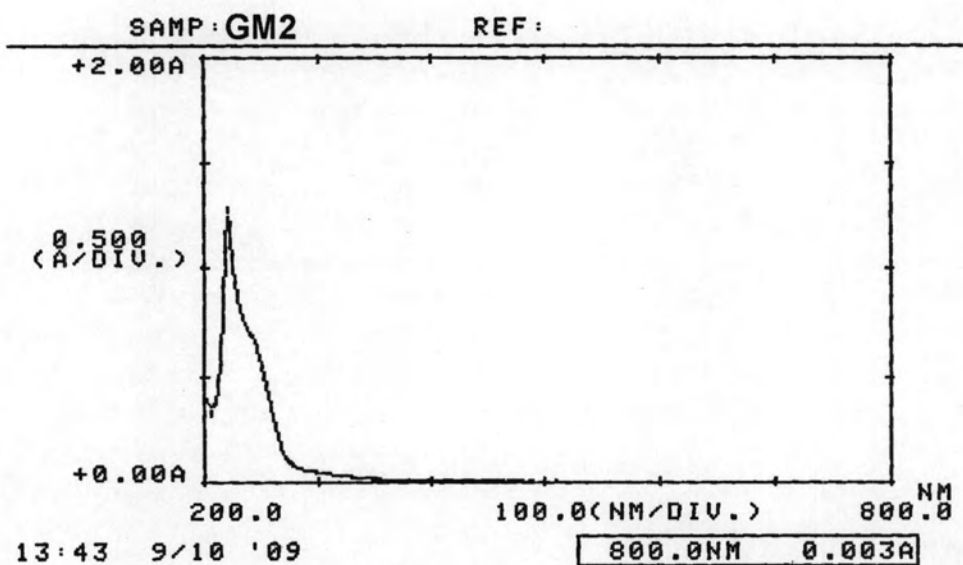


Figure C7 UV spectrum of compound GM2.

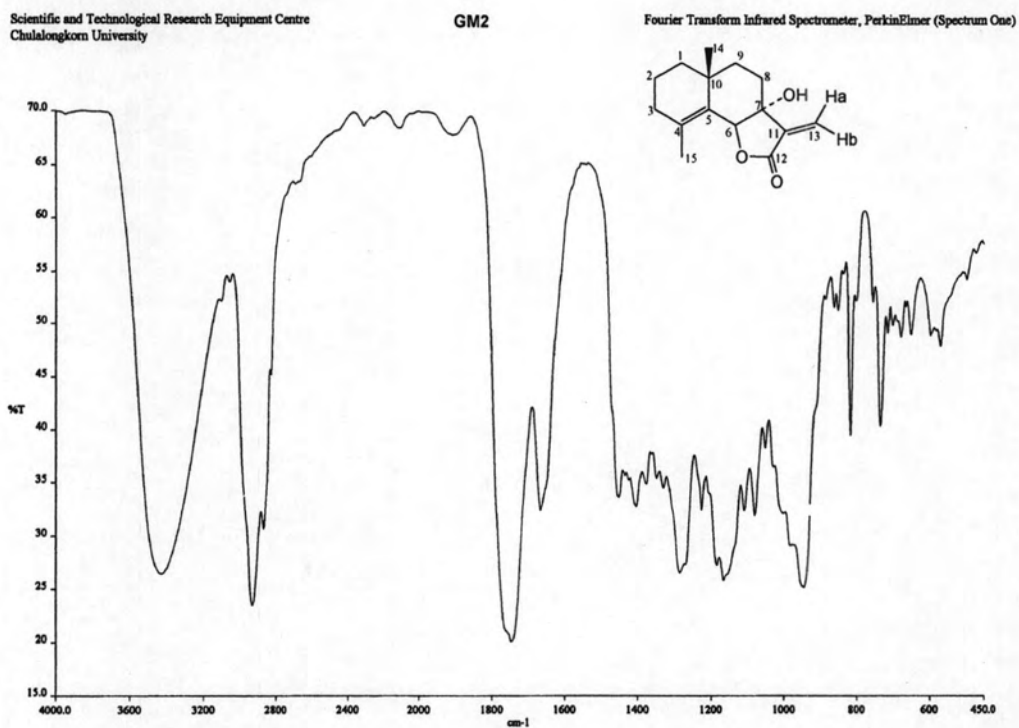


Figure C8 IR spectrum of compound GM2.

C:\galbur\data\EI\data\GM2  
 Dip: Probe EI: temp 30-350, rate 20/min, hold 30sec  
 GS63 #214 RT: 2.36 AV: 1 NL: 1.30E6  
 T: + c Full ms [ 50.00-900.00]

10/6/2552 16:19:20

GM2

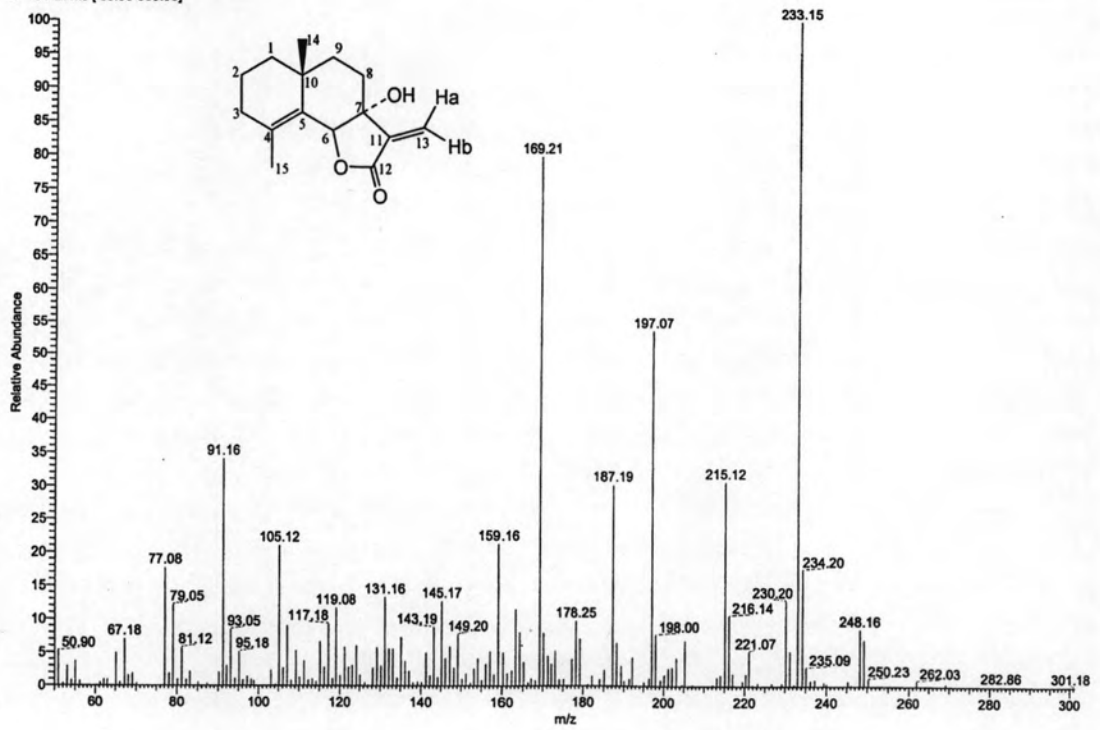
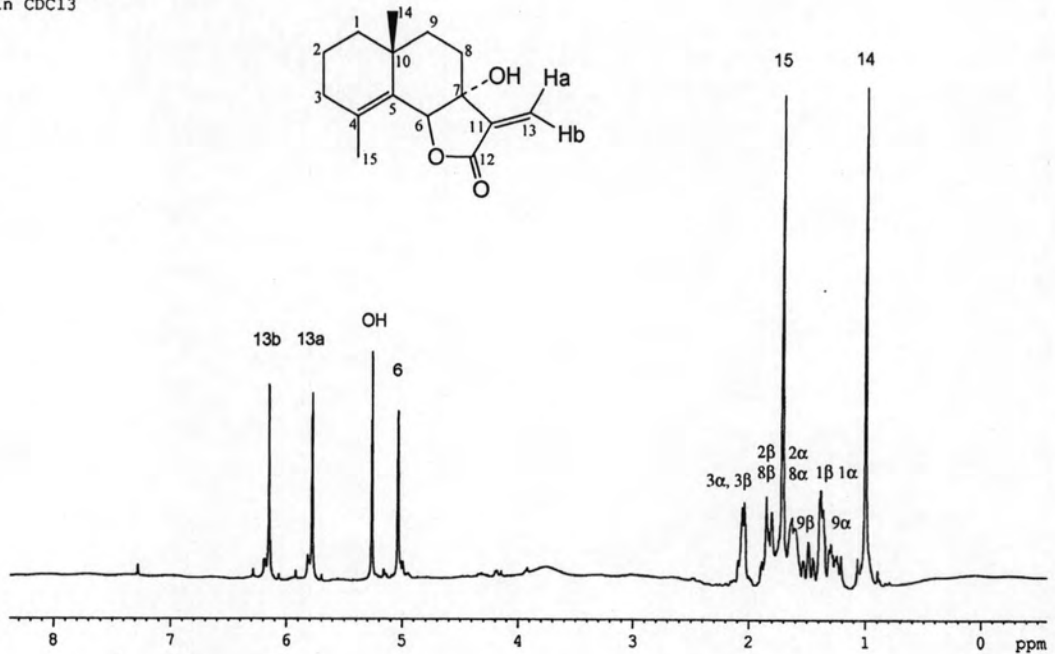


Figure C9 Mass spectrum of compound GM2.

**GM2**  
 $^1\text{H}$  NMR 300 MHz  
 in  $\text{CDCl}_3$

Figure C10  $^1\text{H}$ -NMR (300 MHz) spectrum of compound GM2 ( $\text{CDCl}_3$ ).

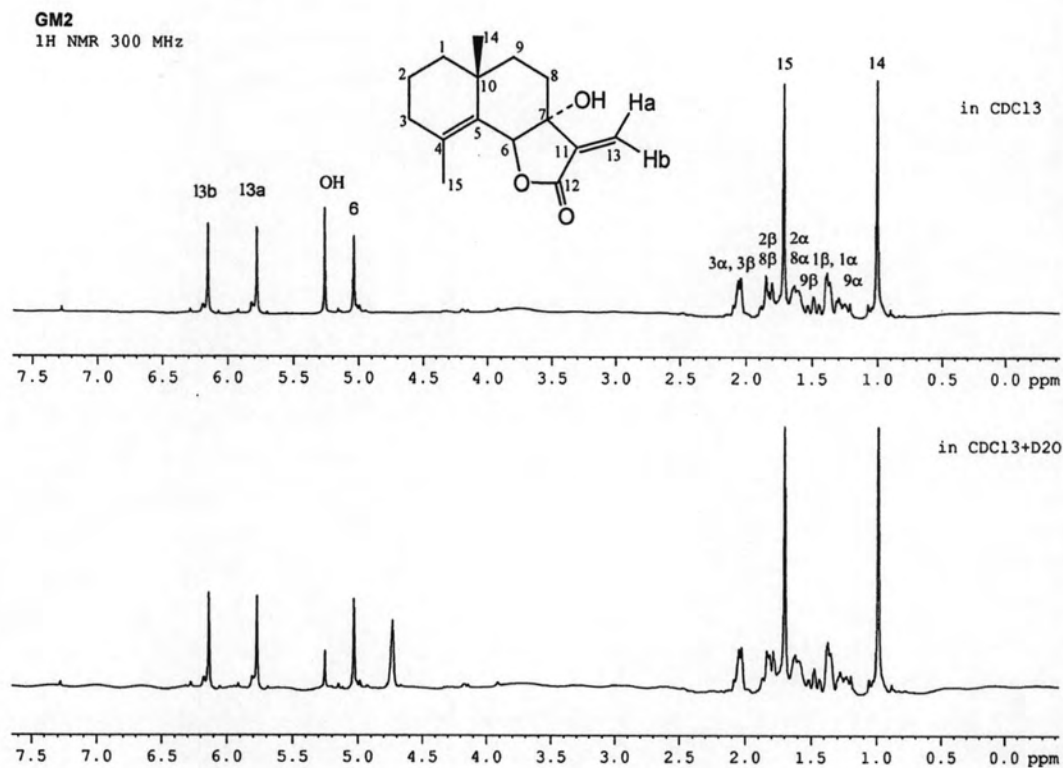


Figure C11 <sup>1</sup>H-NMR (300 MHz) spectrum of compound GM2 (CDCl<sub>3</sub>).

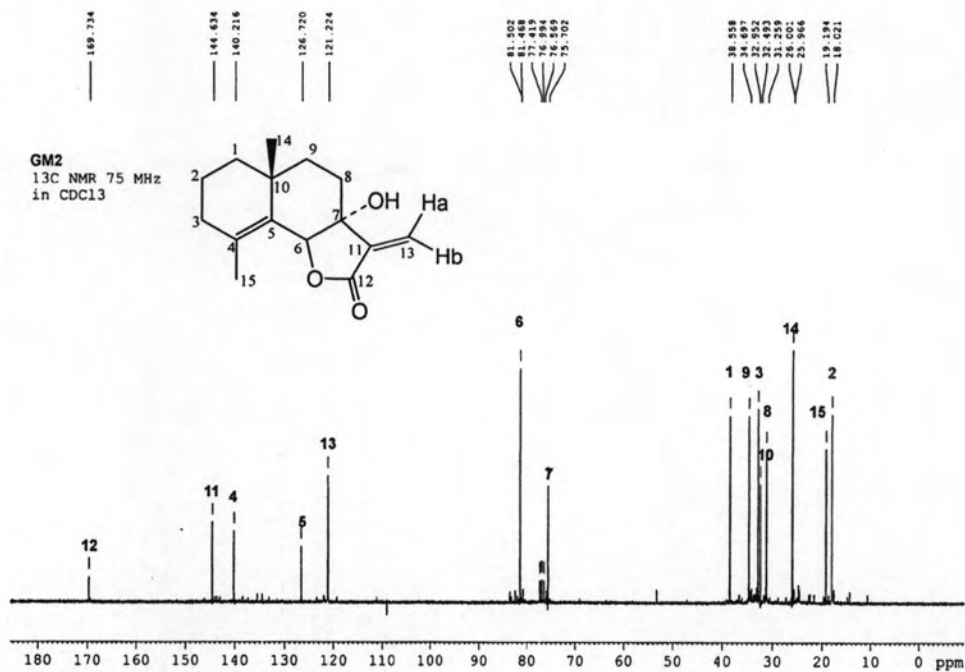


Figure C12 <sup>13</sup>C-NMR (75 MHz) spectrum of compound GM2 (CDCl<sub>3</sub>).

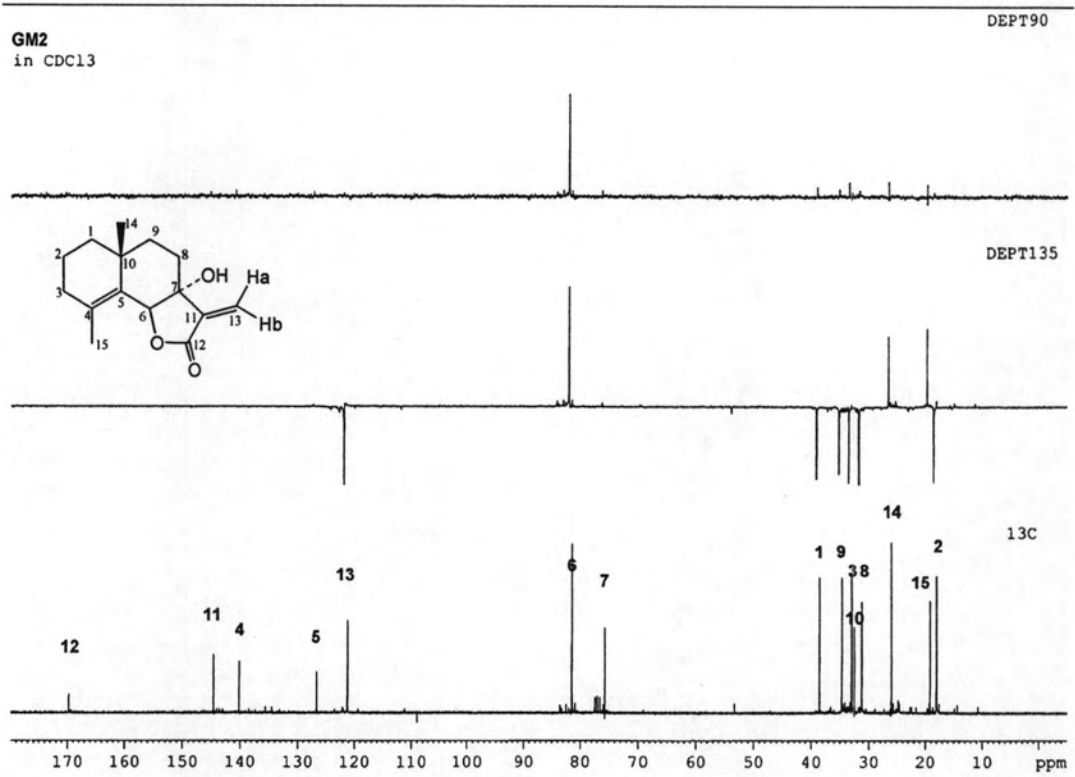


Figure C13 DEPT spectrum of compound GM2 (CDCl<sub>3</sub>).

## VITA

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### Oral presentation

Oral Presentation Awards of the 6<sup>th</sup> Thai Traditional and Alternative Medicine Conference 2009, August 2-4, 2009, Nonthaburi, Thailand.

Uppatanpreecha, P., Laorpaksa, A, Sirikantaramas, S., and Sukrong, S., Screening for topoisomerase I-inhibitory activity from Thai medicinal plants by yeast cell-based assay. Proceeding of the 6<sup>th</sup> Thai Traditional and Alternative Medicine Conference, August 2-4, 2009, IMPACT Exhibition and Convention Center, Nonthaburi, Thailand.

