

# Megathyrin B: A Cytotoxic Diterpene from *Isodon megathyrus*

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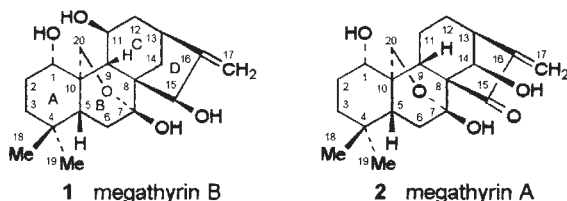
Received: January 28, 1998; Revision accepted: May 3, 1998

**Abstract:** From the leaves of *Isodon megathyrus*, a novel *ent*-kaurene diterpene, megathyrin B, was isolated and its structure determined as  $1\alpha,7\beta,11\beta,15\beta$ -tetrahydroxy-*ent*-7 $\alpha$ ,20-epoxykaur-16-ene by 1D- and 2D-NMR spectral analysis. Additionally, its stereochemistry was unambiguously assigned by X-ray crystallography. This compound was cytotoxic to the KB and KB-V cell lines.

**Key words:** *Isodon megathyrus*, Labiatae, Leaves, *ent*-kaurene diterpene, human cancer lines, cytotoxic activity, 2D-NMR techniques, X-ray crystallography, megathyrin B, electrospray LC/MS.

## Introduction

Phytochemical and biological studies aimed at the discovery and development of novel anticancer agents from natural sources have been conducted in our laboratories for a number of years. A previous bioassay-guided fractionation of the leaves of *Isodon megathyrus* (Diels) H. W. Li (Labiatae) resulted in the isolation of four cytotoxic *ent*-kaurene diterpenes (1). A continued investigation of this material resulted in the isolation of a further novel cytotoxic diterpene, named megathyrin B (1). The present paper reports the isolation, structure elucidation and cytotoxicity of 1.



## Materials and Methods

### General

UV spectra were taken in MeOH on a Beckman DU-7 spectrometer. IR spectra were recorded in a KBr pellet on an MIDAC FT-IR interferometer. The optical rotations were measured with a Perkin-Elmer 241 polarimeter. The <sup>1</sup>H-, <sup>13</sup>C-NMR,

DEPT, DQF-COSY, HOHAHA, HETCOR, and ROESY spectra were recorded at 500.12 MHz for <sup>1</sup>H, and 125.76 MHz for <sup>13</sup>C with a GE OMEGA 500 instrument, using GE standard programs in C<sub>5</sub>D<sub>5</sub>N solution. Selective INEPT spectra were recorded on a Nicolet NT-360 instrument operating at 90.8 Hz, with *J* = 6 Hz for the aliphatic protons. HRFABMS were recorded by direct-inlet on a MAT-95 mass spectrometer (MAT-Finnigan Company) using glycerol as matrix. LC/MS chromatography was performed with a Hewlett Packard (Wilmington, DE, USA) electrospray system comprised of a 1090 series II L high performance liquid chromatograph (HPLC) equipped with a photodiode array detector, a 5987A electrospray, and a 5989B mass spectrometer. The splitter used was a 1:50/1:125 stream splitter from LCPackings (San Francisco, CA, USA), and the postcolumn pump was an ABI MicroGradient pump. TLC was performed on precoated Kieselgel 60 F<sub>254</sub> plates (Merck) and detection was achieved by spraying with 10% H<sub>2</sub>SO<sub>4</sub> followed by heating.

### Plant material

Leaves of *I. megathyrus* (Diels) H. W. Li were collected in Fugong County, Yunnan Province, People's Republic of China, and identified by Prof. H.-W. Li of the Kunming Institute of Botany, Chinese Academy of Sciences, China. A voucher specimen (No. KM-89-1425) was deposited in the Herbarium of the Department of Taxonomy, Kunming Institute of Botany, Chinese Academy of Sciences.

### Extraction and isolation

The milled air-dried leaves (230 g) were extracted with MeOH (0.5 liter × 3) under reflux as previously described (1). The combined MeOH extract was concentrated under vacuum, and the residue (31 g) was chromatographed over silica gel (700 g), using petroleum ether, followed by CHCl<sub>3</sub> and CHCl<sub>3</sub> with increasing concentrations of MeOH (petroleum ether 4 l, CHCl<sub>3</sub> 5 l, 10% MeOH/CHCl<sub>3</sub> 5 l, 20% MeOH/CHCl<sub>3</sub> 4 l, MeOH 2.5 l). Further silica gel (200 g) chromatography of the CHCl<sub>3</sub>:MeOH (8:2) eluted fraction (1.2 g) with a mixture of CHCl<sub>3</sub>-Me<sub>2</sub>CO (8:2, 2 l) yielded a fraction, the workup of which afforded a crystalline complex (designated **DCIM**) comprising compounds 1 and 2 (28 mg, 0.012%), which was shown to be homogenous by TLC [silica gel, solvent systems: 8% MeOH/CHCl<sub>3</sub> (R<sub>f</sub> 0.3); EtOAc/CHCl<sub>3</sub> (1:1) (R<sub>f</sub> 0.4)] and HPLC [column, Zorbax ODS 4.6 × 250 mm; solvent system: MeOH:H<sub>2</sub>O (7.75:2.25; 0.3 ml/min); detection at 230 nm, t<sub>R</sub> 35 min].

**DCIM:** Colorless plates, m.p. 214–216 °C,  $[\alpha]_D^{25}$ : –66° (c 0.0033, MeOH). UV:  $\lambda_{\text{max}}^{\text{EtOH}}$  (log  $\epsilon$ ) = 230 nm (4.01). IR (KBr):  $\nu_{\text{max}}$  = 3440, 3300, 1643, 1420, 1325 and 1060  $\text{cm}^{-1}$ . EIMS:  $m/z$  (rel. intensity) = 350 (5), 348 (18), 332 (62), 330 (45), 314 (25), 249 (28), 203 (10), 181 (73), 167 (65), 152 (83), 133 (75), 121 (97), 105 (75), 91 (100). HRFABMS  $m/z$ : observed 698.40299 for  $\text{C}_{40}\text{H}_{58}\text{O}_{10}$  calcd. 698.40300.

#### Electrospray LC/MS analysis of DCIM

The HPLC separation was performed with a Kromasil C-18 reverse phase column (250 × 3.2 mm i.d.) with 5  $\mu\text{m}$  packing material (Technikrom, Wilmette, IL USA). The gradient began at 20% acetonitrile in water and was held at this concentration for the first 10 min. This was followed by a linear gradient to 40% acetonitrile over the next 8 min, a linear increase to 75% acetonitrile over the following 10 min and then a sharp transition to 100% acetonitrile over the next 2 min. The electrospray (ES) lenses and the skimmers were adjusted to detect  $[\text{M} - \text{H}]^-$  ions in a “normal” negative ion mode.

#### Isolation of megathyrin B (1)

**DCIM** (22 mg) was dissolved in boiling MeOH for crystallization and subsequent X-ray analysis. However, in place of DCIM, compound **1** (3.5 mg) was obtained as fine needles on cooling and standing overnight.

**Megathyrin B (1):** Colorless needles, m.p. 224–226 °C,  $[\alpha]_D^{25}$ : –36° (c 0.05, MeOH). IR (KBr):  $\nu_{\text{max}}$  = 3440, 3300, 1643, 1420, 1325, and 1060  $\text{cm}^{-1}$ .  $\text{C}_{20}\text{H}_{30}\text{O}_5$ , MW = 350, EIMS:  $m/z$  = 350 (5), 332 (62), 314 (25), 249 (28), 203 (10), 181 (73), 167 (65), 152 (83), 133 (75), 121 (97), 105 (75), 91 (100).  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR data see Table 1.

**X-ray crystallography of 1:** Colorless prisms, orthorhombic, space group  $\text{P}2_12_12_1$ , with  $a = 6.114$  (1),  $b = 11.219$  (1),  $c = 29.337$  (8) Å.  $V = 2012.3$  (7) Å<sup>3</sup>,  $Z = 4$ . Data collection was carried out at room temperature on a Siemens P4 diffractometer ( $\lambda = 0.71073$  Å). The structure was solved and refined using the Siemens SHELXTL (version 5.05) software package. Non-hydrogen atoms were refined anisotropically by full-matrix least squares. Hydrogen atoms were included in calculated positions. The refinement converged at  $R_1 = 4.83\%$ ,  $wR_2 = 10.55\%$  and a goodness-of-fit of 1.071. There is one co-crystallized methanol molecule per asymmetric unit.

The atomic coordinates, equivalent isotropic displacement parameters, lengths, bond angles, anisotropic displacement parameters, H-atom coordinates, and isotropic displacement parameters of compound **1** (code name “rm-4”) can be obtained, upon request, from Dr. Olga Kennard, University Chemical Laboratory, 12 Union Road, Cambridge CB2 1EW, UK. Any request should be accompanied by the full literature citation for this paper.

#### Cytotoxicity and antimitotic assays

Test compound was monitored using an astrocytoma (ASK) assay (3), according to a published method (4). Data for compound **1** were obtained using a panel of human cancer cell lines and established protocols (5).

## Results and Discussion

Further chromatographic separation of the cytotoxic fraction described previously (1) gave a crystalline material designated as “**DCIM**”, which displayed a single spot on normal phase TLC developed in several different solvent systems and was not separable by HPLC.

**DCIM** was shown to possess an apparent molecular formula of  $\text{C}_{40}\text{H}_{58}\text{O}_{10}$  by high-resolution FABMS, in which a quasi-molecular ion was observed at  $m/z$  699.4154  $[\text{M} + \text{H}]^+$  in the positive mode mass spectrum. Its IR spectrum showed a conjugated ketone (1710, 1647  $\text{cm}^{-1}$ ) absorbency, which was also supported by the UV absorption at 230 nm. However, the  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectra recorded in deuterated pyridine appeared to be unduly complex. The  $^{13}\text{C}$ -DEPT spectrum of **DCIM** indicated the presence of four methyl, fourteen methylene, eleven methine, and eleven quaternary carbon signals, with two hemiacetals ( $\delta$  96.64 and 98.37), a ketonic, two conjugated olefinic and one pair of terminal methylene carbons. A close inspection of the NMR spectra revealed it to be an equimolecular “mixture” of two related diterpenes of the *ent*-7 $\alpha$ ,20-epoxy-kaurene type skeleton.

In spite of the intrinsic complexity of the  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectra of **DCIM**, the well-resolved signals enabled us to determine the presence of megathyrin A (**2**) and a novel diterpene, designated as megathyrin B (**1**), by deciphering the through-bond connectivity information derived from the DQF-COSY, HOHAHA, HETCOR, and selective INEPT spectra, coupled with the stereochemistry information revealed from the ROESY spectrum. The NMR signals attributable to **1** are presented in Table 1.

**Table 1**  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR assignments of megathyrin B (**1**)\*.

carbon	$^1\text{H}$	$^{13}\text{C}$
1	$\beta$ : 4.23 (dd, 5.0, 12.0)	74.24 (d)
2	$\alpha$ : 2.01 obsc.; $\beta$ : 1.97 obsc.	28.59 (t)
3	$\alpha$ : 1.45 (dd, 13.2, 3.2); $\beta$ : 1.25 (dt, 3.2, 13.2)	39.74 (t)
4	/	34.29 (s)
5	$\beta$ : 1.92 (ddd, 1.5, 7.5, 12.0)	47.91 (d)
6	$\alpha$ : 2.04 obsc.; $\beta$ : 3.11 (t, 12.5)	34.38 (t)
7	/	96.64 (s)
8	/	54.04 (s)
9	$\beta$ : 2.70 (dd, 9.8, 1.5)	50.89 (d)
10	/	41.49 (s)
11	$\alpha$ : 4.78 (q, 9.5)	63.42 (d)
12	$\alpha$ : 1.89 obsc.; $\beta$ : 2.36 (ddd, 12.5, 9.0, 4.5)	43.09 (t)
13	$\alpha$ : 2.78 (br. d, 9.2)	36.99 (d)
14	$\alpha$ : 2.06 (m); $\beta$ : 2.11 (m)	26.97 (t)
15	$\alpha$ : 5.16 (s)	75.45 (d)
16	/	164.15 (s)
17	a: 5.22 (br.s); b: 5.46 (br.s)	106.79 (t)
18	0.78 (s)	31.97 (q)
19	1.16 (s)	21.49 (q)
20	a: 4.88 (br.s, 10.0); b: 4.65 (dd, 10.0, 1.5)	64.76 (t)

\* Recorded in  $\text{C}_5\text{D}_5\text{N}$ , chemical shift values was reported as  $\delta$  values (ppm) from internal TMS at 500 MHz, signal multiplicity and coupling constants (Hz) are shown in parentheses. m = multiplex signals; obsc. = obscured signals.

Recently, we reported the isolation of an inseparable 1:1 complex (**DCRA**) of two diterpenes structurally related to **1** and **2** from *Rabdosia angustifolia* (2). The present mixture (**DCIM**) of **1** and **2**, particularly the observation of an adduct molecular ion of **1** and **2** in the FAB-MS, is reminiscent of the complexation involved in **DCRA** (2), in which two subunits are bound together by an intermolecular hydrogen bond, and the very good hydrophobic close approach of mutual matching, suggested a similar non-covalent interaction between subunits **1** and **2** of **DCIM**, at least in the solid state. However, the connection between the two subunits within **DCIM** was unclear, and confirmation by X-ray crystallography was thought to be needed. However, it is most likely that the subunits **1** and **2** are bound together through the same or similar fashion as the complex **DCRA** (2). Our efforts to prepare suitable single crystals of **DCIM** for X-ray crystallographic analysis were unsuccessful. Unexpectedly, however, a small quantity of megathyrin B (**1**) was obtained as needles from a saturated methanol solution in which crystal growth of **DCIM** for X-ray crystallography was attempted.

The fact that **1** did crystallize as a single entity may imply that in a polar solvent such as methanol, the complexation bond between **1** and **2** that occurs in the solid form of **DCIM** is broken. This hypothesis is supported by our observation that no significant complexation-induced chemical shifts existed

when the  $^1\text{H-NMR}$  spectra of **1** and **2** and **DCIM** (Fig. 1) were compared. Additionally, analysis of **DCIM** by LC/MS resulted in two resolved peaks being detected at  $m/z$  347 and 349, corresponding to the quasi-molecular ions of compounds **1** and **2**, respectively.

Megathyrin B (**1**) exhibited a molecular formula  $\text{C}_{20}\text{H}_{30}\text{O}_5$  ( $M_r = 350$ ) based on its  $^{13}\text{C-NMR}$  (DEPT) and mass spectra. An *ent*-7 $\alpha$ ,20-epoxy-kaurene-hemiacetal skeleton was evident from the characteristic  $^1\text{H-}$  and  $^{13}\text{C-NMR}$  spectra of **1** showing the presence of two tertiary methyl ( $\delta$  31.97, 21.49), seven methylene (including an oxygenated methylene and an exomethylene group,  $\delta$  28.59, 39.74, 34.38, 43.09, 26.97, 106.79, 64.76), six methine (including three oxygenated methines,  $\delta$  74.24, 47.91, 50.89, 63.42, 36.99, 75.45), four quaternary carbons ( $\delta$  34.29, 54.04, 41.49, 164.15), and a hemiacetal carbon ( $\delta$  96.64).

The  $\alpha$ -orientation of the hydroxy group at C-1 was suggested from the large coupling constants of H-1 resonating at  $\delta$  4.23 (dd,  $J = 5.0, 12.0$  Hz), which was further confirmed by the ROE correlation (Fig. 2) between H-1 and H-5, which is  $\beta$ -oriented (axial) for *ent*-kaurene type diterpenes. The hydroxy group at C-11 was deduced conclusively to be  $\beta$ -oriented based on the observation of a ROE cross peak between H-11 at  $\delta$  4.78 ( $q, J = 9.5$  Hz) and one of the C-20 methylene protons resonating at

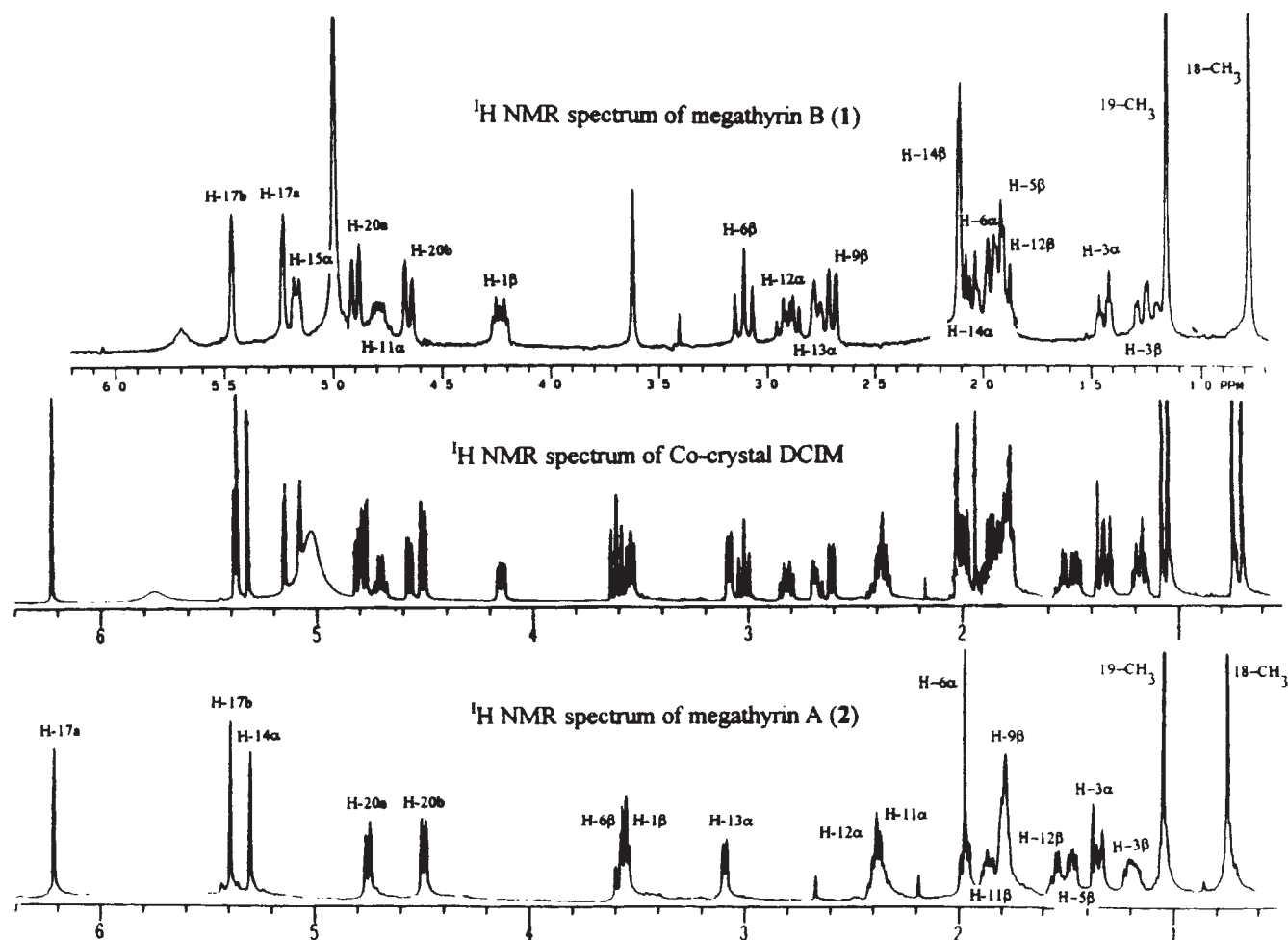


Fig. 1  $^1\text{H-NMR}$  spectral comparison of megathyrin B (**1**) and A (**2**) with that of **DCIM**.

$\delta$  4.88 (br.d,  $J = 10.0$  Hz, H-20a). Although their chemical shifts approximate each other, the signals of the C-14 methylene protons were differentiated [at  $\delta$  2.06 (m) to H-14 $\alpha$  and  $\delta$  2.11 to H-14 $\beta$ ] by use of DQF-COSY, HETCOR, HOHAHA, and especially ROESY experiments, since the former showed a significant ROE cross correlation with H-11 $\alpha$ . Meanwhile, its partner, H-14 $\beta$  showed a significant ROE cross peak with H-15, which allowed assignment of the hydroxy group at C-15 to be a  $\beta$ -configuration. Thus, the structure of compound **1** was deduced as 1 $\alpha$ ,7 $\beta$ ,11 $\beta$ ,15 $\beta$ -tetrahydroxy-*ent*-7 $\alpha$ ,20-epoxy-kaur-16-ene.

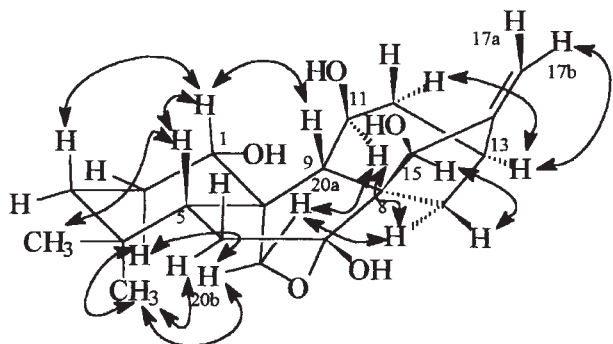


Fig. 2 Significant ROE correlations of megathyrin B (**1**).

The structure of **1** was confirmed by a single crystal X-ray analysis as presented in the ORTEP diagram in Figure 3. All bond distances and angles agree well with generally accepted values for the given bond type. There is an intramolecular hydrogen bond between the oxygen atom of 1-OH and the hydrogen atom of 11-OH and some intermolecular H-bonds. Ring A exhibits a chair conformation, and rings B and C, as well as the other two rings formed by the hemiacetal bridge, adopt boat conformations. Interestingly, a similar diterpene with a molecular formula of  $C_{20}H_{28}O_6$ , ring C has a chair conformation (6); therefore, the boat conformation of ring C in megathyrin B (**1**) may be a consequence of the intramolecular hydrogen bond. Ring D is a five membered ring with an envelope conformation.

Megathyrin B (**1**) was evaluated for cytotoxicity using human cancer cell lines (5). As shown in Table 2, megathyrin B (**1**) showed potential cytotoxicity in the KB and KB-V ( $\pm$  VLB) cell

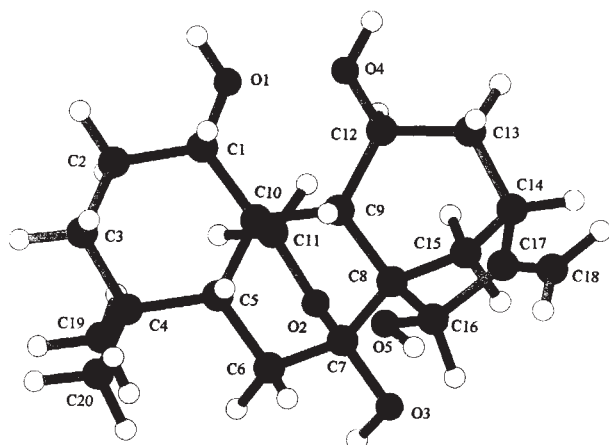


Fig. 3 Perspective drawing of the crystal structure of megathyrin B (**1**) showing hydrogen atoms.

lines. It is notable that the five diterpenes [megathyrin B and those reported in (1)] isolated so far from *I. megathyrsus* have exhibited cytotoxic activity.

Table 2 Cytotoxic activity of megathyrin B (**1**)<sup>a</sup>.

Compound	Cell Line <sup>b</sup>						
	Lu-1	KB	KB-V (+VLB)	KB-V (-VLB)	LNCAp	ZR-75-1	ASK <sup>c</sup>
megathyrin B ( <b>1</b> )	>20	0.9	1.6	1.3	>20	>20	-
colchicine <sup>d</sup>	0.2	0.2	0.6	3.5	0.06	0.1	+
ellipticine <sup>d</sup>	0.2	0.04	0.2	0.3	0.8	0.9	-

<sup>a</sup> Results are expressed as ED<sub>50</sub> values ( $\mu$ g/ml).

<sup>b</sup> Key to cell lines used: Lu-1 = human lung cancer; KB = human oral epidermoid carcinoma; KB-V = vinblastine-resistant KB test in the presence (+VLB) or absence (-VLB) of 1  $\mu$ g/ml vinblastine; LNCAp = hormone-dependent human prostate cancer; ZR-75-1 = hormone-dependent human breast cancer; ASK = rat glioma.

<sup>c</sup> Test compound that reverse astrocyte formation is scored as (+) and non-reverse astrocyte formation is scored as (-) in the ASK test at the concentration at 100  $\mu$ g/ml.

<sup>d</sup> Positive control compounds.

## Acknowledgements

The authors thank Mr. Z.-W. Lin and Ms. F.-D. Niu of the Kunming Institute of Botany, for their assistance in plant material collection and extraction, Mr. James C. Graham of The Program for Collaborative Research in the Pharmaceutical Sciences, College of Pharmacy, UIC, for the LC/MS data; and the Research Resources Center of the University of Illinois at Chicago for the provision of NMR spectroscopic facilities. The Cornell University group would like to thank the National Cancer Institute, Bethesda, MD, USA, for partial financial support.

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