

## A new dimeric neolignan from *Magnolia grandiflora* L. seeds

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**Abstract** Bioassay-guided fractionation of the MeOH extract of *Magnolia grandiflora* seeds resulted in the isolation of a new dimeric neolignan, named bishonokiol A (**1**), as well as two known neolignans magnolol (**2**) and honokiol (**3**). The structures of the compounds were determined on the basis of data obtained using NMR and MS. Bishonokiol A (**1**) showed potent anti-proliferative activities in four human cancer cell lines, with IC<sub>50</sub> values ranging from 5.1 to 7.5 μM. Additionally, bishonokiol A (**1**) induced apoptosis, as well as down-regulated the expression of the anti-apoptotic protein Bcl-2 and caspase-3 cleavage in HepG2 cell line.

**Keywords** *Magnolia grandiflora* · Neolignans · Cytotoxicity · Apoptosis

### Introduction

Natural products are considered as rich sources for the discovery of new therapeutic agents (Baker et al. 2007; Ngo et al. 2013). In particularly, plants contain a variety of functionally relevant secondary metabolites that have

been shown to prevent the development of malignancies (Surh et al. 2003). *Magnolia grandiflora* L., a traditional medicine widely used for the treatment of fever, diarrhea, abdominal diseases, and rheumatic arthritis (Schühly et al. 2001), contains alkaloids, tyramine, flavonoids, terpenes, phenolic alcohols, glycosides, and sesquiterpene lactones (Mohamed et al. 2010; Del Valle Mondragón et al. 2004; Feltenstein et al. 2004). In our search for biologically active agents from medicinal plants, we examined the properties of *M. grandiflora* seed extracts. The EtOAc-soluble extracts (10 μg/mL) exhibited cytotoxic activity in the human liver hepatocellular carcinoma cell line, HepG2 (inhibition rate, 85 %). Bioassay-guided fractionation of the MeOH extract of *M. grandiflora* seeds resulted in the isolation of one new dineolignan, bishonokiol A (**1**), together with two known neolignans magnolol (**2**) and honokiol (**3**) (Fig. 1). In this study, we report the isolation, identification, and biological activities of compound **1**.

### Materials and methods

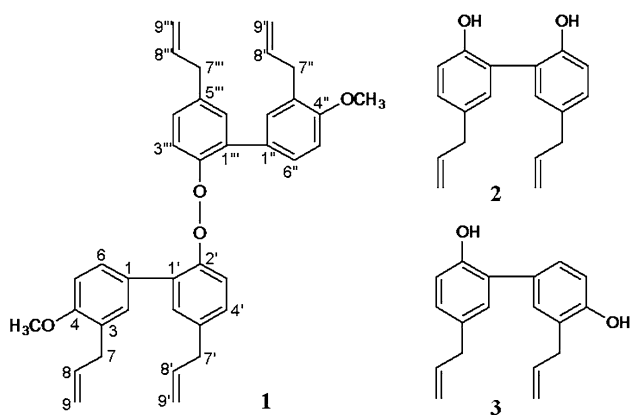
#### General experimental procedures

Optical rotation was measured with a JASCO P-1020 digital polarimeter (Jasco, Japan). UV spectrum was recorded on an UV-1601 spectrometer (Shimadzu, Japan), and IR spectrum was obtained on a FT-IR-650 spectrometer (Gangdong, China). NMR spectra were recorded using a Varian<sup>unity</sup> Ionva-400 NMR instrument (VARI, USA). ESI-MS and HR-MS spectra were recorded using a Finnigan navigator LC/MS/DS system (Finnigan, USA) and a JMS-HX/HX 110A tandem mass spectrometer (JEOL, Japan), respectively. A Waters 2535Q HPLC system fitted

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**Fig. 1** Structures of compounds **1–3**

with a 2998 photodiode array detector (Waters, USA) was used for the semi-preparative separations.

#### Plant material

Fresh seeds of *M. grandiflora* L. were collected in October 2012 from a farm located in Bengbu city, Anhui, China. A voucher specimen (DM. BY0158) has been deposited in our laboratory. The authenticity of the plant was confirmed by Prof. Huizi Lv, College of Pharmacy, Yanbian University.

#### Extraction and isolation

*Magnolia grandiflora* L. seeds (1.4 kg) were extracted with MeOH ( $2 \times 10$  L, 72 h each). MeOH was evaporated in vacuo in order to yield MeOH-soluble extract. The MeOH-soluble extract (100 g) was suspended in H<sub>2</sub>O, and sequentially partitioned with *n*-hexane, and EtOAc. The bioactive EtOAc-soluble material (13.2 g) was subjected to silica gel column chromatography (Merck Kieselgel 60, 500 g; 230–400 mesh; 4.0  $\times$  60 cm). The column was eluted with a stepwise gradient using mixtures of *n*-hexane-EtOAc (100:1, 1,000 mL; 90:1, 1,000 mL; 80:1, 2,000 mL; 75:25, 2,000 mL; 60:40, 2,000 mL; 50:50, 2,000 mL) and 250 mL fractions were collected. The active fraction 4 (150 mg, eluted 80:20 Hexane/EtOAc; cytotoxicity) was further purified by preparative reversed phase HPLC by using a 65 % CH<sub>3</sub>CN in H<sub>2</sub>O over 40 min (YMC-J<sup>s</sup> sphere ODS-H80; 10  $\times$  25 mm; 5  $\mu$ m particle size; absorbance 254 nm; flow rate 3 mL/min; 0.05 % trifluoroacetic acid) to yield bishonokiol A (**1**, 19.1 mg) and magnolol (**2**, 37.7 mg). The active fraction 5 (0.9 g, eluted 75:25 *n*-hexane/EtOAc; cytotoxicity) was subjected to silica gel column chromatography (Merck Kieselgel 60, 80 g; 230–400 mesh; 2.0  $\times$  30 cm) using elution mixtures of *n*-

hexane-EtOAc (85:15, 2,000 mL), and honokiol (**3**, 536.2 mg) was identified.

#### Bishonokiol A (**1**)

Purple oil;  $[\alpha]_D^{25} +1.7$  (c, 0.1, MeOH); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 225 (2.764), 264 (2.768), 304 (2.953) nm; IR  $\nu_{\max}^{\text{KBr}}$  cm<sup>-1</sup> 3,018, 1,521, 1,476, 1,215, 1,046; ESI-MS  $m/z$  557 [M–H]<sup>-</sup>, 559 [M+H]<sup>+</sup>; HRESI-MS  $m/z$  559.2835 calculated 559.2848 for C<sub>38</sub>H<sub>39</sub>O<sub>4</sub>. NMR data are shown in Table 1.

#### Magnolol (**2**)

ESI-MS  $m/z$  [M–H]<sup>-</sup> 265. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>),  $\delta$  7.12–6.76 (6H, m, Ar–H), 5.99 (2H, m, H-8, 8'), 5.09 Ar–OH, 5.12 (4H, m, H-9, 9'), 3.38 (4H, d,  $J = 6.4$  Hz, H-7, 7') ppm (Lee et al. 2008).

#### Honokiol (**3**)

ESI-MS  $m/z$  [M–H]<sup>-</sup> 265. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>),  $\delta$  7.23–6.91 (6H, m, Ar–H), 6.02 (2H, m, H-8, 8'), 5.13 (4H, m, H-9, 9'), 3.48 (2H, d,  $J = 6.4$  Hz, H-7), 3.37 (2H, d,  $J = 6.4$  Hz, H-7') ppm (Lee et al. 2008).

#### Cell viability assay

The human liver hepatocellular carcinoma (HepG2), breast cancer (MDA-MB-231 and MDA-MB-435), and nasopharyngeal carcinoma (HNE1) cell lines were obtained from the Shanghai cell line bank (China). The cells were maintained in Dulbecco's Modified Eagle's Medium (DEME, Hyclone, USA) containing 10 % fetal bovine serum (FBS, Hyclone, USA) and 1 % penicillin/streptomycin (Gibco, USA) in a humidified 5 % CO<sub>2</sub> atmosphere at 37 °C. Upon reaching confluence, the cells were detached using a 0.25 % trypsin–EDTA solution. The cells were seeded in 96-well plate at a density of 8,000 cells/well, 1 day before the start of treatment. We treated the cells with various concentrations of compounds **1–3** for 48 h. Cell viability was evaluated using a MTT assay as described previously. The amount of formazan formed was calculated from the absorbance ( $A$ ) measured at 570 nm by using a microplate reader. Cell viability (%) =  $(A_{\text{sample}} - A_{\text{blank}}) / (A_{\text{control}} - A_{\text{blank}}) \times 100$  %.

#### PI staining

Human liver hepatocellular carcinoma HepG2 cells were seeded at a density of  $5 \times 10^5$  cells/well in six-well plates in order to reach exponential growth for 24 h before treatment. Whole cell lysates from HepG2 cells were treated with drugs for 48 h by using propidium iodide (PI)

**Table 1** NMR data for bishonokiol A (**1**) in CDCl<sub>3</sub> (400 MHz)

	$\delta_c$	$\delta_H$ ( $J$ , Hz)		$\delta_c$	$\delta_H$ ( $J$ , Hz)
1	129.8 (s)		1''	129.8 (s)	
2	130.5 (d)	7.23 (1H, d, $J = 2.4$ Hz)	2''	130.5 (d)	7.23 (1H, d, $J = 2.4$ Hz)
3	129.0 (s)		3''	129.0 (s)	
4	157.0 (s)		4''	157.0 (s)	
5	110.9 (d)	6.96 (1H, d, $J = 8.4$ Hz)	5''	110.9 (d)	6.96 (1H, d, $J = 8.4$ Hz)
6	127.9 (d)	7.28 (1H, dd, $J = 2.4, 8.4$ Hz)	6''	127.9 (d)	7.28 (1H, dd, $J = 2.4, 8.4$ Hz)
7	34.3 (t)	3.43 (2H, d, $J = 6.8$ Hz)	7''	34.3 (t)	3.43 (2H, d, $J = 6.8$ Hz)
8	136.5 (d)	6.0 (m)	8''	136.5 (d)	6.0 (m)
9	115.5 (t)	5.07 (m)	9''	115.5 (t)	5.07 (m)
4-OCH <sub>3</sub>	55.5 (q)	3.88 (3H, s)	4''-OCH <sub>3</sub>	55.5 (q)	3.88 (3H, s)
1'	127.8 (s)		1'''	127.8 (s)	
2'	150.8 (s)		2'''	150.8 (s)	
3'	115.5 (d)	6.90 (1H, d, $J = 8.4$ Hz)	3'''	115.5 (d)	6.90 (1H, d, $J = 8.4$ Hz)
4'	128.8 (d)	7.05 (1H, dd, $J = 2.4, 8.4$ Hz)	4'''	128.8 (d)	7.05 (1H, dd, $J = 2.4, 8.4$ Hz)
5'	132.1 (s)		5'''	132.1 (s)	
6'	130.2 (d)	7.03 (1H, d, $J = 2.4$ Hz)	6'''	130.2 (d)	7.03 (1H, d, $J = 2.4$ Hz)
7'	39.4 (t)	3.35 (2H, d, $J = 6.8$ Hz)	7'''	39.4 (t)	3.35 (2H, d, $J = 6.8$ Hz)
8'	137.8 (d)	6.0 (m)	8'''	137.8 (d)	6.0 (m)
9'	115.8 (t)	5.11 (m)	9'''	115.8 (t)	5.11 (m)

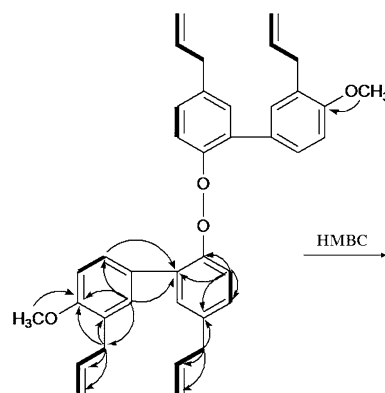
staining, which was then quantified by using flow cytometry (BD Accuri C<sub>6</sub>, USA).

#### Western blot analysis

The HepG2 cells were seeded at a density of  $5 \times 10^5$  cells/well in six-well plates. After incubation of the drugs, the cells were harvested, washed twice in phosphate-buffered saline (PBS), and lysed using a lysis buffer [50 mM Tris-HCl pH = 7.4, 1 % Triton X-100, 150 mM NaCl, and a cocktail of protease inhibitors (Sigma, USA)]. Lysates were loaded onto 12 % SDS-PAGE gel and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5 % skim milk, and were incubated overnight with anti-Bcl-2 (Proteintech, USA), anti-Caspase 3 (Abcam, UK), and anti- $\beta$ -actin (Santa Cruz Biotechnology, USA) antibodies. Mouse and rabbit horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, USA) were used in TBST/5 % skim milk. The amount of bound antibodies was visualized using an enhanced chemiluminescence (ECL) substrate (Intron Biotechnology, Korea) and imaged with gel imaging equipment (Bio-Rad, CA, USA).

#### Results and discussion

Chromatographic purification of the EtOAc-soluble fraction led to the isolation of compounds **1**–**3**. The molecular



**Fig. 2** <sup>1</sup>H–<sup>1</sup>H COSY (*bold*) and key HMBC correlations of **1**

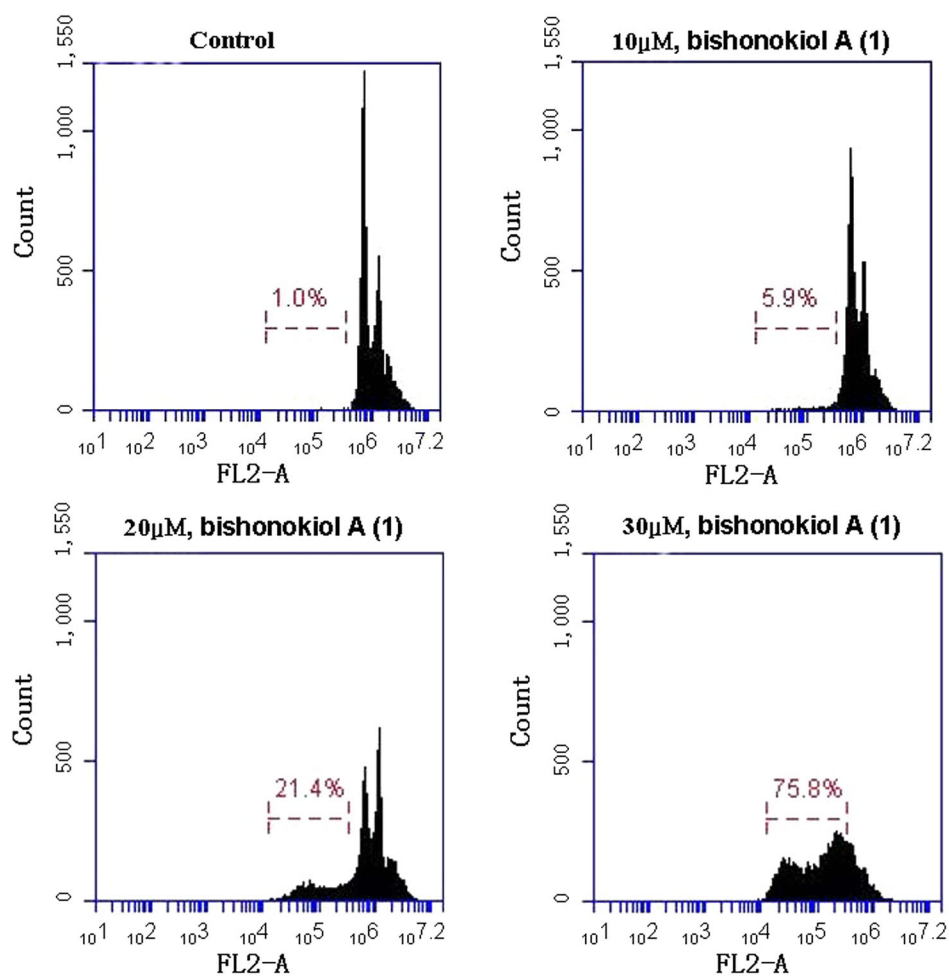
formula of compound **1**, C<sub>38</sub>H<sub>38</sub>O<sub>4</sub> was determined from HR-ESIMS data ( $m/z$  559.2835 calculated 559.2848 for C<sub>38</sub>H<sub>39</sub>O<sub>4</sub>) in conjunction with NMR data. The <sup>1</sup>H-NMR spectrum showed two ABX type protons with signals at  $\delta_H$  7.28 (1H, dd,  $J = 8.4, 2.4$  Hz), 7.23 (1H, d,  $J = 2.4$  Hz), and 6.96 (1H, d,  $J = 8.4$  Hz), as well as 7.05 (1H, dd,  $J = 8.4, 2.4$  Hz), 7.03 (1H, d,  $J = 2.4$  Hz), and 6.90 (1H, d,  $J = 8.4$  Hz), vinyl group signals at  $\delta_H$  6.0 (m) and 5.07 or 5.11 (m), *O*-methyl signals at  $\delta_H$  3.88 (3H, s), and two aliphatic proton signals at  $\delta_H$  3.43 (2H, d,  $J = 6.8$  Hz) and 3.35 (2H, d,  $J = 6.8$  Hz). The <sup>13</sup>C-NMR and distortionless enhancement by polarization transfer (DEPT) experiments only showed the following nineteen

**Table 2** Cell viability data (IC<sub>50</sub>) for compounds **1**, **2**, and **3**

	HepG2	MDA-MB-231	MDA-MB-435	HNE1
Bishonokiol A ( <b>1</b> )	5.1 ± 0.2 μM	4.8 ± 0.3 μM	6.7 ± 0.2 μM	7.5 ± 0.4 μM
Magnolol ( <b>2</b> )	17.3 ± 0.9 μM	NT	26.6 ± 1.2 μM	NT
Honokiol ( <b>3</b> )	6.3 ± 0.5 μM	NT	12.2 ± 0.8 μM	NT

*HepG2* Human liver hepatocellular carcinoma cell, *MDA-MB-231* human breast cancer cell, *MDA-MB-435* human breast cancer cell, *HNE1* human nasopharyngeal carcinoma cell, *NT* not tested

**Fig. 3** Effect of bishonokiol A (**1**) on apoptosis. HepG2 cells were treated with bishonokiol A (10, 20, and 30 μM) or with 0.1 % DMSO. The cells were harvested, fixed, and stained with propidium iodide. Quantitative analysis of sub-G<sub>1</sub> cells was carried out by flow cytometer



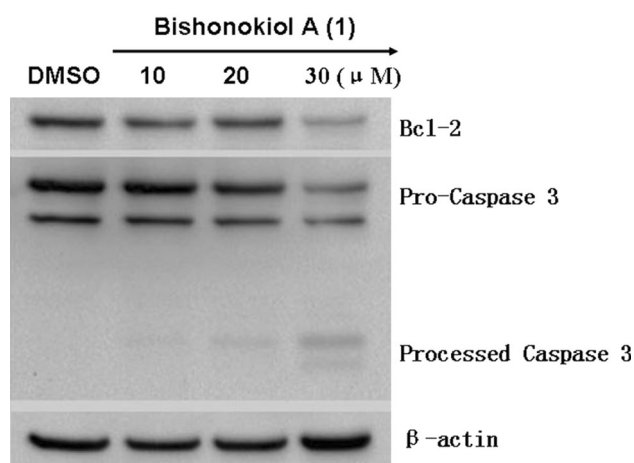
carbon signals, which indicated that compound **1** had complete symmetry: seven aromatic or olefinic quaternary, seven aromatic or olefinic methine, two aromatic or olefinic methane, two aliphatic methane, and one *O*-methyl carbons (Table 1). The physicochemical properties and NMR data suggested that compound **1** was very similar to the known neolignan 4-*O*-methylhonokiol (Lee et al. 2008; Ito et al. 1982; Oh et al. 2009). These results suggested that compound **1** is a dimeric molecule containing two 4-*O*-methylhonokiol moieties. The connectivity between the two 4-*O*-methylhonokiol units through a peroxide bridge (C-2' OH and C-2''' OH) was verified from MS and NMR

data. The <sup>1</sup>H-<sup>1</sup>H COSY showed important correlations between protons at H-5/H-6, H-7/H-8, H-8/H-9, H-3'/H-4', H-7'/H-8', and H-8'/H-9' (Fig. 2). Meanwhile, heteronuclear multiple-bond correlation (HMBC) spectroscopy experiments showed key <sup>1</sup>H-<sup>13</sup>C long range correlations (4-OCH<sub>3</sub>/C-4, H-7/C-3, C-4, C-8, C-9, H-7'/C-5', C-8', C-9', C-2/C-1', and C-6/C-1') (Fig. 2). Therefore, the structure of the new dimeric neolignan, bishonokiol A (**1**), was assigned as shown in Fig. 1.

*Magnolia* species are considered rich sources of lignans (Ito et al. 1982; Schuehly et al. 2010; Schühly et al. 2009; Clark et al. 1981; Shen et al. 2009). Neolignans such as

obovatal, honokiol, and magnolol have been previously shown to possess various biological activities including antitumor, antiviral, anxiolytic-like, and anti-inflammatory effects (Oh et al. 2009; Arora et al. 2012; Yu et al. 2012). To determine the anti-proliferative activity of the isolated compounds **1–3**, MTT assays were carried out using human liver hepatocellular carcinoma HepG2, breast cancer MDA-MB-231 and MDA-MB-435, and nasopharyngeal carcinoma HNE1 cell lines. Our results showed that 48 h after administration, compound **1** exhibited potent anti-proliferative activities against four human cancer cell lines with IC<sub>50</sub> values ranging from 5.1 to 7.5  $\mu$ M, whereas the two other compounds had lower potencies (Table 2).

Apoptosis is an important programmed cell death (PCD) process that occurs in order to maintain the homeostasis between cell proliferation and cell death. We investigated whether compound **1** induced cell death involved apoptosis, and our result indeed showed that **1** was a potent pro-apoptotic compound. We analyzed the effect of compound **1** on apoptosis in the HepG2 cell line by using propidium iodide (PI) staining in combination with flow cytometry. After exposure to 10, 20, and 30  $\mu$ M compound **1** for 48 h, the apoptosis rate were 5.9, 21.4, and 75.8 %, respectively (Fig. 3). To explore the mechanisms underlying the pro-apoptotic action of compound **1**, we also examined the expression level of the antiapoptotic protein Bcl-2, as well as the cleavage of caspase 3, a key down-stream effector of apoptosis. As shown in Fig. 4, western blot analysis indicated that compound **1** down-regulated the expression of Bcl-2 and potentiated the cleavage of caspase-3 in a concentration dependent manner.



**Fig. 4** Effect of bishonokiol A (**1**) on the expression of apoptosis related proteins. HepG2 cells were treated for 24 h with vehicle or with various concentrations of bishonokiol A (**1**). The ability of bishonokiol A (**1**) to regulate the expression of apoptosis-related proteins was evaluated as described in the materials and methods

Taken together, the findings suggest that newly identified dimeric neolignan (**1**) exerted anti-cancer effects through the induction of apoptosis in human cancer cells. Further studies will be required to investigate the in vivo anti-tumor activity of the new compound **1** and to confirm the cellular mechanisms involved.

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