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# A new *ent*-kaurane diterpene derivative from the stems of *Eurya chinensis* R.Br

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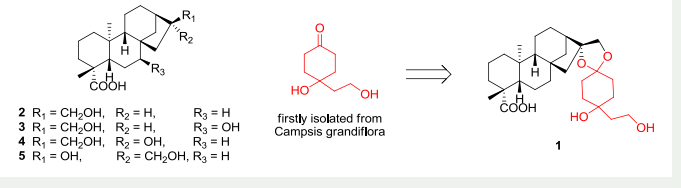
**ABSTRACT**

One new *ent*-kaurane diterpene derivative (**1**), along with four known diterpenes, was isolated from the stems of *Eurya chinensis* R.Br. The structure of the new compound was established by extensive analysis of mass spectrometric and 1D and 2D NMR spectroscopic data. Compound **1** showed moderate anti-inflammatory activities with $IC_{50}$ value of 8.12 μM. This is the first example of diterpenoids with 4-hydroxy-4-(2-hydroxyethyl)-1-hydroxyl-cyclohexanoyl substituent.

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*Eurya chinensis*; diterpene; derivative; anti-inflammatory

## 1. Introduction

The genus *Eurya* (approximately 130 species) belongs to the family Theaceae, which is distributed throughout the tropical areas of Asia and the south-west Pacific Islands (Chung & Epperson 2000). Previous chemical studies on the genus *Eurya* led to the isolation of triterpenes, phenylpropanoids, flavonoids and lignans (Morita et al. 1974; Khan et al. 1992; Oh et al. 2011; Yang Kuo et al. 2013), in addition, many of these compounds possessed different biological properties, including anti-inflammatory, cytostatic and antioxidant activities (Park, Lee et al. 2005; Park, Yang et al. 2005; Yang Kuo et al. 2013).

*Eurya chinensis* R.Br. grows throughout the south of China, its fresh stems and leaves have been used as remedy for a variety of ailments, such as fever, jaundice and trauma (Song et al. 2017). In our previous studies, (±)-euryacoumarin A and 6-demethylobtusinin, two

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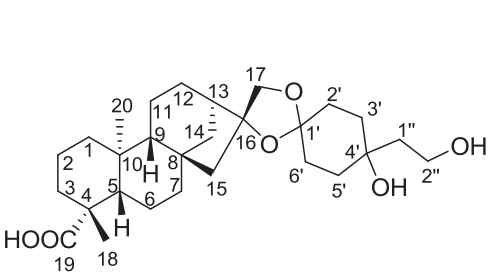
new coumarins, were isolated from the stems of *E. chinensis* (Song et al. 2017). As a continuing search for structurally unique and biologically active compounds, the stems of *E. chinensis* were thoroughly isolated. As a result, one new *ent*-kaurane diterpene derivative, 16*α*,17-[4-hydroxy-4-(2-hydroxyethyl)-cyclohexylidenedioxy]-*ent*-kauran-19-oic acid (**1**), along with four known *ent*-kaurane diterpenoids (**2**–**5**) (Figure 1) have been identified: 17-hydroxy-16*α*-*ent*-kauran-19-oic acid (**2**) (Wu et al. 1996), 7*β*,17-dihydroxy-16*α*-*ent*-kauran-19-oic acid (**3**) (Furtado et al. 2013), 16*α*,17-dihydroxy-*ent*-kauran-19-oic acid (**4**) (Wu et al. 1996) and 16*β*,17-dihydroxy-*ent*-kauran-19-oic acid (**5**) (Wu et al. 1996). Herein, we describes the isolation and structural elucidation of these compounds, as well as the biological evaluation of antitumour and anti-inflammatory activities.

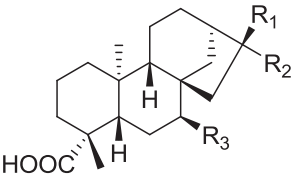
## 2. Results and discussion

Compound **1** was obtained as an amorphous powder, and its molecular formula $C_{28}H_{44}O_6$ was established by the $^{13}C$ NMR data and an HRESIMS ion at *m/z* 475.3027 $[M - H]^-$ (calcd 475.3060), requiring seven degrees of unsaturation.

The $^{13}C$ NMR data and the HSQC spectrum of **1** exhibited 28 carbons, attributing to two tertiary methyls, 16 methylenes, three methines and seven quaternary carbons. Two downfield signals at $\delta_C$ 90.1 and 70.6 suggesting the existence of an oxygenated quaternary carbon and an oxygenated methylene, together with the diagnostic signals of three methines [$\delta_C$ 58.2 (C-5), 56.8 (C-9) and 47.3 (C-13)] and three quaternary carbons [$\delta_C$ 45.7 (C-4), 44.7 (C-8) and 40.8 (C-10)], indicated an *ent*-kaurane diterpene skeleton (Chen et al. 2016; Galala et al. 2016; Xu et al. 2016; Liu et al. 2017), which could be supported by its $^1H$–$^1H$ COSY and HMBC experiments. The $^1H$–$^1H$ COSY spectrum of **1** indicated the correlations from $\delta_H$ 1.88/0.84 ($H_2$-1) to $\delta_H$ 1.93/1.40 ($H_2$-2), then to $\delta_H$ 2.15/1.03 ($H_2$-3), and from $\delta_H$ 1.08 (H-5) to $\delta_H$ 1.85 ($H_2$-6), then to $\delta_H$ 1.56/1.47 ($H_2$-7), and from $\delta_H$ 1.01 (H-9) to $\delta_H$ 1.65/1.40 ($H_2$-11), then to $\delta_H$ 1.56 ($H_2$-12) and $\delta_H$ 2.07 (H-13). Similarly, its HMBC data exhibited that the proton at $\delta_H$ 1.08 (H-5) correlated with the carbons at $\delta_C$ 29.5 (C-18), 181.7 (C-19) and 16.4 (C-20), $\delta_H$ 1.01 (H-9) correlated with $\delta_C$ 42.0 (C-1), 28.0 (C-12), 39.1 (C-14) and 57.7 (C-15), while 2.07 (H-13) correlated with $\delta_C$ 44.7 (C-8), 20.3 (C-11), 57.7 (C-15) and 70.6 (C-17). Therefore, all the above characteristic data were indicative of the same diterpene skeleton with that of 16,17-dihydroxy-*ent*-kauran-19-oic acid (Wu et al. 1996).




**1**




**2** $R_1 = CH_2OH$, $R_2 = H$, $R_3 = H$
**3** $R_1 = CH_2OH$, $R_2 = H$, $R_3 = OH$
**4** $R_1 = CH_2OH$, $R_2 = OH$, $R_3 = H$
**5** $R_1 = OH$, $R_2 = CH_2OH$, $R_3 = H$

**Figure 1.** *Ent*-kaurane diterpenes **1**–**5** isolated from *E. Chinensis* R.Br.

Apart from the signals of the diterpene, one oxygenated methylene ($\delta_H$ 3.76, 2H, t, $J$ = 7.0 Hz), one non-cyclic aliphatic methylene ($\delta_H$ 1.58, 2H, overlapped) and four cyclic methylenes [($\delta_H$ 1.68, 4H, overlapped), ($\delta_H$ 1.82, 2H, overlapped), ($\delta_H$ 1.55, 2H, overlapped)] were exhibited in the $^1$H NMR spectrum. Besides, one ketal ($\delta_C$ 109.8) was readily identified in the $^{13}$C NMR spectrum. Moreover, the presence of three proton sequences of $CH_2$ (2′)–$CH_2$ (3′), $CH_2$ (5′)–$CH_2$ (6′), and $CH_2$ (1″)–$CH_2$ (2″) was disclosed in $^1$H–$^1$H COSY spectrum. Therefore, the left eight carbons indicated the presence of a 4-hydroxy-4-(2-hydroxyethyl)-cyclohexylidene substituent (Guiso et al. 1997; Kim et al. 2007). This residue was assigned to be located at C-16 and C-17 from the HMBC correlation between H-17a ($\delta_H$ 4.10) and C-1′ ($\delta_C$ 109.8).

In the NOESY spectrum, the cross-peaks of $H_3$-18/H-5, H-5/H-9 and H-9/H-15b suggested $H_3$-18, H-5, H-9 and $H_2$-15 were all co-facial. Therefore, they were arbitrarily assigned as $\beta$-orientation based on the comparisons with the spectral data of similar *ent*-kauran-19-oic acids and biogenetic considerations (Wu et al. 1996; Chen et al. 2016; Galala et al. 2016; Xu et al. 2016; Liu et al. 2017). However, due to the missing of key correlations in the NOESY spectrum, the configuration of C-16 in **1** was deduced by hydrolysis of **1** and subsequent LC-MS analysis. Mild acidic hydrolysis of **1** afforded an 16$\alpha$,17-dihydroxy-*ent*-kauran-19-oic acid (**4**) which was confirmed by LC-MS analysis (Figure S9). Therefore, the chiral carbons including C-16 in **1** share the same configurations as those in **4**, and thus, **1** is established to be to 16$\alpha$,17-[4-hydroxy-4-(2-hydroxyethyl)-cyclohexylidenedioxy]-*ent*-kauran-19-oic acid.

*In the previous studies, Eurya* plants were found to be important sources of newly potential anticancer and anti-inflammatory agents (Park, Lee et al. 2005; Park, Yang et al. 2005). Therefore, all the isolated compounds were evaluated for their cytotoxic activities against three human tumour cell lines NCI-H46, SW480 and HepG2 using MTT assay (Mosmann 1983). However, none showed significant activity (cellular proliferation inhibition rate <50% at 20 μM). Besides, all the isolated compounds were evaluated for their activities against the production of NO in RAW264.7 macrophages stimulated by lipopolysaccharide. Compound **1** showed moderate inhibitory effect on NO production in LPS-stimulated RAW264.7 cells with $IC_{50}$ value of 8.12 μM, but the remaining compounds were inactive ($IC_{50}$ > 50 μM). It is interesting that only compound **1** with 4-hydroxy-4-(2-hydroxyethyl)-cyclohexylidene substituent acts as the active compound. On one hand, it seems that the 4-hydroxy-4-(2-hydroxyethyl)-cyclohexylidene substituent is essential for the inhibitory effect on NO production in LPS stimulated RAW264.7 cells. On the other hand, the naked hydroxyl groups of diterpene skeleton in compounds **2–5** might be adverse to the activity and should be protected by some functional groups such as the acetal in compound **1**.

## 3. Experimental

### 3.1. General experimental procedures

Optical rotations were measured on a Perkin-Elmer 341 polarimeter. UV spectra were recorded in MeOH on a Perkin-Elmer Lambda 35 UV–Vis spectrophotometer. IR spectra were acquired using a Bruker Vertex 33 infrared spectrophotometer (Bruker, Karlsruhe, Germany) with KBr disc. Analytical HPLC was performed on a Waters 2690 instrument with a 996 PAD (photodiode array detector) and coupled with an Alltech ELSD 2000 detector. HR-ESI-MS spectra were recorded on a Bruker Bio TOF IIIQ mass spectrometer. NMR spectra were recorded on a Bruker Advance-500 spectrometer using (tetramethylsilane) TMS as an internal standard. All organic solvents used for extraction were of analytical grade (Shanghai Chemical

Plant, Shanghai, People's Republic of China). Methanol used for HPLC was of HPLC-grade (J&K, Beijing, People's Republic of China). Silica gel (200–300 mesh) (Qingdao Haiyang Chemical Plant, Qingdao, People's Republic of China), C18 reversed-phase silica gel (150–200 mesh, Merck), MCI gel (CHP20P, 75–150 μM, Mitsubishi Chemical Industries Ltd.) and Sephadex LH-20 gel (Amersham Biosciences) were used for column chromatography. TLC spots were visualised under UV light and by dipping into 5% $H_2SO_4$ in alcohol followed by heating. Purification by means of HPLC was conducted using Alltima C18 column (250 mm × 10 mm, 5 μM, Tong Heng Innovation Technology Co., Ltd., Beijing, People's Republic of China).

### 3.2. Plant material

The stems of *E. chinensis* were collected in Nankang County, Jiangxi Province, China, in June 2013 and identified by prof. Fu-Wu Xing, South China Botanical Garden. A voucher specimen (No. 201309) has been deposited at the Laboratory of Natural Product Chemistry Biology (SCBG), South China Botanical Garden.

### 3.3. Extraction and isolation

Dried-powdered stems of *E. chinensis* (50.0 kg) were extracted by 95% ethanol at room temperate and filtered, then the filtrate was concentrated under vacuum to give a crude residue (2.1 kg). The residue (2.1 kg) was suspended in $H_2O$ and extracted with *n*-hexane and EtOAc. The EtOAc-soluble fraction (1.2 kg) was subjected to column chromatography (CC) over silica gel eluting with *n*-hexane/acetone in a stepwise manner to give six fractions (E1–E6). Fraction E4 (237 g) was then subjected to CC over silica gel eluting with $CH_2Cl_2$/EtOAc in a stepwise manner, affording four fractions (E4A–E4F). Fraction E4C was subjected to CC over MCI gel (MeOH/$H_2O$, 30–70%) to give five subfractions (E4C1–E4C5). Subfraction E4C2 was further subjected to CC over silica gel eluting with $CH_2Cl_2$/EtOAc in a stepwise manner to yield **2** (70 mg). Fraction E5 (98.3 g) was subjected to CC over MCI gel (MeOH/$H_2O$, 30–70%) to give 10 fractions (E5A–E5 J). E5H (2.6 g) was subjected to CC over Sephadex LH-20 (MeOH) to afford five subfractions (E5H1–E5H5). E5H3 (78 mg) was subjected to CC over silica gel eluting with $CH_2Cl_2$/EtOAc in a stepwise manner to yield **1** (12 mg). E5H4 (213 mg) was subjected to CC over silica gel eluting with $CH_2Cl_2$/EtOAc in a stepwise manner to yield **4** (60 mg) and yield **5** (93 mg). E5I (3.8 g) was subjected to CC over Sephadex LH-20 (MeOH) to afford six subfractions (E5I1–E5I6). E5I3 (240 mg) was subjected to CC over silica gel eluting with $CH_2Cl_2$/EtOAc in a stepwise manner to yield **3** (6 mg).

*Ent-16α,17-[4-hydroxy-4-(2-hydroxyethyl)-cyclohexylidenedioxy]-(–)-19-oic acid* (**1**): Amorphous powder; $[\alpha]_D^{25}$–28.8 (*c* 1.70; $CH_3OH$); CD $\Delta\varepsilon$ (*c* 1.03, $CH_3OH$) −0.18 (224.3 nm); IR (KBr) $\nu_{max}$ 3409, 2929, 2853, 1699, 1449, 1371, 1263, 1106, 1030, 957 $cm^{-1}$; HR-ESI-MS $[M - H]^-$ at *m/z* 475.3027 (Calcd for $C_{28}H_{44}O_6$–H, 475. 3060); $^1H$ NMR (500 MHz, $CD_3OD$): $\delta_H$ 1.88 (1H, m, H-1a), 0.84 (1H, m, H-1b), 1.93 (1H, m, H-2a), 1.40 (1H, m, H-2b), 2.15 (1H, m, H-3a), 1.03 (1H, m, H-3b), 1.08 (1H, m, H-5), 1.85 (2H, m, H-6), 1.56 (1H, overlapped, H-7a), 1.47 (1H, m, H-7b), 1.01 (1H, brs, H-9), 1.65 (1H, overlapped, H-11a), 1.40 (1H, overlapped, H-11b), 1.56 (2H, overlapped, H-12), 2.07 (1H, m, H-13), 2.01 (1H, overlapped, H-14a), 1.52 (1H, m, H-14b), 1.74 (1H, d, *J* = 4.2 Hz, H-15a), 1.79 (1H, d, *J* = 4.2 Hz, H-15b), 4.10 (1H, d, *J* = 8.6 Hz, H-17a), 3.96 (1H, d, *J* = 8.6 Hz, H-17b), 1.20 (3H, s, H-18), 0.99 (3H, s, H-20), 1.68 (2H, overlapped, H-2′),

1.82 (1H, overlapped, H-3′a), 1.55 (1H, overlapped, H-3′b), 1.82 (1H, overlapped, H-5′a), 1.55 (1H, overlapped, H-5′b), 1.68 (2H, overlapped, H-6′), 1.58 (2H, overlapped, H-1″), 3.76 (2H, t, $J$ = 7.0 Hz, H-2″). $^{13}$C NMR (125 MHz, $CD_3OD$): $\delta_C$ 42.0 (C-1), 20.0 (C-2), 39.2 (C-3), 45.7 (C-4), 58.2 (C-5), 23.3 (C-6), 42.9 (C-7), 44.7 (C-8), 56.8 (C-9), 40.8 (C-10), 20.3 (C-11), 28.0 (C-12), 47.3 (C-13), 39.1 (C-14), 57.7 (C-15), 90.1 (C-16), 70.6 (C-17), 29.5 (C-18), 181.7 (C-19), 16.4 (C-20), 109.8 (C-1′), 35.8 (C-2′), 32.8 (C-3′), 71.2 (C-4′), 32.9 (C-5′), 35.8 (C-6′), 43.0 (C-1″), 59.2 (C-2″).

### 3.4. Acid hydrolysis of 1

Compound **1** (2 mg) was hydrolysed with 5% HCl (3 mL) at 50 °C for 2 h. The mixture was neutralised with 10% $NaHCO_3$ and extracted with $CH_2Cl_2$ (3 × 5 mL). Mild acidic hydrolysis of **1** afforded an 16*α*,17-dihydroxy-*ent*-kauran-19-oic acid (**4**) which was confirmed by LC-MS analysis (Figure S9).

### 3.5. Cytotoxic activity against NCI-H46, SW480 and HepG2

Compounds **1–5** were evaluated for inhibitory activity against human lung cancer cell (NCI-H46), human colorectal cancer cells (SW480) and human liver cancer cells (HepG2) using the MTT method reported previously (Mosmann 1983)**.** Doxorubicin was used as positive control. Cells were plated in 96-well tissue plates at a density of $1 \times 10^4$ cells/well. Adherent cell lines were previously incubated for 24 h to ensure adhesion to the wells in an atmosphere of 5% $CO_2$. Compounds **1–5** were applied at various concentrations (0.01, 0.1, 1, 10, 100 μM) and control cells were treated with DMSO at the highest concentration used in test wells (0.5%). 1 h prior to the end of the incubation period, 20 mL of MTT (5 mg/mL in PBS, 5% MTT) were added to each well and further incubated at 37 °C for another 4 h. Supernatants were removed and 150 μL DMSO were afterwards added to each well in order to dissolve the formazan crystals. The mixture was oscillatored for 10 min at room temperature and its absorbance was measured at 490 nm (Genios, Tecan, Austria). The concentration resulting in 50% of cell-growth inhibition ($IC_{50}$) was calculated using the Probit program in SPSS 19 for windows XP (SPSS Inc.Chicago)

### 3.6. Measurement of nitric oxide (NO) production in RAW264.7 macrophages

Compounds **1–5** were examined for inhibition on NO production in LPS-stimulated RAW264.7 cells according to previously described method (Park et al. 2013; Zhong et al. 2015). Murine monocytic RAW264.7 macrophagesispensed into 96-well plates ($5 \times 10^5$ cells/well) containing DMEM medium (Gibco) with 10% FBS under a humidified atmosphere of 5% $CO_2$ at 37 °C. After 24 h preincubation, cells were treated with serial dilutions of the compounds, with the maximum concentration of 200 μM, in the presence of 1 μM LPS for 18 h. Each compound was dissolved in DMSO and further diluted in medium to produce different concentrations. NO production in each well was assessed by adding 100 μL of Griess reagent (Reagent A & Reagent B, respectively, Biotine) to 100 μL of each supernatant from LPS (Sigma)-treated or LPS- and compound-treated cells in triplicate. After 5-min incubation, the absorbance was measured at 420 nm with a Multiscan Spectrum (TECAN Genios). L-NMMA ($N^G$-Monomethyl-L-arginine, Monoacetate Salt, beyotime biotechnology) was used as a positive control.

## 4. Conclusions

One new *ent*-kaurane diterpene derivative (**1**), along with four known diterpenes, were isolated from the stems of *E. chinensis*. To the best of our knowledge, compound **1** was the first example of diterpenoids with 4-hydroxy-4-(2-hydroxyethyl)-1-hydroxyl-cyclohexanoyl substituent. The cytotoxic and anti-inflammatory effect of these isolates were evaluated, compound **1** showed moderate anti-inflammatory activities with $IC_{50}$ value of 8.12 μM.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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