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Original article

6,7-*seco*-*ent*-Kaurane diterpenoids from *Isodon sculponeatus* and their bioactivity

Hua-Yi Jiang [a,b], Wei-Guang Wang [a], Min Zhou [a,b], Hai-Yan Wu [a,b], Rui Zhan [a], Xue Du [a], Jian-Xin Pu [a,*], Han-Dong Sun [a,*]

[a] *State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China*
[b] *University of Chinese Academy of Sciences, Beijing 100049, China*

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ABSTRACT

One new 6,7-*seco*-*ent*-kaurane diterpenoid, sculponin T (**1**), was isolated from the aerial parts of *Isodon sculponeatus*, along with four known analogs, sculponeatin J (**2**), sculponeatin K (**3**), sculponeatin C (**4**), and sculponeatin Q (**5**). Their structures were elucidated by extensive spectroscopic analysis and by comparison with data reported in the literature. Significant cytotoxic activity was observed for compound **2** against five human tumor cell lines with IC_{50} values ranging from 1.8 μmol/L to 3.3 μmol/L, and it also inhibited NO production in LPS-stimulated RAW264.7 cells, with IC_{50} value of 3.3 μmol/L.
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1. Introduction

The genus *Isodon*, which includes about 150 species, is a cosmopolitan and important genus of the family Lamiaceae and has attracted considerable attention as a prolific source of diterpenoids with diverse structures and biological activities [1,2]. *Isodon sculponeatus* (Vaniot) Kudo, a perennial herb, is distributed widely in southern China, and has been used as a folk medicine for treatment of dysentery and beriberi [3,4]. Previous phytochemical investigations of this species proved that it was a rich source of bioactive diterpenoids, mainly *ent*-kauranoids [5–15], such as sculponeatin I [11], sculponin A [12], and sculponeatin O [15]. In our efforts to seek structurally interesting and potential bioactive diterpenoids from the genus *Isodon*, we investigated the chemical constituents of the aerial parts of *I. sculponeatus* collected from Muli County of Sichuan Province, China, and have found a series of *ent*-kaurane diterpenoids, of which some have antitumor and anti-inflammatory activities [16]. A continued phytochemical investigation on this plant led to the isolation of an additional one new 6,7-*seco*-*ent*-kaurane diterpenoid, sculponin T (**1**), together with four known analogs, sculponeatin J (**2**) [10], sculponeatin K (**3**) [10], sculponeatin C (**4**) [6], and sculponeatin Q (**5**) [15] (Fig. 1). Herein, we report the isolation and structure elucidation of the new compound, as well as the *in vitro* cytotoxicity evaluation of all isolates obtained against five human tumor cell lines (HL-60, SMMC-7721, A-549, MCF-7, and SW-480), and the inhibitory activity against NO production in LPS-stimulated RAW264.7 cells of compounds **1**, **2**, and **4**.

2. Experimental

2.1. Plant material

The aerial parts of *I. sculponeatus* were collected in August 2011 and identified by Prof. Xi-Wen Li, Kunming Institute of Botany. A voucher specimen (KIB 20110813) has been deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

2.2. Extraction and isolation

The dried and powdered aerial parts of *I. sculponeatus* (8 kg) were extracted with 70% aqueous acetone (30 L) four times (two days each time) at room temperature, then filtered. The filtrate was evaporated under reduced pressure and then partitioned between EtOAc and H_2O. The EtOAc soluble portion (700 g) was subjected to silica gel CC (3 kg, 100–200 mesh), eluted with a $CHCl_3$–Me_2CO

* Corresponding authors.
E-mail addresses: pujianxin@mail.kib.ac.cn (J.-X. Pu), hdsun@mail.kib.ac.cn (H.-D. Sun).

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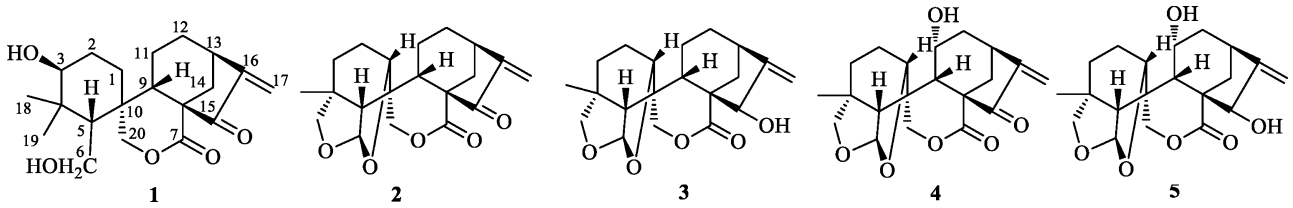

Fig. 1. Structures of compounds **1**–**5**.

gradient system (1:0–0:1) that afforded fractions A–G. The fractions were then decolorized using MCI gel and eluted with 90% MeOH–H_2O. Fraction B (120 g) was chromatographed *via* silica gel CC (200–300 mesh), eluted with $CHCl_3$–MeOH gradient (150:1–1:1) to yield fractions B1–B5. Compound **4** (139 mg) was crystallized from fraction B5. Fraction B1 was purified by repeated chromatography over silica gel CC (petroleum ether–Me_2CO gradient, 12:1–0:1), followed by semipreparative HPLC (30% MeCN–H_2O), to yield compounds **5** (40 mg), **2** (47 mg), and **3** (19 mg). Fraction D (100 g) was subjected to RP-18 CC (MeOH–H_2O gradient, 10%–100%) to give fractions D1–D6. Then fraction D2 was separated by CC on silica gel (200–300 mesh), eluted with $CHCl_3$–MeOH (gradient system: 100:1–1:1), to give compound **1** (4 mg).

2.3. The cytotoxicity assay

The human tumor cell lines HL-60, SMMC-7721, A-549, MCF-7, and SW-480 were used, which were obtained from ATCC (Manassas, VA, USA). All the cells were cultured in RPMI-1640 or DMEM medium (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (Hyclone) at 37 °C in a humidified atmosphere with 5% CO_2. Cell viability was assessed by conducting colorimetric measurements of the amount of insoluble formazan formed in living cells based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfopheny)-2*H*-tetrazolium (MTS) (Sigma, St. Louis, MO, USA) [17]. Briefly, 100 μL of adherent cells were seeded into each well of a 96-well cell culture plate and allowed to adhere for 12 h before drug addition, while suspended cells were seeded just before drug addition, both with an initial density of 1×10^5 cells/mL in 100 μL medium. Each tumor cell line was exposed to the test compound at various concentrations in triplicate for 48 h, with cisplatin and paclitaxel (Sigma) as positive controls. After the incubation, MTS (100 μg) was added to each well, and the incubation continued for 4 h at 37 °C. The cells were lysed with 100 μL of 20% SDS–50% DMF after removal of 100 μL medium. The optical density of the lysate was measured at 490 nm in a 96-well microtiter plate reader (Bio-Rad 680). The IC_{50} value of each compound was calculated by the Reed and Muench's method [18].

2.4. Nitric oxide production in RAW264.7 macrophages

Murine monocytic RAW264.7 macrophages were dispensed into 96-well plates (2×10^5 cells/well) containing RPMI 1640 medium (Hyclone) 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 25 μmol/L, in the presence of 1 μg/mL 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 and Reagent B, respectively, Sigma) to 100 μL of each supernant from LPS (Sigma)-treated or LPS- and compound-treated cells in triplicate. After 5 min incubation, the absorbance was measured at 570 nm with a 2104 Envision Multilabel Plate Reader (Perkin-Elmer Life Sciences, Inc., Boston, MA, USA). MG-132 was used as a positive control [19].

3. Results and discussion

Compound **1**, $[\alpha]_D^{26.0} + 12.1$ (*c* 0.06, MeOH), UV (MeOH) λ_{max} (log ε): 232 (3.67), 201 (3.62) nm, obtained as a white, amorphous powder, displayed a molecular ion peak at *m*/*z* 348.1933 ($[M]^+$, calcd. 348.1937) in its HREIMS, in accordance with a molecular formula $C_{20}H_{28}O_5$. Its IR absorptions at 3430, 1739, 1710, and 1643 cm^{-1} implied the existence of OH, C=O, and C=C groups. The ^1H NMR spectrum (Table 1) indicated the presence of two tertiary methyl groups (δ_H 1.34, 0.98), and two olefinic protons (δ_H 5.91, 5.26). The ^{13}C NMR and DEPT data (Table 1) suggested that **1** was a diterpenoid with a total of 20 carbons, consisting of two methyls, eight methylenes (two oxygenated and one olefinic), four methines (one oxygenated), and six quaternary carbons (two carbonyl and one olefinic). Analysis of the HSQC and ^1H–^1H COSY spectra (Fig. 2) revealed the spin systems for the molecular fragments of C-1–C-2–C-3, C-5–C-6, and C-9–C-11–C-12–C-13–C-14. Their connectivity

Table 1
NMR data of sculponin T (**1**) (C_5D_5N, TMS, δ in ppm, *J* in Hz).[a,b]

No.	^1H NMR	^{13}C NMR	HMBC (H → C)	No.	^1H NMR	^{13}C NMR	HMBC (H → C)
1	2.36 (overlap)	19.8 (t)	C-2, 10	11	1.71 (overlap)		C-9, 12, 13
	1.47 (m)		C-10	12	1.99 (overlap)	30.0 (t)	C-9, 13, 16
2	2.05 (overlap)	25.6 (t)	C-1, 3	13	2.85 (m)	35.6 (d)	C-8, 11, 16, 17
	1.75 (overlap)		C-1, 3, 10	14	2.51 (dd, 12.3, 4.5)	30.0 (t)	C-7, 8, 9, 13
3	3.57 (br s)	74.6 (d)			2.20 (d, 12.3)		C-8, 9, 12, 13, 15, 16
4		38.9 (s)		15		203.6 (s)	
5	2.36 (overlap)	46.0 (d)	C-6, 9, 10	16		151.4 (s)	
6	3.95 (m)	58.5 (t)	C-4, 5, 10	17	5.91 (br s)	117.5 (t)	C-13, 15, 16
7		171.7 (s)			5.26 (br s)		C-13
8		58.5 (s)		18	1.34 (s)	30.1 (q)	C-3, 4, 5, 19
9	3.15 (dd, 14.7, 5.0)	45.3 (d)	C-1, 8, 10, 12, 14	19	0.98 (s)	24.1 (q)	C-3, 5, 18
10		42.5 (s)		20	4.91 (d, 8.3)	71.1 (t)	C-1, 7, 9, 10
11	1.85 (m)	17.8 (t)	C-9, 12				

[a] ^1H NMR and ^{13}C NMR data were recorded at 500 MHz and 125 MHz, respectively.
[b] The assignments were based on HSQC, ^1H–^1H COSY, and HMBC experiments.

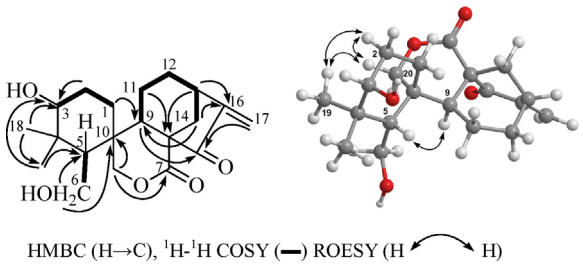

Fig. 2. Key HMBC, ^{1}H–^{1}H COSY, and ROESY correlations of **1**.

was deduced from the HMBC experiment. In the HMBC spectrum (Fig. 2), Me-18 and Me-19 correlated to C-3 (δ_C 74.6, d) and C-5 (δ_C 46.0, d), and H-5 (δ_H 2.36, overlap) correlated to C-4 (δ_C 38.9, s), suggesting that C-4 quaternary carbon was connected to Me-18 and Me-19, and C-3 was connected to C-5 through C-4. The HMBC correlations from H_2-6 (δ_H 3.95, m) to C-4, C-5, and C-10 (δ_C 42.5, s), established the attachment of an oxygenated methylene carbon (C-6) to C-5. Correlations from H-9 (δ_H 3.15, dd, J = 14.7, 5.0 Hz) to C-1 (δ_C 19.8, t) and C-10, and from H_2-20 (δ_H 4.91, d, J = 8.3 Hz) to C-1, C-9 (δ_C 45.3, d), and C-10 suggested that C-10 was connected with C-1, C-9, and C-20. In addition, HMBC correlations from H-11 (δ_H 1.85, m) and H-13 (δ_H 2.85, m) to C-8 (δ_C 58.5, s), from H-14 (δ_H 2.51, dd, J = 12.3, 4.5 Hz) to C-9, from H-17 (δ_H 5.26, br s) to C-13 (δ_C 35.6, d), C-15 (δ_C 203.6, s), and C-16 (δ_C 151.4, s), and from H-9 to C-15 suggested that C-8, C-9, and C-11–C-14 constituted the six-membered ring C, and allowed the connection of C-13 and C-16, and of C-8 and C-15 to form a five-membered ring D. Thus, the basis skeleton of **1** was characteristic of a 6,7-*seco*-*ent*-kaurane diterpenoid. Finally, the presence of an OH group at C-3 was evident from the HMBC correlations from H-2 (δ_H 1.75, overlap), Me-18, and Me-19 to C-3.

The relative configurations of **1** were established by the ROESY experiment (Fig. 2). The ROESY correlation between H-5 and H-9β was in agreement with the β-orientation of H-5. The α-orientation for CH_2-20 was evident from the ROESY correlations from H_2-20 to H-2α and Me-19α. In addition, the HO-3 of **1** was assigned to be β-oriented for the upfield shifts of C-1 (δ −19.4 ppm) and C-5 (δ −10.5 ppm) compared with those of C-1 and C-3 in macrocalyxoformin D [20], which was caused by the γ-steric compression effect between HO-3 and H-1β and between HO-3 and H-5β. Thus, the structure of compound **1** was determined as 3β,6-dihydroxy-6,7-*seco*-*ent*-kaur-16-en-15-one-7,20-olide and given the name sculponin T.

^{1}H NMR, ^{13}C NMR, DEPT, HSQC, HMBC, COSY, ROESY, HREIMS, IR and UV spectra of compound **1** are supplied in Supporting information.

Compounds **2**–**5** were identified by comparison of their physical constant data with data in the literature [6,10,15].

Considering the cytotoxicity of diterpenoids previously isolated from the plants of the genus *Isodon* [1], all isolates were evaluated for their *in vitro* cytotoxicity against five human tumor cell lines (HL-60, SMMC-7721, A-549, MCF-7, and SW-480) according to a previously described procedure [17]. The results are given in Table 2. Compound **2**, with the existence of an α-exomethylene-cyclopentanone group, exhibited the highest potency against all the five cancer cell lines with IC_{50} values of 2.9, 3.3, 3.3, 2.8, and 1.8 μmol/L, respectively. Compounds **1** and **4**, with the existence of an α-exomethylene-cyclopentanone group, showed higher cytotoxicity against the five human tumor cell lines, compared with those of compounds **3** and **5** without an α-exomethylene-cyclopentanone group, which were inactive in the tested system (IC_{50} > 40 μmol/L). This finding suggests that the α-exomethylene-cyclopentanone group might be important in mediating the cytotoxicity of *ent*-kauranoids, and the result is in good agreement with conclusions of previous structure-activity relationship research [1].

Table 2
IC_{50} values (μmol/L) of diterpenoids from *I. sculponeatus* for human tumor cell lines.

Compound[a]	HL-60	SMMC-7721	A-549	MCF-7	SW-480
1	17.1	22.2	>40	27.7	13.4
2	2.9	3.3	3.3	2.8	1.8
4	14.4	15.4	17.5	15.8	7.3
DDP[b]	2.0	16.2	17.5	17.8	12.8
Paclitaxel[b]	<0.008	<0.008	1.36	<0.008	0.04

[a] Compounds **3** and **5** were inactive for all cell lines (IC_{50} > 40 μmol/L).
[b] DDP (cisplatin) and paclitaxel were used as positive controls.

In addition, due to the folk use of *I. sculponeatus* [3,4], and since NO is an essential component of the host innate immune and inflammatory responses to a variety of pathogens [21], the anti-inflammatory assay in LPS-stimulated RAW264.7 cells was carried out on compounds **1**, **2**, and **4**. As a result, compound **2** exhibited strong inhibitory activity against LPS-induced NO production with IC_{50} value of 3.3 μmol/L, while compounds **1** and **4** showed weak inhibitory activity with respective IC_{50} value of 18.6 μmol/L and 21.2 μmol/L. At the highest concentration used, none of the tested compounds exhibited inhibitory activities, which suggested that the inhibitory activities against NO production in LPS-stimulated RAW264.7 cells were not induced by the cytotoxicity of the compounds tested.

4. Conclusion

In summary, one new 6,7-*seco*-*ent*-kaurane diterpenoid and another four known analogs were isolated from the aerial parts of *I. sculponeatus*. All isolates were evaluated for their *in vitro* cytotoxicity against HL-60, SMMC-7721, A-549, MCF-7, and SW-480 cell lines. Compound **2** exhibited significant cytotoxic activity against all the cell lines with IC_{50} values ranging from 1.8 μmol/L to 3.3 μmol/L, while compounds **1** and **4** showed moderate cytotoxicity. Selected compounds were evaluated for their inhibitory activity on NO production in LPS-stimulated RAW264.7 cells, and compounds **1**, **2**, and **4** showed intriguing activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cclet.2014.01.041.

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