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Three New 11,20-Epoxy-*ent*-kauranoids from *Isodon rubescens*

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Three rare and new 11,20-epoxy-*ent*-kaurane diterpenoids, named jianshirubesins D-F (**1**-**3**), along with one known analogue (**4**), were isolated from the aerial parts of *Isodon rubescens*. Their structures were established by analysis of spectroscopic data. Found in the MTT assay to evaluate the cytotoxicity of compounds **1**, **2**, and **4**, only **1** could selectively inhibit certain cell lines from proliferating. In addition, a simple structure-activity relationship discussion might suggest a new bioactive moiety, different from the α,β-unsaturated ketone group.

Key words: Jianshirubesins D-F, 11,20-Epoxy-*ent*-kauranoids, *Isodon rubescens*, Cytotoxicity

INTRODUCTION

More than one thousand *ent*-kauranoids have been reported as the natural products thus far, of which, the majority were identified from *Isodon* species (Sun et al., 2006; Jung et al., 2009; Qin et al., 2009; Park, 2011; Zhao et al., 2011b). These *ent*-kauranoids isolated from the *Isodon* genus are classified into eight types. Among these types, the C-20 oxygenated type can be further divided into nine subtypes, depending on the number and position of epoxy rings (Sun et al., 2006). To the best of our knowledge, only five *ent*-kauranoids belonging to the 11,20-epoxy subtype, macrocalyxin B (Wang et al., 1995), parvifoline B (Li and Chen, 1992), pseudoirroratin A (Zhang et al., 2002), rubescensin W (Han et al., 2004), and maoyecrystal I (Han et al., 2004), have been identified from *Isodon* species or even from nature.

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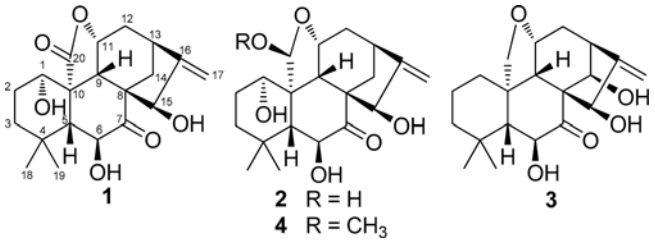

Fig. 1. Structures of compounds **1**-**4**.

Isodon rubescens (Hemsley) Hara is a famous and well-studied folk medicine in China (Han et al., 2003; Huang et al., 2007; Luo et al., 2010; Zou et al., 2011, 2012). In our first-time phytochemical research on this species collected in Hubei province, three rare and new 11,20-epoxy-*ent*-kauranoids, jianshirubesins D-F (**1**-**3**), and a known one, rubescensin W (**4**) (Fig. 1) were obtained. In this paper, the isolation, structure elucidation, and cytotoxic properties of these compounds, together with a brief discussion about structure-activity relationship (SAR), are reported.

MATERIALS AND METHODS

General experimental procedures

Optical rotations: JASCO P-1020 polarimeter. UV spectra: Shimadzu UV-2401A spectrophotometer. IR spectra: BioRad FtS-135 spectrophotometer; KBr

pellets; in cm^{-1}. 1D and 2D NMR spectra: Bruker DRX-500 and DRX-600 spectrometers; δ in ppm relative to TMS; *J* in Hz. HR-EI-MS: Waters AutoSpec Premier spectrometer P776; in *m/z*. Semi-preparative HPLC: Agilent 1100 liquid chromatography; Zorbax SB-C_{18} column (9.4 mm × 25 cm). Column chromatography (CC): silica gel (SiO_2, 100-200 mesh; Qingdao Marine Chemical, Inc.); RP-18 gel (40-63 μm, Lichroprep, Merck); MCI gel (75-150 μm, Mitsubishi Chemical Corporation). TLC: visualization by heating Silica gel plates sprayed with 5% H_2SO_4 in EtOH.

Plant materials

The aerial parts of *Isodon rubescens* (Hemsley) Hara were collected in Jianshi County, Hubei Province, P. R. China in September 2010, and identified by Prof. Ying-Ming Wang. A voucher specimen (No.IP20081006) has been deposited in Hubei Key Laboratory of Natural Medicinal Chemistry and Resource Evaluation, Tongji School of Pharmacy, Huazhong University of Science and Technology.

Cytotoxicity assay

MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay was performed to evaluate the cytotoxicity of **1**, **2**, and **4**. The HL-60 (human promyelocytic leukemia), SMMC-7721 (liver cancer), A-549 (lung cancer), MCF-7 (breast cancer) and SW480 (colon cancer) cell lines were seeded in a 96-well plate at a density of 5,000 to 10,000 cells per well in 100 μL of medium, treated with 5 μL of different concentrations of studied compounds in triplicates, and then cultivated for 48 h at 37°C. Then 20 μL of an MTT solution in medium was added into each well, before the termination of the experiment. The plates were incubated in an incubator (37°C, 5% CO_2) for 4 h. The medium was removed. SDS (10%, 200 μL) was added into each well to dissolve the dark blue crystal. Absorbance values with a test wavelength of 595 nm were recorded. The rates of cell-growth inhibition were calculated. The IC_{50} were calculated by the Reed and Muench method, and cisplatin was used as a positive control.

Extraction and isolation

The air-dried and powdered aerial parts of *I. rubescens* (10 kg) were extracted four times with 95% aqueous EtOH at room temperature to yield an extract, which was dissolved in H_2O and extracted with EtOAc. The EtOAc partition (550 g) was applied to SiO_2 CC, eluting with $CHCl_3$-MeOH (1:0-0:1 gradient system) to give fractions A-F. Fraction D (90 g) was decolored with MCI gel column (90% MeOH-H_2O), and then was applied repeatedly to SiO_2 CC to give Fr.D1-6. Fr.D5 (3.5 g) was then subjected to RP-18 CC with gradient elution (MeOH-H_2O 30%-100%) to get eight sub-fractions. Further semi-preparative HPLC (acetonitrile-H_2O, 3 mL/min, 40%) of Fr.D2/2 (140 mg) led to compounds **1-4** (6 mg, 4 mg, 0.8 mg, and 40 mg, respectively).

Jianshirubesin D (1)

White amorphous powder. $[\alpha]_D^{22} = -23.5$ (*c* 0.28, MeOH). UV λ_{max} (MeOH) nm: 207, 254. IR (KBr) cm^{-1}: 3446, 2934, 2874, 1727, 1709, 1629, 1461, 1388, 1133, 1109, 1076. ^{1}H- and ^{13}C-NMR data, see Table I. HR-EI-MS (pos.) *m/z*: 362.1711 ($[M]^+$, $C_{20}H_{26}O_7$; calcd. 362.1729).

Jianshirubesin E (2)

White amorphous powder. $[\alpha]_D^{25} = -4.2$ (*c* 0.11, MeOH). UV λ_{max} (MeOH) nm: 207. IR (KBr) cm^{-1}: 3441, 2925, 1633, 1456, 1121, 1023, 582. ^{1}H- and ^{13}C-NMR: see Table I. HR-EI-MS (pos.) *m/z*: 364.1898 ($[M]^+$, $C_{20}H_{28}O_7$; calcd. 364.1886).

Jianshirubesin F (3)

White amorphous powder. $[\alpha]_D^{22} = -25.3$ (*c* 0.06, MeOH). UV λ_{max} (MeOH) nm: 204. IR (KBr) cm^{-1}: 3428, 2939, 2926, 1696, 1633, 1454, 1386, 1313, 1048. ^{1}H- and ^{13}C-NMR: see Table I. HR-EI-MS (pos.) *m/z*: 348.1945 ($[M]^+$, $C_{20}H_{28}O_5$; calcd. 348.1937).

RESULTS AND DISCUSSION

Jianshirubesin D (**1**) was obtained as white amorphous powder. The molecular formula $C_{20}H_{26}O_6$ was determined from the positive HR-EI-MS molecular ion peak at *m/z* 362.1711 $[M]^+$, indicating eight degrees of unsaturation. In ^{13}C-NMR and DEPT spectra, 20 carbon signals were observed, which included two methyls, five methylenes (including an olefinic one), seven methines (of which four were oxygenated), and six quaternary carbons (of which one was olefinic, two were carbonyl carbons) (Table I). These data suggested that compound **1** was similar to an *ent*-kauranoid. Meanwhile, the maximum absorption wavelength in UV spectrum at 207 nm indicated that ketone (δ_C 211.6) and olefine (δ_C 158.2 and δ_C 109.5) groups were not conjugated (Huang, 2012). Detail analyses of ^{1}H-^{1}H COSY, HSQC, and HMBC spectra helped to confirm the gross structure of compound **1** (Fig. 2). The HMBC correlations from H_2-3 (δ_H 1.56 and 1.31-1.36), H-5 (δ_H 1.82), and H-9 (δ_H 3.82) to C-1 (δ_C 80.1), from H-6 (δ_H 4.98) to C-5 (δ_C 52.3) and C-8 (δ_C 59.9), from H-15 (δ_H 5.67) to C-16 (δ_C 158.2) and C-17 (δ_C 109.5), and from H-11 (δ_H 6.07) to C-10 (δ_C 57.1) and C-13 (δ_C 36.9), together with the ^{1}H-^{1}H COSY correlations from three active hydrogens

Table I. ^{1}H- and ^{13}C-NMR data (in C_5D_5N) of compounds **1-3** (δ in ppm, *J* in Hz)

No.	**1**		**2**		**3**	
	δ_H[a]	δ_C[b]	δ_H[a]	δ_C[c]	δ_H[a]	δ_C[c]
1	4.10 d, 10.3	80.1 d	4.02 dd, 12.8, 4.5	81.7 d	1.86 d, 12.9 1.16-1.20 m	40.0 t
2	2.41-2.50 m 1.84-1.89 m	30.3 t	2.73-2.83 m 1.78-1.83 m	31.2 t	1.48 t, 15.1 1.24-1.27 m	19.2 t
3	1.56 dt, 13.4, 3.7 1.31-1.36 m	42.0 t	1.36-1.42 m 1.19-1.24 m	43.0 t	1.20-1.25 m 1.06 t, 16.3	43.8 t
4		35.4 s		36.1 s		35.8 s
5	1.82 d, 13.2	52.3 d	1.64 d, 13.2	53.9 d	1.66 d, 12.5	54.8 d
6	4.98 dd, 13.2, 4.3	75.2 d	4.92 d, 13.2	73.8 d	4.99 d, 12.5	75.0 d
7		211.6 s		213.1 s		211.5 s
8		59.9 s		57.8 s		63.3 s
9	3.82 d, 5.2	49.3 d	3.59 d, 4.1	50.6 d	3.17 d, 5.0	52.6 d
10		57.1 s		59.7 s		51.4 s
11	6.07 dd, 8.0, 5.2	75.2 d	5.36 dd, 7.5, 4.1	73.2 d	4.25 t, 5.0	73.1 d
12	2.38-2.41 m 1.84-1.88 m	38.5 t	2.37-2.40 m 1.81-1.86 m	38.1 t	2.69-2.73 m 2.03-2.06 m	40.1 t
13	2.65 br. t, 6.2	36.9 d	2.64 br. t, 6.3	36.7 d	3.04 d, 7.5	47.2 d
14	2.14 d, 11.9 1.47 dd, 11.9, 6.2	35.7 t	2.53 d, 11.2 1.38-1.44 m	36.0 t	5.23 s	75.0 d
15	5.67 d, 5.2	76.1 d	5.61 br. s	76.2 d	6.50 s	74.1 d
16		158.2 s		158.9 s		160.1 s
17	5.56 s 5.12 s	109.5 t	5.56 s 5.16 s	109.4 t	5.67 s 5.34 s	109.4 t
18	1.30 s	36.1 q	1.32 s	34.9 q	1.29 s	35.2 q
19	1.93 s	23.1 q	1.45 s	21.1 q	0.94 s	21.5 q
20		177.5 s	6.34 d, 5.6	100.2 d	4.36 d, 8.9 3.88 d, 8.9	70.3 t
HO-1	7.60 s					
HO-6	6.09 d, 4.3		5.79 s			
HO-15	7.96 d, 5.2		5.16 br. s			
HO-20			8.12 d, 5.6			

[a]Recorded in 600 MHz. [b]Recorded at 125 MHz. [c]Recorded at 150 MHz.

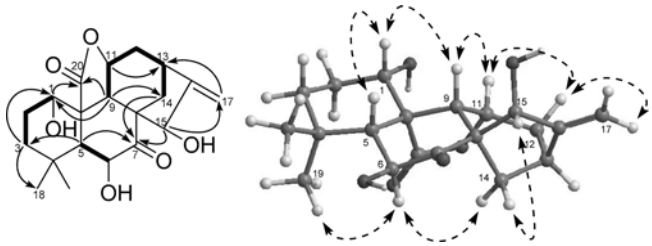

Fig. 2. Key HMBC (H⌒C), ^{1}H-^{1}H COSY (—), and ROESY (⌒) correlations of compound **1**.

(δ_H 7.60, 6.09, and 7.96) to H-1 (δ_H 4.10), H-6, and H-15, respectively, revealed that three of four oxymethines, namely C-1, C-6, and C-15, were substituted by free OH groups, but not the remaining C-11. In the HMBC spectrum, correlations from H-5, H-14, and H-15 to the ketone carbonyl (δ_C 211.2), and from H-1, H-5, and H-9 to the lactone carbonyl (δ_C 177.0) permitted to locate the ketone and lactone carbonyls at C-7 and C-20, respectively. The non-HO-substituted C-11 at δ_C 75.2, which shifted downfield by nearly δ_C 12.0 relative to characteristic C-11 at δ_C 63.5 in normal *ent*-kaurane (Zhao et al., 2011a), and the remaining one double bond equivalent both supported the presence of a lactone ring between C-11 and C-20. As depicted in Fig. 2, ROESY correlations of H-1/H-5β, H-6/H-14α/H_3-19, H-11/H-9β/H-12β, and H-15/H-14β revealed H-1, H-6, H-11, and H-15 to be β-, α-, β-, and α-oriented, respectively. Thus, compound **1** was elucidated to be 1α,6β,15β-trihydroxy-11α,20-olide-*ent*-kaur-16-en-7-one.

Jianshirubesin E (**2**) was isolated as white amorphous powder. The molecular formula was determined as $C_{20}H_{28}O_6$ by HR-EI-MS (molecular ion peak at *m/z* 364.1898 $[M]^+$). Comparison of the NMR data of **1** and

rubescensin W (**4**) (Han et al., 2004) showed that **2** was quite similar to **4**. The only difference was HO-20 in **2** instead of CH_3O-20 in **4**, which was deduced by HMBC correlations from the proton of semiacetal at C-20 (δ_H 6.34; δ_C 100.2) to C-5, C-9, C-10, and C-11. Further, ROESY correlations of H-20/H-6/H-19 assigned the same *S** configuration to C-20 of two analogues. So compound **2** was easily established as (20*S**)-1α,6β,15β,20β-tetrahydroxy-11α,20-epoxy-*ent*-kaur-16-en-7-one.

In the HR-EI-MS spectrum of jianshirubesin F (**3**), the peak at *m/z* 348.1945 $[M]^+$ confirmed the molecular formula of **3** as $C_{20}H_{28}O_5$. Comparison of NMR data of **3** with those of **2** exhibited the most significant difference that the methylene at C-14 (δ_H 2.53 and 1.38-1.44; δ_C 36.0) and the semiacetal (δ_H 6.34; δ_C 100.2) at C-20 in **2** were replaced with the oxymethine (δ_H 5.23; δ_C 75.0) and one more oxymethylene (δ_H 4.36, 3.88; δ_C 70.3) in **3**, respectively. In detail, the position and orientation of HO-14 were established by key HMBC correlations of H-14 with C-12 (δ_C 40.1), C-15 (δ_C 74.1), and C-16 (δ_C 160.1), and by the ROESY correlation between H-14/H_2-20. Compound **3** was, therefore, deduced as 6β,14β,15β-trihydroxy-11α,20-epoxy-*ent*-kaur-16-en-7-one.

Among a large number of natural *ent*-kauranes, 11,20-epoxy-*ent*-kauranes were so rare to be found that the only five known ones were isolated from five different *Isodon* species. Herein, jianshirubesins D-F (**1**-**3**) and rubescensin W (**4**) bearing 11,20-epoxy moiety were reported to be obtained from *I. rubescens*. Moreover, compounds **1**-**4** were all substituted by hydroxyl and ketone groups at C-6 and C-7, respectively. These characteristics indicated that these compounds might be biosynthetically related to 6β,7β-dihydroxy-7α,20-epoxy-*ent*-kauranes, which are the most common example of over 200 7α,20-epoxy-*ent*-kauranes (Huang et al., 2006).

For the quantity limit of **3**, only compounds **1**, **2**, and **4** were evaluated for their *in vitro* cytotoxicity by MTT assay, using cisplatin as the positive control (Alley et al., 1988). As showed in Table II, only compound **1** exhibited selective inhibition on the proliferation of HL-60 cells with IC_{50} value of 5.00 μM and of MCF-7 cells with IC_{50} value equivalent to that of the positive control. Abundant *ent*-kauranoids have been evaluated for their cytotoxicity so that the SAR research concluded that the α,β-unsaturated ketone group was responsible for cytotoxicity of this type of compounds (Sun et al., 2006). It was rather strange that neither compound **1** nor maoyecrystal I possess the α,β-unsaturated ketone group, but both of them exhibited certain cytotoxicity (Han et al., 2004). These uncommon results might suggest the possible existence of another cytotoxic moiety, rather than the α,β-unsaturated ketone group in 11,20-epoxy-*ent*-kauranes. Further studies to find more cytotoxic 11,20-epoxy-*ent*-kauranes and to disclose the mechanism of their cytotoxicity are necessary to confirm the above suggestion.

Table II. *In vitro* cytotoxicity data (IC_{50} in μM) of compounds **1**, **2**, and **4** by the MTT method

	HL-60	SMMC-7721	A-549	MCF-7	SW480
1	5.00	> 40	30.11	16.22	22.62
2	> 40	> 40	> 40	> 40	> 40
4	> 40	> 40	> 40	> 40	> 40
cisplatin	1.81	8.86	11.68	15.92	16.65

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