Cytotoxicity of Labdane-Type Diterpenoids from *Hedychium forrestii*

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Two new labdane-type diterpenoids, hedyforrestin D (1) and 15-ethoxy-hedyforrestin D (2), and three known compounds, yunnancoronarin A (4), B (3) and C (5) were isolated from the rhizomes of *Hedychium forrestii*. The structure of the new diterpenoids was established as 6β,15ξ-dihydroxylabda-8(17),11,13-trien-15,16-olide (1), and 6β-hydroxy-15ξ-ethoxylabda-8(17),11,13-trien-15,16-olide (2) on the basis of spectroscopic analyses. In addition, the isolated compounds were evaluated for their cytotoxicity against the lung adenocarcinoma cells A549 and leukemia cells K562 through 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assays. Of these, compounds 3 and 4 exhibited the most activity with IC_{50} values of 0.92 and 2.20 μM, respectively, whereas 5 was inactive against A549 cells and 1 was inactive against both cell lines up to a concentration of 300.81 μM. This shows that both the hydroxy substitution and orientation of unsaturated lactone group in the five-membered ring of C-13 to C-16 seem to play an important role in the anti-tumor activities of human lung adenocarcinoma and leukemia.

Key words *Hedychium forrestii*; Zingiberaceae; labdane-type diterpenoid; hedyforrestin D; cytotoxicity

The genera *Hedychium* KOEN. (Zingiberaceae) are known for their strong aromatic odor and used as hot natured drugs in traditional Chinese medicine (TCM). Some species, especially *H. spictatum* HAM. and *H. coronarium* KOEN., have been used for the treatment of stomach ailments such as pain, indigestion, swelling, diarrhea, hernia and for liver diseases.[1)] Since 1975, a series of labdane-type diterpenoids have been identified and some of them showed significant cytotoxicities against cloned Chinese hamster V-79 cells.[2—7)] In the course of our continuous search for anti-tumor potential entities from TCM, we have aimed at cytotoxic diterpenoids of the type in this genus.[8—11)]

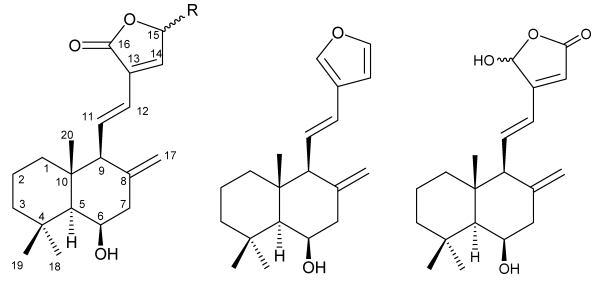
H. forrestii DIELS is a perennial rhizomatous herb distributed in southwest China. A previous phytochemical study on the plant found some cytotoxic labdane-type diterpenoids against oral cavity cancer KB cells.[8)] Recently, a further investigation led us to obtain novel promising active compounds. This paper reports the characterization of two new diterpenoids as well as the *in vitro* anti-tumor activity assays of five labdane-type diterpenoids isolated from this plant, and their cytotoxicities against the lung adenocarcinoma cells A549 and leukemia cells K562 through 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assays.

Results and Discussion

Repeated column chromatography of the EtOAc-soluble fraction of the ethanol extract of *H. forrestii* rhizomes resulted in the isolation of five diterpenoids (**1**—**5**) as shown in Fig. 1. Structures of the three known compounds (Fig. 1) were identified as yunnancoronarin B (**3**), A (**4**) and C (**5**) by comparing their spectral (IR, ^{1}H- and ^{13}C-NMR, EI-MS) and physicochemical data with those reported in the literature.[8,9)]

One of the two new compounds (**1**) was obtained as colorless oil with a negative optical rotation $[\alpha]_D^{24}$ −6.90° (c=0.3, $CHCl_3$). The molecular formula of **1** was assigned as $C_{20}H_{28}O_4$ by the molecular ion peak at m/z 332.1992 $[M]^+$ in HR-EI-MS. Its IR spectrum showed the absorption peaks of hydroxyls at 3450 cm^{-1} and a carbonyl at 1766 cm^{-1} as well as an *exo*-methylene (2932, 1715, 934 cm^{-1}). The ^{1}H-NMR spectrum (Table 1) indicated the presence of three methyl groups (δ_H 1.02, 1.19, 1.24) and the *exo*-methylene (δ_H 4.41, 4.76) which were characteristic of a labdane-type diterpenoid. The ^{13}C-NMR (DEPT) spectrum (Table 2) gave 20 carbon signals, including one carbonyl (δ_C 169.9, C-16) attributed to an α,β-unsaturated lactone ring, four conjugated olefinic carbons [δ_C 138.1/138.3 (d) (C-11), 120.6/120.7 (d) (C-12), 132.3 (s) (C-13), and 140.0/141.3 (d) (C-14)] and the two methylene carbons [δ_C 144.9/145.0 (s) (C-8) and 111.7/112.0 (t) (C-17)]. The other two *trans* olefinic proton signals were further observed at δ_H 6.97 (1H, dd, J=10.1, 15.4 Hz) and 6.12 (1H, d, J=15.4 Hz) which were assignable to H-11 and H-12, respectively, with the former one being downfield shifted because the *trans* olefinic group was linked directly to the α,β-unsaturated γ-lactone.[12)] The latter showed key ^3J correlations with C-14, -16 in HMBC spectrum, and a NOE effect with H-17, respectively (Fig. 2).

Compound **1** was found to have the same lower cyclic



1 R = OH
2 R = OCH_2CH_3
3 R = H

4 **5**

Fig. 1. Chemical Structures of **1**—**5**

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moiety as those in **2**—**5** by comparing their NMR data in Tables 1 and 2, in addition to C(H)-6 signals which were shown by δ_H 4.41 (1H, br s) and δ_C 69.0 (d).[8,9] Another C-15ξ proton in the upper cyclic moiety of hemiacetal lactone was shown at δ_H 6.14 (1H, br s) and δ_C 96.3 (d). As compound **1** was isolated as a 1 : 1 mixture of diastereoisomers at the C-15 position, doubled signals of both δ_H and δ_C were observed at several positions (Tables 1, 2). The situation is similar to those reported in the literature.[5,7,8,11—13]

It was further noted that the hydroxy and carbonyl were positioned conversely between C-15 and C-16 in compounds **1** and **5**. This was confirmed by the distinguished 1D NMR shifts at δ_H 6.90 (s, H-14) and δ_C 140.0/141.3 (d) (C-14) of **1**, in contrast to δ_H 5.79 s/5.80 s (H-14)[9] and δ_C 115.7 (d) (C-14) of **5** (Tables 1, 2), respectively. The final conclusion of this structure was made by its other significant chemical shift differences compared with those of compound **5** and 2D NMR spectral experiments, especially the HMBC and NOE correlations (Fig. 2). Therefore, the structure of compound **1** was elucidated as 6β,15ξ-dihydroxylabda-8(17),11,13-trien-15,16-olide, which was a new compound and has been assigned the trivial name of hedyforrestin D.

Compound **2** was also a new compound and was obtained as white powder. HR-EI-MS showed the molecular formula as $C_{22}H_{32}O_4$ (*m/z* 360.2292, $[M]^+$), an increase of 28 as compared with that of compound **1**. Its IR, ^{1}H- and ^{13}C-NMR spectra (Tables 1, 2) were very similar to those of **1**. ^{1}H-NMR spectrum indicated exceptionally the presence of an ethoxy group at δ_H 3.94 (1H, m), 3.76 (1H, m), and 1.28 (3H, t, J=7.1 Hz), which was confirmed by ^{13}C-NMR (DEPT) (δ_C 65.9, 15.1). All the spectral data revealed that compound **2** bore a 15-ethoxy group instead of a hydroxy group in **1**. So the structure of compound **2** was determined to be 6β-hydroxy-15ξ-ethoxylabda-8(17),11,13-trien-15,16-olide as shown in Fig. 1.

Considering it was obtained from the ethanol extract, this compound might be an artifact of **1**.[7] We trivially it named 15ξ-ethoxy-hedyforrestin D.

Table 1. ^{1}H-NMR Spectral Data for Compounds **1** and **2** (δ_H in $CDCl_3$, J in Hz)[a]

Position	**1**	**2**
1	1.07 m (α), 1.47 m (β)	1.08 m (α), 1.53 m (β)
2	1.40 m (α), 1.58 m (β)	1.44 m (α), 1.59 m (β)
3	1.19 m (α), 1.38 m (β)	1.20 m (α), 1.41 m (β)
5	1.11 br s	1.10 br s
6	4.41 br s	4.40 br s
7	2.41 d (2.7)	2.40 d (2.9)
9	2.44 d (10.1)	2.43 d (10.0)
11	6.95 dd (10.1, 15.4)/ 6.97 dd (10.1, 15.4)[b]	6.97 dd (10.1, 15.4)/ 7.00 dd (10.0, 15.9)[b]
12	6.11 d (15.4)/6.12 d (15.4)[b]	6.10 d (15.9)/6.12 d (15.9)[b]
14	6.90 s	6.82 s
15	6.12 s/6.14 s[b]	5.83 s/5.84 s[b]
17	4.75 s (a)/4.76 s (a)[b] 4.40 s (b)/4.41 s (b)[b]	4.92 s (a)/4.94 s (a)[b] 4.76 s (b)/4.77 s (b)[b]
18	1.02 s	1.02 s
19	1.24 s	1.23 s
20	1.18s/1.19 s[b]	1.18s/1.19 s[b]
1′	—	3.94 m/3.76 m[b]
2′	—	1.28 t (7.1)

a) Multiplicity signals were overlapped in the range 1.0—2.0 ppm; assignments made on the basis of COSY, HMQC and HMBC. *b*) Doubled signals due to epimer (1 : 1) at C-15.

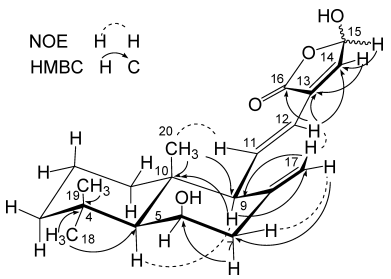

Fig. 2. Selected NOE and HMBC Correlation of Compound **1**

Table 2. ^{13}C-NMR (DEPT) Spectral Data for Compounds **1**—**5** (δ_C in $CDCl_3$)

Position	**1**	**2**	**3**	**4**	**5**
1	43.8 (t)	43.8 (t)	43.7 (t)	43.7 (t)	43.7 (t)
2	19.0/19.2 (t)[a]	19.1/19.2 (t)[a]	19.2 (t)	19.3 (t)	19.1/19.2 (t)[a]
3	44.0/44.1 (t)[a]	43.9/44.1 (t)[a]	44.1 (t)	44.1 (t)	43.9/44.0 (t)[a]
4	34.5 (s)	34.5 (s)	34.5 (s)	34.4 (s)	34.5 (s)
5	56.8 (d)	56.8 (d)	56.8 (d)	56.9 (d)	56.5 (d)
6	69.0 (d)	68.9 (d)	68.9 (d)	68.9 (d)	68.8 (d)
7	46.4 (t)	46.5 (t)	46.5 (t)	46.6 (t)	46.1 (t)
8	144.9/145.0 (s)[a]	145.0/145.1 (s)[a]	145.3 (s)	146.0 (s)	144.6/144.8 (s)[a]
9	62.6 (d)	62.7 (d)	62.6 (d)	61.9 (d)	62.5 (d)
10	40.4/40.5 (s)[a]	40.3/40.4 (s)[a]	40.3 (s)	40.2 (s)	40.4/40.6 (s)[a]
11	138.1/138.3 (d)[a]	138.3/138.4 (d)[a]	135.9 (d)	122.2 (d)	143.1 (d)
12	120.6/120.7 (d)[a]	120.6/120.7 (d)[a]	121.0 (d)	127.3 (d)	123.1/123.0 (d)[a]
13	132.3 (s)	132.6 (s)	129.4 (s)	124.3 (s)	161.3 (s)
14	140.0/141.3 (d)[a]	139.6/139.8 (d)[a]	142.6 (d)	107.6 (d)	115.7 (d)
15	96.3 (d)	101.0 (d)	69.5 (t)	139.9 (d)	172.0 (s)
16	169.9 (s)	169.7 (s)	72.2 (s)	143.2 (d)	98.0 (d)
17	111.7/112.0 (t)[a]	111.8/112.0 (t)	111.8 (t)	111.5 (t)	112.4/111.9 (t)[a]
18	33.5 (q)	33.6 (q)	33.5 (q)	33.6 (q)	33.5 (q)
19	23.8 (q)	23.8 (q)	23.8 (q)	23.8 (q)	23.8 (q)
20	18.0 (q)	18.1 (q)	18.0 (q)	17.9 (q)	18.1 (q)
1′	—	65.9 (t)	—	—	—
2′	—	15.1 (q)	—	—	—

a) Doubled signals due to epimer (1 : 1) at C-15 in compounds **1** and **2** or C-16 in compound **5**.

Table 3. Cytotoxicity Data of Compounds **1**—**5** against Cancer Cell Lines A549 and K562

Compounds	Cell lines IC_{50} (μM)[a]	
	A549	K562
1	>300.81	>300.81
2	>277.40	77.92
3	0.92	9.54
4	11.08	2.19
5	>300.81	28.69
Cisplatin[b]	0.77	3.43

a) Concentration of compound required to inhibit cell growth by 50%, as determined by MTT assay (see Experimental). Data are expressed as means of three independents. *b*) Cisplatin was used as the positive control.

Compounds **1**—**5** were evaluated for their *in vitro* anti-tumor activity using the cytotoxicity represented classical MTT method.[14] The IC_{50} values of these compounds against the lung adenocarcinoma A549 and leukemia K562 cell lines are shown in Table 3. The most active compounds were **3** and **4** with IC_{50} values of 0.92 μm and 2.19 μm, respectively. Compound **3** was also more active than compound **4** against A549 cells, while those compounds against K562 cells were **4**>**3**>**5**>**2**≫**1**, respectively. All the five labdane-type diterpenoids exhibited generally stronger cytotoxic effect against K562 cells than those against A549 cells, but the two new compounds showed weak to little effect on both cell lines. These results suggested that a hydroxyl substitution at C-15 greatly reduced the effect, whereas an ethoxy substitution showed weak effect against K562 cells. The change of five-membered ring structural moieties among C-13—C-16 in compounds **1**—**4** made the corresponding cytotoxicities against K562 cells in the order of furan nucleus>α,β-unsaturated γ-lactone>cyclic acetal α,β-unsaturated γ-lactone≫cyclic hemiacetal α,β-unsaturated γ-lactone. This is similar to their cytotoxicities against other cancer cell lines like KB and V-79 which were reported previously.[5,8] The orientation of cyclic hemiacetal α,β-unsaturated γ-lactone in **1** and **5** also influenced the activity against K562 cells. Therefore, it could be proposed that the furnan nucleus and its ketonic lactone derivatives of labdane-type diterpenoids showed a promising broad spectrum of *in vitro* anti-tumor effects. Their *in vivo* effects and mechanisms need to be further investigated.

In conclusion, although the structure–activity relationships of these compounds were not conclusively determined, this finding suggests that labdan-type diterpenoids may have potential for further development as anti-tumor substances.

Experimental

General Experimental Procedures Melting point determinations were performed using a Kofler micro-hotstage. Optical rotations were measured on a SEPA-300 polarimeter. IR spectra (KBr) were obtained on a Perkin-Elmer 577 spectrometer. EI-MS and HR-EI-MS (high resolution EI-MS) were registered using a VG Autospec-3000 spectrometer. NMR spectra including ^{1}H- and ^{13}C-NMR, ^{1}H–^{1}H COSY, HMBC, HMQC were recorded at 400 and 100 MHz, respectively, on a DRX-500 NMR spectrometer in $CDCl_3$. Analytical TLC was performed on precoated silica gel GF_{254} plates (Qingdao Marine Chemical, Ltd., China). For column chromatography, silica gel (H and 200—300 mesh, Qingdao Marine Chemical, Ltd., China) were used.

Plant Material The rhizomes of *H. forrestii* were collected in Xishuangbanna, Yunnan Province, China in April, 2003, and identified by Prof. Tong Shaoquan, Kunming Institute of Botany. A voucher specimen is deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, the Chinese Academy of Sciences.

Extraction and Isolation Dried and powdered plant material (3.0 kg) was extracted twice with hot 95% ethanol and filtered. The ethanol extract was concentrated and suspended in H_2O (1.2 l) that was partitioned against EtOAc (1.5 l×3) to give EtOAc-soluble fraction as a brown oil (101 g) and a water layer. The EtOAc fraction was subjected to column chromatography (C.C.) by silica gel and eluted with gradient petroleum ether (60—90 °C)–EtOAc (P–E=10 : 0, 10 : 1, 5 : 1 to 1 : 1) to afford five fractions A—E. Fraction B was further subjected to C.C. and eluted with P–E=20 : 1 to give compound **4** (700 mg), similar C.C. of fraction C (P–E=5 : 1 and $CHCl_3$–EtOAc=30 : 1) to yield compounds **2** (23 mg) and **3** (68 mg). Fraction E was subjected to C.C. (P–E=2 : 1—1 : 1) to afford three sub-fractions E1—E3, repeated C.C. of E2 ($CHCl_3$–MeOH=80 : 1) to furnish compound **5** (116 mg), and E3 ($CHCl_3$–EtOAc=5 : 1—1 : 1 and petroleum ether–acetone=4 : 1) to afford compound **1** (59 mg).

Hedyforrestin D (**1**): Colorless oil; ^{1}H- and ^{13}C-NMR: see Tables 1 and 2; IR (KBr) cm^{-1}: 3450, 2932, 2858, 1766, 1715, 1460, 1386, 1203, 1092, 1019, 934; HR-EI-MS: 332.1992 $[M]^+$ (Calcd for $C_{20}H_{28}O_4$: 332.1988); EI-MS *m*/*z*: 332 (6), 314 (19), 153 (44), 123 (49), 109 (91), 69 (100); $[\alpha]_D^{24}$ −6.90° (*c*=0.3, $CHCl_3$).

15ξ-Ethoxy-hedyforrestin D (**2**): White powder; mp 139—141 °C; ^{1}H- and ^{13}C-NMR see Table 1; IR (KBr) cm^{-1}: 3475, 3083, 2900, 1754, 1345, 1097, 1026, 941; HR-EI-MS: 360.2292 (Calcd for $C_{22}H_{32}O_4$: 360.2301); EI-MS *m*/*z*: 360 (22), 342 (26), 314 (33), 190 (37), 162 (100), 69 (61). $[\alpha]_D^{23}$ +25.71° (*c*=0.4, $CHCl_3$).

Cytotoxicity Assay The *in vitro* anti-tumor activity of the isolated compounds was examined for their cytotoxicities against the lung adenocarcinoma A549 cell lines and leukemia K562 cell lines by MTT method according to a reported protocol.[14] In brief, freshly trypsinized cell suspensions were seeded in 96-well microtitre plates at densities of 1×10^4 cells per well with tested compounds added from DMSO-diluted stock. After 3 d in culture, attached cells were incubated with MTT and subsequently solubilized in DMSO. The absorbance at 550 nm was then measured using a microplate reader. The IC_{50} is the concentration of agent that reduced cell growth by 50% under the experimental conditions with cisplatin as the positive control.

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