New Cytotoxic 6-Oxygenated 8,9-Dihydrofurocoumarins, Hedyotiscone A – C, from *Hedyotis biflora*

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Abstract

Using the bioactivity-guided fractionation method, three new 6-oxygenated 8,9-dihydrofurocoumarin-type compounds, hedyotiscones A (**1**), B (**2**), and C (**3**) were isolated from the methanol extract of *Hedyotis biflora* together with seven known compounds. The structures of all isolated compounds were determined on the basis of mass and spectroscopic evidence. Compounds **1 – 3**, oleanolic acid, and 6′-(*β*-sitosteryl-3-*O*-*β*-D-glucopyranosidyl) pentadecanoate showed marginal cytotoxicity against Hep G2 cells (human liver cancer cells) with IC_{50} values of 14.4, 17.4, 4.9, 8.0, and 9.2 *μ*g/mL, respectively. Ursolic acid showed significant cytotoxicity against MDA-MB-231 cells (human breast carcinoma cells) with an IC_{50} value of 1.49 *μ*g/mL.

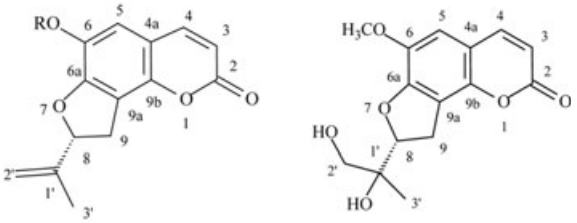
The *Hedyotis* genus (Rubiaceae) is used in traditional Chinese medicine for various purposes, such as tonics and agents for the treatment of appendicitis, boils, dysentery, hepatitis, and tonsillitis [1]. For example, *H. corymbosa* and *H. diffusa* are popular in Taiwan/China and have been used in folk medicine to treat gynecological diseases and cancer [2]. Previous studies on this genus have reported the isolation of triterpenoids [1], flavonoids [3], [4], iridoids [5], [6], and anthraquinones [7], as well as their biological activities, e. g., anti-inflammatory [8], neuroprotective [3], and cytotoxic properties [1]. However, no furocoumarin-type compound has been reported from these plants.

In bioassays for screening the crude extracts of Formosan plants, we found that the $CHCl_3$ extract of the whole herbs *Hedyotis biflora* (L.) showed effective growth inhibition in a human liver cancer cell line Hep G2 (IC_{50} = 9.42 *μ*g/mL). This plant is distributed in sunny to slightly shaded areas of low elevation in Taiwan [9] and has been used for common cold relief. Further investigation using bioactivity-directed fractionation and isolation methods led to the isolation of three new dihydrofurocoumarin-type compounds (Fig. **1**), hedyotiscones A (**1**), B (**2**), and C (**3**), together with seven known ones, 6,7-dimethoxycoumarin [10], ursolic acid [11], oleanolic acid [12], 6′-(*β*-sitosteryl-3-*O*-*β*-D-glucopyranosidyl) pentadecanoate [13], a mixture of *β*-sitosterol/stigmasterol [14], *β*-sitosterol 3-*O*-*β*-D-glucoside [14], and scandoside methyl ether [15]. This is the first report of dihydrofurocoumarins from Rubiaceae. The structures of the isolates were determined on the basis of spectroscopic data. All compounds have been assayed for their cytotoxicity in Hep G2, MDA-MB-231, A549, and MCF-7 cancer cell lines.

The HR-ESI-MS of **1** showed an ion at *m/z* = 259.0968 $[M + H]^+$, corresponding to a molecular formula $C_{15}H_{15}O_4$. The UV (λ_{max} = 229 and 345 nm) data indicated the existence of a coumarin nucleus [16]. The IR spectrum indicated the presence of a lactone carbonyl group (ν_{max} = 1725 cm^{-1}). The ^{1}H-NMR spectrum of **1** (Table **1**) revealed two doublets at δ = 7.60 and δ = 6.27, each with J = 9.6 Hz, characteristic of an α,β-unsaturated δ-lactone moiety of a coumarin ring [17]. A singlet signal at δ = 6.78 (1H) indicated the presence of a penta-substituted aromatic ring. Another singlet signal at δ = 3.92 (3H) was assigned to a methoxy group attached to the aromatic ring. In addition, geminal methylene proton signals at δ = 3.21 (1H, dd, J = 16.2 and 8.1 Hz) and δ = 3.54 (1H, dd, J = 16.2 and 8.1 Hz), terminal olefinic proton signals at δ = 5.13 (1H, s) and δ = 4.99 (1H, s), a vinylic methyl proton signal at δ = 1.79 (3H, s), and an allylic oxymethine proton at δ = 5.42, (1H, t, J = 8.1 Hz) were consistent with the proposed 2-isopropenyldihydrofurocoumarin skeleton [17].

The connectivity of each proton to the respective carbon was confirmed by HMQC. The assignments of ^{13}C-NMR data were confirmed by the HMBC technique (Fig. **2**). The HMBC spectrum revealed the correlations of C-8/H-9, C-8/H-2′, and C-8/H-3′ confirmed that it is a 2-isopropenyl-2,3-dihydrofuran moiety. Furthermore, the NOESY correlations (Fig. **2**) between OCH_3/H-5 and H-5/H-4 suggested the 6-methoxy substitution, and the correlations between H-8/H-9 and H-3′/H-9 indicated that the isopropenyl group was attached to C-8. Therefore, the furan function will be formed at C-6a and C-9a. The negative optical rotation value indicted that **1** had an *R* configuration at C-8 [18]. Thus, the proposed structure of 6-methoxy-8-(prop-1-en-2-yl)-8, 9-dihydrofuro[2,3-*h*]chromen-2-one was determined unambiguously and named hedyotiscone A.

The molecular formula of **2** was established as $C_{14}H_{12}O_4$ by HR-ESI-MS, which indicated 14 amu less than **1**. The similar UV spec-



Hedyotiscone A (**1**) R=CH_3 Hedyotiscone C (**3**)

Hedyotiscone B (**2**) R=H

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

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Received: March 15, 2005 · **Accepted:** June 20, 2005

Bibliography: Planta Med 2006; 72: 75–78 · © Georg Thieme Verlag KG Stuttgart · New York · DOI 10.1055/s-2005-873178 · Published online November 10, 2005 · ISSN 0032-0943

Table **1** NMR data of compounds **1** and **2**[a] in $CDCl_3$ and **3**[a] in acetone-d_6 [δ in ppm, J in Hz]

	δ (H)			δ (C)		
Position	***1***	***2***	***3***	***1***	***2***	***3***
2	–	–	–	161.2, s	161.0, s	161.0, s
3	6.27, d (9.6)	6.23, d (9.6)	6.25, d (9.6)	112.7, d	113.8, d	112.8, d
4	7.60, d (9.6)	7.60, d (9.6)	7.60, d (9.6)	143.6, d	144.8, d	144.9, d
4a	–	–	–	112.5, s	112.9, s	113.3, s
5	6.78, s	6.86, s	6.77, s	109.4, d	112.6, d	110.9, d
6	–	–	–	142.4, s	144.5, s	142.6,s
6a	–	–	–	152.7, s	152.1, s	153.8, s
8	5.42, t (8.1)	5.42, t (8.1)	5.04, t (8.1)	88.7, d	88.6, d	88.6, d
9	3.21, dd (16.2, 8.1)	3.24, dd, (16.2, 8.1)	3.45, dd (16.2, 8.1)	31.8, t	32.4, t	27.7, t
	3.54, dd (16.2, 8.1)	3.56, dd (16.2, 8.1)	3.40, dd (16.2,8.1)	–	–	–
9a	–	–	–	114.8, s	115.1, s	116.2, s
9b	–	–	–	146.1, s	146.1, s	146.9, s
1′	–	–	–	141.9, s	139.5, s	74.8, s
2′	5.13, s	5.13, s	3.80, d (10.8)	113.2, t	114.4, t	67.8, t
	4.99, s	4.99, s	3.57, d (10. 8)	–	–	–
3′	1.79, s	1.79, s	1.25, s	16.9, d	17.12, d	19.9, d
OCH_3	3.92, s	–	3.89, s	56.4, q	–	56.6 q

[a] All spectra were recorded at 400 MHz (^1H) and 100 MHz (^{13}C); assignments were confirmed by 2D NMR COSY, NOESY, HMQC, and HMBC.

trum suggested **2** having a similar coumarin core as **1**. The presence of a hydroxy group and a carbonyl group was identified by its IR (ν_{max} = 3439 and 1727 cm^{-1}) bands. The ^1H- and ^{13}C-NMR data of **2** (Table **1**) were comparable to those of **1**, except for the absence of the methoxy signals (δ_H = 3.92, δ_C = 56.4) as in **1**. In addition, the molecular weight difference (*m/z* = 258 for **1** and *m/z* = 244 for **2**) and the ^{13}C-NMR data ($\delta_{C\text{-}6}$ = 142.4 for **1** and $\delta_{C\text{-}6}$ = 144.5 for **2**) suggested a hydroxy group in **2** instead of the methoxy group in **1**. The assignments of the ^1H- and ^{13}C-NMR were also confirmed by HMQC and HMBC. A NOESY correlation between H-5/H-4 was observed. This evidence suggested that the hydroxy group was attached at C-6. Similarly, the stereochemistry of C-8 was confirmed by the negative optical rotation value. Thus, the structure of 6-hydroxy-8-(prop-1-en-2-yl)-8,9-dihydro-furo[2,3-*h*]chromen-2-one was concluded, and named hedyotiscone B.

The molecular formula of **3** was deduced as $C_{15}H_{17}O_6$ by HR-ESI-MS. UV absorptions were observed that suggested a coumarin skeleton. The presence of hydroxy and carbonyl groups was indicated by its IR absorptions at ν_{max} = 3433 and 1721 cm^{-1}. The ^1H- and ^{13}C-NMR spectra (Table **1**) revealed signals for a methoxy group and a 8-(1,2-dihydroxy-1-methylethyl)-8,9-dihydrofurocoumarin skeleton. The 1,2-diol-isopropane moiety was confirmed by ^1H-NMR signals at δ = 3.80 (1H, d, J = 10.8 Hz, H-2′), δ = 3.57 (1H, d, J = 10.8 Hz, H-2′), and δ = 1.25 (3H, s, H-3′), and ^{13}C-NMR signals at d = 74.8, d = 67.8 (for a diol system) and d = 19.9 (for the quaternary methyl group).

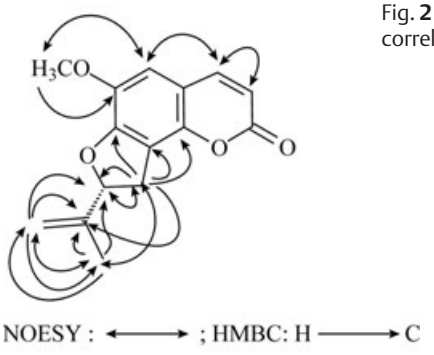

Fig. **2** Key NOESY and HMBC correlations of compound **1**.

Thus, the difference between **1** and **3** is the presence of a 1′,2′-diol system in **3** instead of a terminal double bond at C-1′ and C-2′ in **1**. The NMR assignments were confirmed by 2D-NMR spectra and summarized in Table **1**. The methoxy group was determined as being attached at C-6 by NOESY, and the configuration of C-8 was predicted to be an *R* configuration as in **1** and **2**. However, the stereochemistry of C-1′ remained to be defined. Thus, the structure of 8-(1,2-dihydroxy-1-methylethyl)-6-methoxy-8,9-dihydro-furo[2,3-*h*]chromen-2-one was elucidated, which we named hedyotiscone C.

Many furocoumarins have been studied for their interesting bioactivity, e.g., anti-AIDS and antibacterial properties [19], [20]. Some 8-isopropyl-8,9-dihydrofuro[2,3-*h*]chromen-2-ones have been identified [16], [21]; however, 6-oxygenated substituted compounds are rare and no activity has been reported. The purified compounds were evaluated for their cytotoxicity in Hep G2 (human liver cancer), A549 (human lung carcinoma), and two human breast carcinomas, MDA-MB-231 and MCF-7, cell lines. Based on the criteria of NCI, IC_{50} values $\leq 4\,\mu g/mL$ were considered significant. Compounds 1 – 3, oleanolic acid, and 6′-(β-sitosteryl-3-*O*-β-D-glucopyranosidyl) pentadecanoate exhibited marginal cytotoxicity against Hep G2 cells with IC_{50} values of 14.4, 17.4, 4.9, 8.0, and 9.2 $\mu g/mL$, respectively, and they were weakly cytotoxic (5 – 30% inhibition) at 20 $\mu g/mL$ against A549, MDA-MB-231, and MCF-7 cell lines. Ursolic acid

exhibited significant cytotoxicity against MDA-MB-231 cells with an IC_{50} of 1.49 μg/mL and a marginal effect in A549 and MCF7 cells with IC_{50} values of 19.8 and 4.7 μg/mL. However, it showed only weak cytotoxicity against Hep G2 cells (31% inhibition at 20 μg/mL). As described in the introduction, the crude $CHCl_3$ extract showed good activity in Hep G2 cells. It is interesting that the major compound, ursolic acid [ca. 20 g yield from the crude materials (7.3 kg)], showed weak activity and most of the minor active compounds showed significant activity in the Hep G2 cell line.

Materials and Methods

General experimental procedures: Optical rotations were measured on a JASCO DIP-370 digital polarimeter. UV spectra were obtained on a Hitachi 220 – 20 spectrophotometer. IR spectra were measured on a Hitachi 260 – 30 spectrophotometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian Inova 500, Varian Unity Plus 400 MHz, or Varian Gemini 200 MHz spectrometers using TMS as internal standard. Chemical shifts are reported in parts per million (δ), and coupling constants (J) are expressed in Hertz. LR-EI-MS were collected on a Bruker APEX II mass or a Quattro GC-MS spectrometer having a direct inlet system. HR-ESI-MS was measured on a Bruker APEX II mass spectrometer. Silica gel 60 (Merck, 230 – 400 mesh) was used for column chromatography. Shimadzu LC-10AT pumps, a SPD-10A UV-vis detector, Hypersil ODS 5 μm (250 × 4.6 mm i. d.), and preparative ODS 5 μm (250 × 21.2 mm i. d.) columns were employed for the HPLC analysis. Pre-coated silica gel plates (Merck, Kieselgel 60 F254, 0.25 mm) were used for preparative TLC. Spots were detected by spraying with 50% H_2SO_4 followed by heating on a hot plate.

Plant material: Plant materials of *Hedyotis biflora* Lev. were collected from Pintung County, Taiwan in November 2002. The voucher specimen (Hedyotis 001) was identified by Dr. Ming-Hong Yen (associate professor of the Graduate Institute of Natural Products, Kaohsiung Medical University,) and a sample was deposited in the Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan.

Extraction and Isolation: Dried whole plants (7.3 kg) of *H. biflora* were cut into small pieces and extracted with MeOH (20 L × 3) and partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ layer (90 g) showed significant cytotoxic activity against the Hep G2 cancer cell line. The active layer was chromatographed over silica gel (2700 g) and eluted with (each1.5 L, *n*-hexane-EtOAc, 1 : 0, 1 : 1, 9 : 1) to (each1.5 L, $CHCl_3$-MeOH, 20 : 1, 10 : 1, 8 : 1, 6 : 1, 4 : 1) to give 9 frs (H1 – 9). Crystalline fr. H3 (2.6 g) was filtered and washed with MeOH to give a mixture of β-sitosterol and stigmasterol (1.6 g) (*n*-hexane-EtOAc, 1 : 1, R_f = 0.4). Crystalline fr. H5 (32.5 g) was filtered and washed with $CHCl_3$ to give ursolic acid (20.2 g) ($CHCl_3$, 100%, R_f = 0.2). The fr. H6 (9.7g) was rechromatographed over silica gel (1500 g) using (each 1.0 L, *n*-hexane-EtOAc, 3 : 2, 1 : 1, 1 : 4) mixtures to obtain subfrs (H6.1 – 10). The subfrs H6 – 3 (100 mg) were further separated by preparative reverse-phase HPLC (MeOH-H_2O, 50 : 50, flow rate = 3.5 mL/min) to give oleanolic acid (12 mg, t_R = 40.0 min) and ursolic acid (70 mg, t_R = 43.0 min). The subfr. H6 – 4 (102 mg) was further purified by repeated silica gel chromatography to yield **1** (10 mg) ($CHCl_3$, 100%, R_f = 0.5), **2** (3 mg) ($CHCl_3$-MeOH, 100 : 1, R_f = 0.3), **3** (3 mg) ($CHCl_3$-MeOH, 20 : 1, R_f = 0.6), 6,7-dimethoxycumarin (3 mg) ($CHCl_3$-MeOH, 100 : 1, R_f = 0.5), and 6′-(β-sitosteryl-3-*O*-β-D-glucopyranosidyl) pentadecanoate (6 mg) ($CHCl_3$-MeOH, 20 : 1, R_f = 0.6). The fr. H7 (8.2 g) was chromatographed over silica gel (1200 mg) using (5 L, $CHCl_3$-MeOH, 10 : 1) mixtures for elution to afford β-sitosterol-3-*O*-β-D-glucoside (120 mg) ($CHCl_3$-MeOH, 20 : 1, R_f = 0.4) and scandoside methyl ether (20 mg) ($CHCl_3$-MeOH, 10 : 1, R_f = 0.2).

Hedyotiscone A (**1**): colorless plates, m. p. 149 – 150 °C; $[\alpha]_D^{25}$: –19.5° (*c* 0.62, EtOH); UV (MeOH): λ_{max} (log ε) = 229 (3.57), 345 (3.55) nm; IR (neat): ν_{max} = 2925, 2855, 1725, 1614, 1578 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz), see Table **1**; LR-ESI-MS: *m/z* (rel. int.) = 258 (M^+, 32), 243.2 (M – CH_3^+, 100); HR-ESI-MS: found: 259.0968 $[M + H]^+$; calcd. for $C_{15}H_{15}O_4$: 259.0965.

Hedyotiscone B (**2**): colorless plates, m. p. 149 – 150 °C; $[\alpha]_D^{25}$: –12.5° (*c* 0.16, EtOH); UV (MeOH): λ_{max} (log ε) = 229 (3.83), 345 (3.59) nm; IR (neat): ν_{max} = 3439, 2925, 2868, 1727, 1618, 1588 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz), see Table **1**; LR-ESI-MS: *m/z* (rel. int.) = 244.2 (M^+, 20), 229 (M – CH_3^+, 100); HR-ESI-MS: found: 245.0810 $[M + H]^+$; calcd. for $C_{14}H_{12}O_4$: 245.0808.

Hedyotiscone C (**3**): colorless powder; m. p. 160 – 161 °C; $[\alpha]_D^{25}$: –15.6° (*c* 0.2, EtOH); UV (MeOH): λ_{max} (log ε) = 229 (3.73), 345 (3.55) nm; IR (neat): ν_{max} = 3433, 2920, 2858, 1721, 1614, 1579 cm^{-1}; ^{1}H-NMR (acetone-d_6, 400 MHz) and ^{13}C-NMR (acetone-d_6, 100 MHz), see Table **1**; LR-ESI-MS: *m/z* (rel. int.) = 259 (M – H_2O – CH_3^+,10), 217 (M – 1,2-diol-isopropane$^+$, 100), HR-ESI-MS: found: 293.1023 $[M + H]^+$; calcd. for $C_{15}H_{17}O_6$: 293.1020.

Cytotoxicity assay [22]: Compounds were assayed for cytotoxicity against Hep G2, A549, MCF-7 and MDA-MB-231 cell lines using the MTT method. Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 5,000 – 10,000 cells per well, and tested compounds were added from DMSO stock solutions. After 3 days in culture, attached cells were incubated with MTT (0.5 μg/mL, 1 h) and subsequently solubilized in DMSO. The absorbance was measured at 550 nm using a microplate reader. The IC_{50} is the concentration of agent that reduced cell growth by 50% under the experimental conditions. Results represent the mean two to three separate experiments, each performed in triplicate.

Acknowledgements

We gratefully acknowledge financial support for the project from National Science Council and National Science Technology Program/Biotechnology and Pharmaceuticals, Taiwan, R:O:C.

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