J Nat Med (2013) 67:212–216
DOI 10.1007/s11418-012-0647-x

NOTE

Haworforbins A–C, new phenolics from *Haworthia cymbiformis*

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Received: 23 January 2012 / Accepted: 16 February 2012 / Published online: 16 March 2012

Abstract Two new isocoumarin glucosides, haworforbins A (**1**) and B (**2**), and a new chromone, haworforbin C (**3**), have been isolated from *Haworthia cymbiformis*. Their structures and absolute configurations were elucidated on the basis of NMR and CD data. Haworforbin C (**3**) exhibited moderate inhibition of nitric oxide production in lipopolysaccharide-stimulated RAW264.7 cell line.

Keywords *Haworthia cymbiformis* · Haworforbins A-C · iNOS production inhibition activity

Introduction

Haworthia cymbiformis (Haw.) Duval (Xanthorrhoeaceae) is a succulent plant of South African origin which is usually grown as an ornamental plant. Recently, the aerial parts of *H. cymbiformis* have also been used in foods and drinks, though there are no reports on the constituents of this plant. In our screening program for bioactive natural products [1–5], the chloroform-soluble fraction from the whole plants of *H. cymbiformis* showed inhibition of nitric oxide (NO) production in RAW264.7 cell line stimulated by lipopolysaccharide (LPS). Investigation of the constituents of the chloroform fraction led to the isolation of three new compounds, haworforbins A–C (**1**–**3**), together with uncinoside A [6]. Structure elucidation and NO production inhibition activity of the isolated compounds are reported herein (Fig. 1).

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Results and discussion

Haworforbin A {**1**, $[\alpha]_D^{28}$ −20 (*c* 1.0, MeOH)} was isolated as a white amorphous solid, with molecular formula $C_{17}H_{22}O_9$ as determined by HRESITOFMS [*m/z* 371.1338 $(M+H)^+$, Δ −0.4 mmu]. IR absorptions suggested the presence of a carbonyl (1654 cm^{-1}) and a hydroxy (3397 cm^{-1}) group. ^1H and ^{13}C NMR data (Table 1) revealed 17 carbon resonances due to six sp^2 quaternary carbons, one sp^2 methine, six sp^3 methines, two sp^3 methylenes, and two methyls. Of them, three sp^2 quaternary carbons (δ_C 161.3, 161.5, and 170.8), six sp^3 methines (δ_C 70.2–100.6), and one sp^3 methylene (δ_C 61.9) are connected to an oxygen atom. These chemical shifts suggested the structure of **1** to be an isocoumarin glycoside.

Analysis of the 2D NMR data (HSQC, ^1H–^1H COSY, and HMBC) supported the structure of **1** as a new isocoumarin glycoside. The existence of two partial structures **a** (from C-9 to C-4) and **b** (from C-1′ to C-6′) were deduced from ^1H–^1H COSY analysis of **1** in CD_3OD/$CDCl_3$ (1:1, Fig. 2). HMBC correlations of H-3 to C-4a, H_2-4 to C-5 and C-8a suggested the connectivity of partial structure **a** to C-4a. HMBC correlations of H-5 to C-6 and C-7, and C-7-Me to C-6, C-7, and C-8 confirmed the position of the methyl group at C-7. Finally, HMBC correlations of H-1′ to C-6 and C-5′ suggested the pyranose

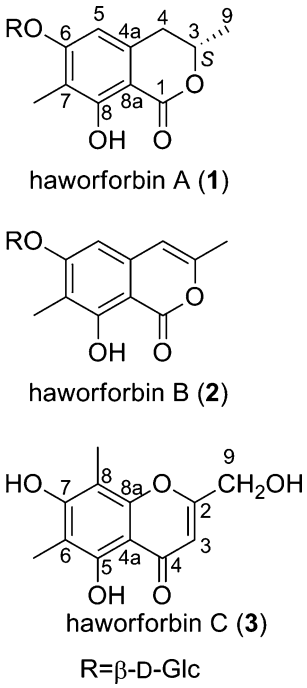

Fig. 1 Structures of compounds **1–3**

form of the sugar moiety and the connectivity of C-6 and C-1′ through an oxygen atom.

The ROESY correlations for H-1′/H-3′ and H-5′, and H-3′/H-5′ suggested that the hexopyranose took a chair form with axial orientations for H-1′, H-3′, and H-5′. Anti-relationships for H-1′/H-2′ and H-3′/H-4′ were deduced from the $^{3}J_{H-1'/H-2'}$ and $^{3}J_{H-3'/H-4'}$ values (7.7 and 8.8 Hz, respectively). Thus, the hexopyranose unit was assigned as a *β*-glucopyranoside. The glucose moiety was determined as the D-form on the basis of HPLC analysis with chiral detector of the acid hydrolysate of **1**. In addition, the absolute configuration of C-3 was suggested to be *S* by the positive Cotton effect at 270 nm in the CD spectrum of **1** and its aglycone [CD (MeOH) λ_{max} 271 ($\Delta\varepsilon$ 1.98) nm] [7, 8]. Thus, the structure of **1** was deduced to be a *β*-D-glucopyranoside derivative of monaschromone [9].

Haworforbin B {**2**, $[\alpha]_D^{28}$ −104 (*c* 0.3, MeOH)} was isolated as a white amorphous solid with molecular formula of $C_{17}H_{20}O_9$ as determined by HRESIMS [*m/z* 369.1175 $(M+H)^+$, Δ −1.1 mmu]. Analysis of the ^{1}H and ^{13}C NMR data (Table 1) suggested the structure of **2** to be a 3,4-dehydroderivative of **1**, since an exchange of an sp^3 methylene and an sp^3 methine in **1**, an sp^2 methine and an sp^2 quaternary carbon were observed in **2**.

The structure of **2** was further confirmed by analysis of the 2D NMR correlations (Fig. 2) and acid hydrolysis. HMBC correlations of H_3-9 to C-3 and C-4, and H-4 to C-4a, C-5, and C-8a confirmed the double bond between C-3 and C-4. In addition, HPLC analysis with chiral detection of the acid hydrolysate of **2** identified the sugar unit as D-glucose.

Haworforbin C (**3**) was isolated as a white amorphous solid with molecular formula of $C_{12}H_{12}O_5$ as determined by HRESIMS [*m/z* 237.0765 $(M+H)^+$, Δ +0.2 mmu]. ^{1}H and ^{13}C NMR data (Table 1) revealed 12 carbon resonances due to eight sp^2 quaternary carbons, one sp^2 methine, one sp^3 methylene, and two methyls. Of them, five sp^2 quaternary carbons (δ_C 153.9, 156.9, 160.7, 170.1, and 183.9) and one sp^3 methylene (δ_C 61.0) are connected to an oxygen atom. Further analysis of the ^{1}H and ^{13}C NMR revealed that, except for the observation of a methylene signal instead of a methyl, the NMR data of **3** are similar to the aglycone unit of uncinoside A [6]. Thus, **3** was assumed to be 5,7-dihydroxy-2-(hydroxymethyl)-6,8-dimethylchromone.

The structure of **3** was further confirmed through analysis of the 2D NMR data (Fig. 2). HMBC correlations of C-6-Me to C-5, C-6, and C-7, and C-8-Me to C-7, C-8, and C-8a confirmed the methyl positions to be at C-6 and C-8, and the hydroxymethyl position at C-2 was deduced from the HMBC correlations of H_2-9 to C-2 and C-3. Thus, **3** was determined to be 5,7-dihydroxy-2-(hydroxymethyl)-6,8-dimethylchromone.

Compounds **1–3** and their aglycones were tested for the inhibition of NO production in LPS-stimulated RAW264.7 cell line, and only **3** was found to show a moderate inhibitory activity (IC_{50} 87.5 μM).

To summarize, two new isocoumarin glucosides, haworforbins A (**1**) and B (**2**), and a new chromone, haworforbin C (**3**), have been isolated from *H. cymbiformis*. Their structures and absolute configurations were elucidated on the basis of NMR and CD data. Haworforbin C (**3**) exhibited moderate inhibition of NO production in LPS-stimulated RAW264.7 cell line.

Experimental section

General experimental procedures

Optical rotations were measured on a JASCO DIP-1000 polarimeter. UV spectra were recorded on a Shimadzu UVmini-1240 spectrophotometer and IR spectra on a JASCO FT/IR-4100 spectrophotometer. CD spectra were recorded on a JASCO J-820 polarimeter. High-resolution ESI MS were obtained on a LTQ Orbitrap XL (Thermo Scientific). ^{1}H and 2D NMR spectra were measured on a 700 MHz spectrometer at 300 K, while ^{13}C NMR spectra were on a 175 MHz spectrometer. The residual CD_2HOD chemical shifts used as an internal standard are δ_H 3.31 and δ_C 49.0. Standard pulse sequences were used for the 2D NMR experiments.

Table 1 ^{1}H and ^{13}C NMR data of haworforbins A–C (**1–3**) in $CD_3OD/CDCl_3$ (1:1) at 300 K

	1		**2**		**3**	
	δ_H (*J*, Hz)	δ_C	δ_H (*J*, Hz)	δ_C	δ_H (*J*, Hz)	δ_C
1		170.8		167.4		
2						170.1
3	4.65 (1H, m)	76.4		153.8	6.28 (1H, s)	105.7
4	2.83 (1H, dd, 16.3, 11.2)	35.0	6.29 (1H, s)	105.4		183.9
	2.89 (1H, dd, 16.3, 3.1)					
4a		138.5		137.4		104.9
5	6.43 (1H, s)	104.9	6.54 (1H, s)	100.8		156.9[a]
6		161.3		162.8		102.8[b]
7		113.7		113.7		160.7
8		161.5		160.5		108.1[b]
8a		102.8		101.1		153.9[a]
9	1.46 (3H, d)	20.7	2.23 (3H, s)	19.3	4.49 (2H, s)	61.0
6-Me					2.18 (3H, s)[a]	7.9[c]
7-Me	2.09 (3H, s)	7.8	2.15 (3H, s)	8.2		
8-Me					2.10 (3H, s)[a]	7.7[c]
1′	4.95 (1H, d, 7.7)	100.6	5.01 (1H, d, 7.7)	100.6		
2′	3.55 (1H, br. t, 7.7)	73.5	3.57 (1H, dd, 8.9, 7.7)	73.6		
3′	3.48 (1H, br. t, 8.8)	76.8	3.49 (1H, t, 8.9)	77.1		
4′	3.46 (1H, m)	70.2	3.45 (1H, t, 8.9)	70.3		
5′	3.44 (1H, m)	76.9	3.48 (1H, m)	77.1		
6′	3.72 (1H, dd, 12.0, 4.7)	61.9	3.73 (1H, dd, 12.1, 5.1)	61.9		
	3.86 (1H, dd, 12.0, 2.0)		3.89 (1H, dd, 12.1, 2.3)			

[a,b,c] Interchangeable

Material

The botanical identification of the *Haworthia cymbiformis* sample was made by Naoyuki Hagane, Sabotensoudan-shitsu, Japan. A voucher specimen (no. SABO100909) has been deposited in Sabotensoudanshitsu.

Extraction and isolation

The whole plants of *H. cymbiformis* (240.4 g) were extracted with methanol to obtain 6.2 g of extract which was successively partitioned with *n*-hexane, chloroform, ethyl acetate, *n*-butanol and water. The chloroform-soluble materials (286.5 mg) were further separated with a silica gel column (hexane/EtOAc, 1:0 → 1:1, and then $CHCl_3$/MeOH, 1:0 → 0:1) to give 13 fractions. Fraction 13 was further separated by ODS HPLC (YMC Pack Pro C18 RS 250 × 10 mm; eluted with CH_3CN/H_2O 22:78, 0.1% HCO_2H; flow rate 2.0 mL/min; UV detection at 254 nm) to yield **1** (2.2 mg, 0.00091%, t_R 21.8 min, purity >99%), **2** (0.6 mg, 0.00024%, t_R 30.6 min, purity >99%), and uncinoside A (2.1 mg, 0.00087%, t_R 24.5 min, purity >99%), and fraction 5 was further separated by silica gel column ($CHCl_3$/MeOH/EtOAc, 50:1:1 → 10:1:1) to give **3** (0.2 mg, 0.00008%).

*Haworforbin A (**1**)*

White amorphous solid; UV (MeOH) λ_{max} 219 (ε 24300), 267 (12500), and 308 (3300) nm; $[\alpha]_D^{28}$ −20 (*c* 1.0, MeOH); CD (MeOH) λ_{max} 211 ($\Delta\varepsilon$ 5.48), 222 (1.01), 224 (0), 228 (−3.82), 245 (−6.74), 267 (0), and 270 (7.40) nm; IR (KBr) ν_{max} 3397 and 1654 cm^{-1}; ^{1}H and ^{13}C NMR data (Table 1); ESIMS *m/z* 371 $(M+H)^+$; HRESITOFMS *m/z* 371.1338 ($[M+H]^+$; calcd for $C_{17}H_{23}O_9$, 371.1342).

*Haworforbin B (**2**)*

White amorphous solid; UV (MeOH) λ_{max} 237 (ε 25100) and 334 (3400) nm; $[\alpha]_D^{28}$ −104 (*c* 0.3, MeOH); IR (KBr) ν_{max} 3396 and 1689 cm^{-1}; ^{1}H and ^{13}C NMR data (Table 1); ESIMS *m/z* 369 $(M+H)^+$; HRESIMS *m/z* 369.1175 ($[M+H]^+$; calcd for $C_{17}H_{21}O_9$, 369.1186).

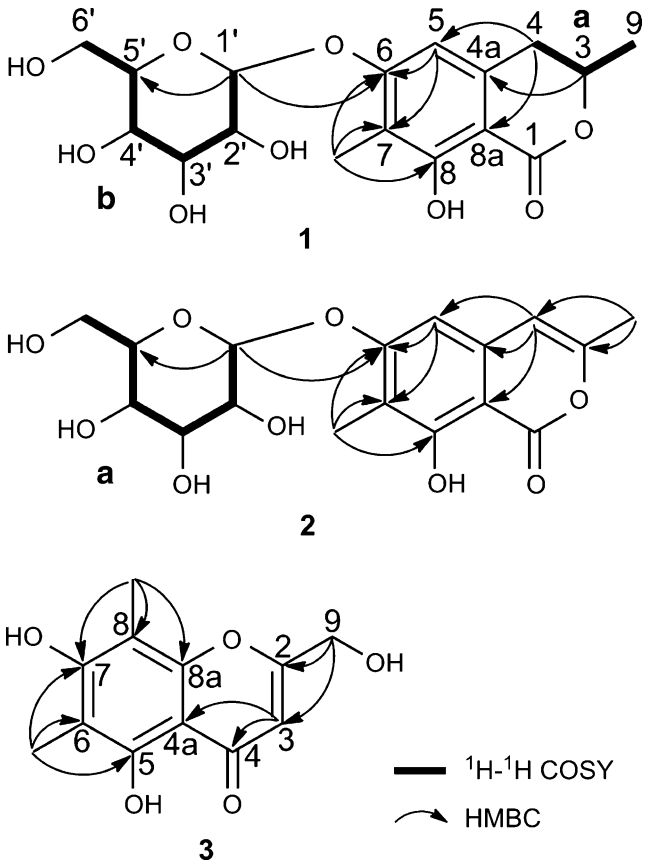

Fig. 2 Selected 2D NMR correlations for haworforbins A–C (**1–3**)

*Haworforbin C (**3**)*

White amorphous solid; UV (MeOH) λ_{max} 205 (ε 12700), 262 (7100) and 306 (3200) nm; IR (KBr) ν_{max} 3476 and 1648 cm^{-1}; ^{1}H and ^{13}C NMR data (Table 1); ESIMS *m/z* 237 $(M+H)^+$; HRESIMS *m/z* 237.0765 ($[M+H]^+$; calcd for $C_{12}H_{13}O_5$, 237.0763).

Acid hydrolysis of **1** and **2**

1 (1.0 mg) was treated with 2 M aqueous HCl (50 μL) at 100°C for 1 h. After neutralization with 2 M aqueous NaOH, the mixture was extracted with $CHCl_3$. The aqueous layer was submitted to HPLC analysis (GL science NH2 column φ 4.6 × 250 mm, eluent: 70% aqueous MeCN, flow rate 1.0 mL/min, JASCO OR-1590 chiral detector). Retention times of authentic L- and D-glucose were as follows: L (9.1 min with negative intensity) and D (9.1 min with positive intensity). The retention time of glucose in the aqueous layer of hydrolysate of **1** was 9.1 min with positive intensity. The $CHCl_3$ layer was dried and the NMR data of the obtained compound is identical to monaschromone [9]. **2** (1.0 mg) was subjected to a similar treatment as **1**, and the retention time of glucose in the aqueous layer of hydrolysate of **2** was 9.1 min with positive intensity. The $CHCl_3$ layer was also dried and the NMR data of the obtained compound is identical to 6,8-dihydroxy-3,7-dimethylisocoumarin [10].

NO production by RAW264.7 cells

The RAW264.7 cells were cultured in α-MEM medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells were seeded onto a 96-well microtiter plate at 1.5×10^5 cells in 100 μL solution per well and were preincubated for 12 h at 37°C in a humidified atmosphere containing 5% CO_2. The cells were cultured in the medium containing LPS (5 μg/mL) with or without the test sample at different concentrations for 24 h. NO production was then determined by the Griess assay. Supernatant of the cultured medium (100 μL) was transferred to a 96-well microtiter plate, and then 100 μL of Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% H_3PO_4 was added). After incubation at room temperature for 15 min, the absorbance at 540 and 620 nm was measured with a microplate reader (Benchmark Plus microplate spectrometer, Bio-Rad). L-NMMA, an NO synthase inhibitor, was used as a positive control (IC_{50} 13.8 μg/mL).

Acknowledgments This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and a grant from The Open Research Center Project in Hoshi University.

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