Anemhupehins A–C, Podocarpane Diterpenoids from *Anemone hupehensis*

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Abstract

Three new podocarpane diterpenoids, namely anemhupehins A–C (1–3), together with four known analogues (4–7), have been isolated from aerial parts of *Anemone hupehensis*. Their structures were characterized based on extensive spectroscopic data. Compounds **1** and **4** showed certain cytotoxicities against human cancer cell lines.

Graphical Abstract

Keywords *Anemone hupehensis* · Podocarpane diterpenoids · Cytotoxicity

1 Introduction

Podocarpane diterpenoids usually possessed a backbone of seventeen carbons arranged in a tricyclohexane system close to abietane and pimarane [1–8]. They do not occur

extensively in nature but are present in several genera, such as *Azadirachta* [1–5], *Humirianthera* [6], *Micrandropsis* [7], and *Podocarpus* [8]. Previous studies have disclosed a few biologically active podocarpane molecules, such as 13-hydroxy-8,11,13-podocarpatriene-19-oic acid, a highly fungistatic agent [9, 10], and 7-oxo-13-hydroxy-14-amino-8,11,13-podocarpatriene, a potent 5-lipoxygenase inhibitor [11].

Anemone hupehensis is a flowering herbaceous perennial in the Ranunculaceae family. It is about one meter high and native in central China. In previous chemical studies on this plant, a few triterpenoids and their saponins were reported [12–15]. In our continuous searching for new and bioactive natural products, we investigated the chemical constituents in aerial parts of *A. hupehensis*, which resulted

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in the isolation of three new podocarpane diterpenoids, anemhupehins A–C (**1–3**), as well as four known ones **4–7** (Fig. 1). Compound **4** was isolated as a new natural product for the first time. Their structures were established by extensive spectroscopic methods. Compounds **1** and **4–7** were evaluated for their cytotoxicities against five human cancer cell lines. We report here the isolation, structure elucidation, and cytotoxicities of these isolates.

2 Results and Discussion

Compound **1** was isolated as a colorless oil. The HRESIMS data at m/z 283.1668 $[M + Na]^+$ (calcd for $C_{17}H_{24}O_2Na$: 283.1669) indicated a molecular formula $C_{17}H_{24}O_2$, requiring six degrees of unsaturation. The IR absorption bands at 3438, 1608, and 1480 cm^{-1} were attributable to hydroxy and phenyl groups. In the 1H NMR spectrum (Table 1), three singlets from at δ_H 1.04 (3H), 1.27 (3H), and 1.54 (3H) were readily identified signals for three methyls, while three signals at δ_H 6.49 (1H, d, $J = 2.8\text{ Hz}$, H-14), 6.67 (1H, dd, $J = 8.6, 2.8\text{ Hz}$, H-12), and 7.17 (1H, d, $J = 8.6\text{ Hz}$, H-11) displayed a 1,3,4-trisubstituted aromatic ring. The ^{13}C NMR data, with the aid of DEPT and HSQC spectra, revealed 17 carbon resonances ascribable for three methyl, four methylene, five methine, and five quaternary carbons (Table 1). Analyses of 1H – 1H COSY data disclosed three spin-coupling systems shown in Fig. 2. These data, together with preliminary analyses of HMBC correlations, suggested that **1** possessed a podocarpane skeleton. A phenolic hydroxy group at δ_H 5.22 (1H, s) was deduced to be placed at C-13 by analyses of HMBC correlations and NMR data. In addition, another hydroxy

group was suggested to be at C-6, as revealed by HMBC correlations from δ_H 4.67 (1H, br d, $J = 5.0\text{ Hz}$, H-6) to δ_C 53.4 (d, C-5), 41.3 (t, C-7), 34.3 (s, C-4), and 37.2 (s, C-10), as well as 1H – 1H COSY cross peaks of H-6/ δ_H 1.40 (1H, br s, H-5) and H-6/ δ_H 3.10 and 2.85 (2H, H_2 -7). These data elucidated the structure of **1** to be 6,13-dihydroxy-8,11,13-podocarpatriene. An ROESY experiment revealed the relative configuration of **1** (Fig. 2), in which the cross peak of H-19/H-20 indicated that CH_3 -19 and CH_3 -20 were in the same side (β orientation), while the cross peaks of H-18/H-5, H-5/H-6, and H-6/H-18 indicated that H-6 should be α oriented. As established by the ROESY data, the energy minimized 3D structure of **1** revealed stereo-configuration of **1**. A Newman projection of C-5 and C-6 indicated the dihedral angle between H-5 and H-6 to be close to $\theta = 90^\circ$ (Fig. 2), which resulted in the coupling constants of $J_{5,6} = 0\text{ Hz}$, that is why H-5 presented as a singlet in the 1H NMR spectrum. All these data elucidated the structure of **1** to be 6 β ,13-dihydroxy-8,11,13-podocarpatriene, named anemhupehin A.

Compound **2** was isolated as a colorless oil. The HRESIMS established the molecular formula to be $C_{17}H_{24}O_2$ on the basis of a molecular ion peak at m/z 259.1704 (calcd for $C_{17}H_{23}O_2$: 259.1704 $[M - H]^-$). IR absorption bands ($3432, 1612, 1481\text{ cm}^{-1}$) also indicated the presence of hydroxy and phenyl functions. The ^{13}C NMR and DEPT disclosed 17 carbon resonances including three methyl, four methylene, five methine, and five quaternary carbons (Table 1). These data showed similar patterns to those of **1**. The 1H – 1H COSY cross peak between δ_H 4.75 (1H, m, H-7) and 2.24/1.64 (each 1H, m, H-6), as well as HMBC correlations from H-7 to δ_C 139.5 (s, C-8) and 30.3 (t, C-6) suggested that a hydroxy should be placed at C-7 in **2**,

Fig. 1 Structures of compounds **1–7**

Table 1 ^1H and ^{13}C NMR data for compounds **1–3** (δ in ppm, J in Hz)^a

No.	1		2		3	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}
1	2.15 (1H, br d, 12.6) 1.36 (1H, m)	42.4 (t)	2.21 (1H, br d, 12.7) 1.31 (1H, m)	39.1 (t)	2.21 (1H, br d, 12.6) 1.31 (1H, m)	38.7 (t)
2	1.83 (1H, m) 1.60 (1H, m)	19.7 (t)	1.72 (1H, m) 1.58 (1H, m)	19.3 (t)	1.72 (1H, m) 1.58 (1H, m)	19.4 (t)
3	1.46 (1H, m) 1.24 (1H, m)	43.2 (t)	1.48 (1H, m) 1.21 (1H, m)	41.4 (t)	1.48 (1H, m) 1.21 (1H, m)	41.6 (t)
4		34.3 (s)		33.3 (s)		33.1 (s)
5	1.40 (1H, br s)	53.4 (d)	1.35 (1H, dd, 13.1, 3.8)	49.4 (d)	1.60 (1H, m)	44.9 (d)
6	4.67 (1H, br d, 5.0)	66.2 (d)	2.24 (1H, m) 1.64 (1H, m)	30.3 (t)	1.98 (2H, m)	28.7 (t)
7	3.10 (1H, dd, 17.4, 5.0) 2.85 (1H, d, 17.4)	41.3 (t)	4.75 (1H, m)	71.4 (d)	4.76 (1H, m)	68.4 (d)
8		133.1 (s)		139.5 (s)		137.6 (s)
9		141.3 (s)		142.5 (s)		142.6 (s)
10		37.2 (s)		38.1 (s)		37.7 (s)
11	7.17 (1H, d, 8.6)	126.7 (d)	7.10 (1H, d, 8.7)	126.1 (d)	7.14 (1H, d, 8.4)	126.1 (d)
12	6.67 (1H, dd, 8.6, 2.8)	113.9 (d)	6.72 (1H, dd, 8.7, 2.7)	115.1 (d)	6.74 (1H, dd, 8.4, 2.6)	116.0 (d)
13		153.3 (s)		153.7 (s)		153.7 (s)
14	6.49 (1H, d, 2.8)	115.5 (d)	6.99 (1H, d, 2.7)	113.3 (d)	6.82 (1H, d, 2.6)	116.0 (d)
18	1.04 (3H, s)	33.9 (q)	0.95 (3H, s)	33.2 (q)	0.97 (3H, s)	33.3 (q)
19	1.27 (3H, s)	23.9 (q)	0.92 (3H, s)	21.7 (q)	0.92 (3H, s)	21.7 (q)
20	1.54 (3H, s)	27.3 (q)	1.22 (3H, s)	25.6 (q)	1.10 (3H, s)	24.2 (q)
OH	5.22 (1H, br s)		4.96 (1H, br s)		5.37 (1H, br s)	

The assignments were based on DEPT, ^1H - ^1H COSY, HSQC, and HMBC experiments

^aData (δ) were measured in CDCl_3

Fig. 2 Key 2D NMR correlations of **1** and stereoconfiguration analyses

rather than at C-6 in **1**. Detailed analyses of 2D NMR data indicated that the other parts of **2** were the same to those of **1**. In the ROESY spectrum, cross peaks of H-5/H-7 suggested that OH-7 should be β oriented. Therefore, compound **2** was elucidated as 7 β ,13-dihydroxy-8,11,13-podocaratriene, named anemhupehin B.

Compound **3** was initially isolated as a mixture with **2**. A further isolation of this mixture only obtained pure compound **2**, while the amount of compound **3** is too less to obtain the clear NMR spectra. However, careful analyses of MS data of **3** and NMR spectra of the mixture (see Supporting Information), with the aid of NMR spectrum of **2**, can unambiguously elucidate the structure of **3**. The

HRESIMS of **3** displayed a molecular ion peak at m/z 259.1704 (calcd for $C_{17}H_{23}O_2$: 259.1704 $[M - H]^-$), revealed the molecular formula $C_{17}H_{24}O_2$, the same to that of **2**. The ^{13}C NMR data also showed good agreements with those of **2**. Analyses of 1D and 2D NMR data suggested that compound **3** should have the same planar structure to that of **1**. However, the ^{13}C NMR shift of C-7 at δ_C 68.4 in **3** was significantly different from that in **2**, indicating that compound **3** might be an epimer of **2**. In the ROESY spectrum of the mixture, the cross peaks of 19/H-20 and H-18/H-5 suggested that C-5 and C-10 possessed the same configurations to those in compounds **2**. Therefore, compound **3** was determined to be 7-epimer of **2**. The 3D structure analyses of **3** and **2** also supported that OH-7 in **3** should be α oriented. Therefore, compound **3** was elucidated as 7 α ,13-dihydroxy-8,11,13-podocarpatriene, named anemhupehin C.

Four known analogues were identified as 13,14-dihydroxy-8,11,13-podocarpatriene (**4**) [16], 13-hydroxy-8,11,13-podocarpatriene (**5**) [17], 13,14-dihydroxy-7-oxo-8,11,13-podocarpatriene (**6**) [17], and 13-hydroxy-7-oxo-8,11,13-podocarpatriene (**7**) [18]. Compound **4** was isolated as a natural product for the first time.

Compounds **1** and **4–7** were evaluated for their cytotoxicities to five human cancer cell lines. As a result, compounds **1** and **4** showed moderate activities as shown in Table 2. The other compounds were inactive to cancer cell lines ($IC_{50} > 40 \mu M$).

3 Experimental

3.1 General Experimental Procedures

Optical rotations were measured on a Jasco-P-1020 polarimeter. IR spectra were obtained using a Bruker Tensor 27 FT-IR spectrometer with KBr pellets. NMR spectra were acquired with a Bruker DRX-600 with tetramethylsilane (TMS) used as an internal standard. HRESIMS were recorded on an API QSTAR Pulsar spectrometer. Silica gel (200–300 mesh), Sephadex LH-20 and RP-18 gel (20–45 μm) were used for column chromatography (CC). Medium pressure liquid chromatography (MPLC) was performed on a Biotage system. Preparative

high performance liquid chromatography (prep-HPLC) was performed on an Agilent 1260 liquid chromatography system equipped with Zorbax SB-C18 columns (5 μm , 9.4 mm $\times$ 150 mm or 21.2 mm $\times$ 150 mm) and a DAD detector. Fractions were monitored by TLC and spots were visualized by heating silica gel plates immersed in H_2SO_4 in EtOH, in combination with the Agilent 1200 series HPLC system (Eclipse XDB-C18 column, 5 μm , 4.6 $\times$ 150 mm).

3.2 Plant Material

Aerial parts of *A. hupehensis* were collected from Shen-LongJia of Hubei province, central China in September 2016 and identified by Prof. Ming-Qing Pan of Wuhan University. A specimen (No. COP-HF20160912.4) was deposited at South-Central University for Nationalities.

3.3 Extraction and Isolation

The air dried sample of *A. hupehensis* (8 kg) were extracted with methanol (24 h $\times$ 3) to afford an extract. The extract was partitioned with water and EtOAc (1:1). The extract of EtOAc lay (202 g) was subjected to silica gel CC using $CHCl_3$ -MeOH (from 1:0 to 0:1) to give eight fractions (A–H). Fraction C (23.8 g) was separated by MPLC with gradient mixture of MeOH and H_2O (20:80–100:0, v/v) to afford ten sub-fractions (C1–C10). Fraction C2 (500 mg) was isolated by silica gel CC using petroleum ether/ Me_2CO (6:1) to afford several subfractions, and compound **6** (13 mg) crystallized from one subfraction. Fraction C4 (280 mg) was purified by Sephadex LH-20 (MeOH) to give compounds **1** (7.2 mg) and **4** (8.6 mg). Compounds **2** and **3** was initially isolated as a mixture (2.8 mg) as obtained from fraction C5 (120 mg) by silica gel CC, and was re-purified by HPLC (MeCN: H_2O (v/v) from 2:8 to 4:6 in 25 min) to afford pure compounds **2** (1.1 mg) and **3** (less than 0.5 mg). Fraction C6 (300 mg) was isolated by Sephadex LH-20 (MeOH) and then purified by HPLC (MeCN: H_2O (v/v) from 3:7 to 4:6 in 25 min) to afford **5** (6.8 mg) and **7** (3.4 mg).

3.3.1 Anemhupehin A (**1**)

Colorless oil, $[\alpha]_D^{22} + 17.6$ (c 0.18 MeOH); IR (KBr) ν_{max} 3443, 3438, 2928, 1624, 1608, 1480, 1367, 1201, 862 cm^{-1} ; for 1H (600 MHz) and ^{13}C NMR (150 MHz) data ($CDCl_3$), see Table 1; HRESIMS: m/z 283.1668 (calcd for $C_{17}H_{24}O_2Na$, $[M + Na]^+$, 283.1669).

Table 2 Cytotoxicities of compounds **1** and **4** (IC_{50} , μM)

Entry	HL-60	SMMC-7721	A-549	MCF-7	SW480
1	> 40	33.8	> 40	28.8	13.2
4	16.8	22.7	> 40	12.2	14.1
Cisplatin	4.4	12.4	16.4	17.1	12.8

3.3.2 Anemhupehin B (2)

Colorless oil, $[\alpha]_D^{22} +12.7$ (*c* 0.08 MeOH); IR (KBr) $\nu_{\max}$ 3441, 3432, 2926, 1612, 1481, 1381, 1211, 882 cm^{-1} ; for ^1H (600 MHz) and ^{13}C NMR (150 MHz) data (CDCl_3), see Table 1; HRESIMS: m/z 259.1704 (calcd for $\text{C}_{17}\text{H}_{23}\text{O}_2$, $[\text{M} - \text{H}]^-$, 259.1704).

3.3.3 Anemhupehin C (3)

Colorless oil, $[\alpha]_D^{22} +8.7$ (*c* 0.02 MeOH); for ^1H (600 MHz) and ^{13}C NMR (150 MHz) data (CDCl_3), see Table 1; HRESIMS: m/z 259.1704 (calcd for $\text{C}_{17}\text{H}_{23}\text{O}_2$, $[\text{M} - \text{H}]^-$, 259.1704).

3.4 Cytotoxicity Assay

Human myeloid leukemia HL-60, hepatocellular carcinoma SMMC-7721, lung cancer A-549 cells, breast cancer MCF-7 and colon cancer SW480 cell lines were used in the cytotoxic assay. All cell lines were cultured in RPMI-1640 or DMEM medium (Hyclone, USA), supplemented with 10% fetal bovine serum (Hyclone, USA) in 5% CO_2 at 37 °C. The cytotoxicity assay was performed according to the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method in 96-well microplates [19]. Cisplatin was used as a positive control.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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