Meroterpenoids and a Naphthoquinone from *Arnebia euchroma* and Their Cytotoxic Activity

DOI 10.1055/s-0035-1545693
Planta Med 2015; 81: 320–326

Publisher and Copyright:
© 2015 by
Georg Thieme Verlag KG
Rüdigerstraße 14
70469 Stuttgart
ISSN 0032-0943

Reprint with the permission by the publisher only

Thieme

Meroterpenoids and a Naphthoquinone from *Arnebia euchroma* and Their Cytotoxic Activity

Authors Lun Wang[1,2], Fu Li[1], Xin Liu[3], Bin Chen[1], Kai Yu[4], Ming-Kui Wang[1]

Affiliations The affiliations are listed at the end of the article

Key words
- *Arnebia euchroma*
- Boraginaceae
- meroterpenoids
- arnebinol
- cytotoxicity

Abstract

Four new meroterpenoids, arnebinols A–D (**1**–**4**), and one new prenylated naphthoquinone, 5,8-*O*-dimethyl-11-deoxyalkannin (**5**), together with seven known meroterpenoids (**6**–**12**) were isolated from the roots of *Arnebia euchroma*. The structures of the isolated compounds were elucidated unambiguously by spectroscopic data analysis, as well as X-ray-single crystal diffraction analysis. Arnebinol A (**1**) and B (**2**) are rare meroterpenoids possessing a 6/10/5 tricyclic ring system. Compounds **1**–**12** were evaluated for their cytotoxicities against MG-63 and SNU387 human cancer cell lines. Compound **5** exhibited the most potent activity with IC_{50} values of 2.69 µM and 6.08 µM, respectively.

Supporting information available online at http://www.thieme-connect.de/products

Introduction

Arnebia euchroma (Royle) Jonst. (family Boraginaceae) is a small genus of annual or perennial herbs, distributed in Asia and the drier regions of Northern Africa [1]. *A. euchroma* ("Zicao" in Chinese) is a traditional Chinese herbal medicine (TCM) recorded in the Pharmacopoeia of China and has been extensively used in China and other countries for the treatment of various diseases [2–4]. According to previous studies, *A. euchroma* produces many types of secondary metabolites including naphthoquinones [3–6], phenolic acids [7, 8], alkaloids [9, 10], and meroterpenoids [11–14]. Meroterpenoids were defined as natural products of mixed biosynthetic origin (polyketide-terpenoid) featuring a quinone, hydroquinone, or closely related subunit linked to a terpenoid moiety through a C–C bond, which have been widely found in fungi and marine organisms, and also in higher plants [15–17]. Some of these compounds possess interesting biological and pharmacological activities, including cytotoxic, anti-inflammatory, antifungal, antibacterial, and antioxidant effects [18–24]. Meroterpenoids isolated from *A. euchroma* contained a phenolic or a quinone unit connected with a monoterpene moiety by one or two C–C bonds. As part of our ongoing search for bioactive constituents, especially for new antitumor agents from TCMs, an EtOH extract of the roots of *A. euchroma* was investigated, and four new meroterpenoids (**1**–**4**), one new prenylated naphthoquinone (**5**), together with seven known meroterpenoids (**6**–**12**) were isolated. We report the isolation and structural elucidation of these compounds, as well as their cytotoxic activities against MG-63 and SNU387 tumor cells.

Results and Discussion

Compound **1** was isolated as a white amorphous powder. The molecular formula $C_{16}H_{16}O_4$ implied nine degrees of unsaturation. The IR spectrum suggested the presence of hydroxyl groups, an α,β-unsaturated-γ-lactone (1716 cm^{-1}), and aromatic absorptions (1488 cm^{-1}). The ^1H NMR spectrum (**Table 1**) showed a pair of *ortho*-coupled aromatic protons, suggesting the presence of one tetrasubstituted benzene ring. Two olefinic proton signals indicated the existence of two trisubstituted double bonds. The ^{13}C NMR (**Table 1**), DEPT, and HSQC spectra revealed the presence of 16 carbon resonances, including one methyl, three methylenes, and five methines along with seven quaternary carbons [including one ester carbonyl at δ_C 177.9 (C-15)]. These functionalities accounted for eight out of the nine degrees of unsaturation, indicating that **1** has a tricyclic core in-

received June 18, 2014
revised Dec. 31, 2014
accepted January 10, 2015

Bibliography
DOI http://dx.doi.org/10.1055/s-0035-1545693
Planta Med 2015; 81: 320–326

ISSN 0032-0943

Correspondence
Prof. Dr. Ming-Kui Wang
Chengdu Institute of Biology
Chinese Academy of Sciences
No. 9, Section 4, Renmin South Road
Chengdu 610041
People's Republic of China
Phone: + 86 28 82 89 08 21
wangmk@cib.ac.cn

Table 1 ^{1}H and ^{13}C NMR data for compounds **1, 2**, and **6**.

Position	1[a]		2[a]		6[b]	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1		150.9 (s)		151.0 (s)		187.7 (s)
2	6.72, d (8.8)	118.4 (d)	6.63, d (8.6)	117.0 (d)	5.97, s	107.4 (d)
3	6.65, d (8.8)	115.4 (d)	6.55, d (8.6)	115.0 (d)		158.0 (s)
4		150.8 (s)		149.8 (s)		182.0 (s)
5		118.6 (s)		127.2 (s)		141.5 (s)
6	6.86, s	78.8 (d)	6.67, br s	83.1 (d)	6.37, br s	79.4 (d)
7	6.95, s	152.5 (d)	5.21, s	126.8 (d)	5.10, s	123.9 (d)
8		129.5 (s)		135.2 (s)		136.2 (s)
9α	2.74, m	26.1 (t)	2.29, br d (12.5)	27.9 (t)	2.29, d (10.5)	26.8 (t)
9β	1.90, m[c]		1.90, td (12.5, 2.3)		1.92, t (12.4)	
10α	2.19, m	25.6 (t)	2.12, m	25.0 (t)	2.12, m	24.1 (t)
10β	1.90, m[c]		2.07, m		2.01, q (11.3)	
11	5.10, t (7.8)	121.6 (d)	5.04, t (7.9)	121.2 (d)	5.07, t (8.3)	122.0 (d)
12		142.2 (s)		143.3 (s)		139.5 (s)
13α	3.38, d (14.6)	27.3 (t)	3.40, d (14.2)	27.9 (t)	3.38, d (13.0)	26.3 (t)
13β	2.42, d (14.6)		2.92, d (14.2)		2.74, d (13.0)	
14		127.8 (s)		128.5 (s)		147.5 (s)
15		177.9 (s)	4.75, dd (11.9, 5.5) 4.69, dt (11.9, 3.0)	77.4 (t)	4.74, br s	77.5 (t)
16	1.58, s	22.1 (q)	1.59, s	22.2 (q)	1.48, s	23.3 (q)
-OCH_3					3.81, s	56.3 (q)

[a] Data were measured in CD_3OD at 600 MHz (^{1}H) and 150 MHz (^{13}C); [b] Data were measured in $CDCl_3$ at 600 MHz (^{1}H) and 150 MHz (^{13}C); [c] Overlapped signal

cluding the benzene ring and α,β-unsaturated-γ-lactone ring. Overall consideration of the NMR data suggested that compound **1** was a meroterpenoid similar to the known co-occurring compound clavilactone A (**7**), which represents a novel class of tyrosine kinase inhibitors [25], previously isolated from a culture of the non-toxigenic fungus *Clitocybe clavipes* [23]. The difference occurred in the positions C-7 and C-8. In the ^{13}C NMR spectrum of **1**, the carbon chemical shifts of C-7 and C-8 were observed downfield at δ_C 152.5 and 129.5, respectively. At the same time, the significant downfield shifts for H-6 (δ_H 6.86) and H-7 (δ_H 6.95) were observed in the ^{1}H NMR spectrum. These differences, together with the MS data, suggested that the 7,8-epoxide group in **7** was replaced by a trisubstituted carbon double bond between C-7 and C-8 in **1**, which was determined by the HMBC correlations between H-7/C-15, H-9/C-7 and H-10/C-8. In the HMBC spectrum (**Fig. 2**), the correlations between H-6 and C-15, C-8, and C-7 confirmed the identity of the α,β-unsaturated-γ-lactone moiety. Accordingly, the planar structure of **1** was proposed. The assignments of all proton and carbon signals could be established by 2D ^{1}H-^{1}H COSY, HSQC, and HMBC experiments. The absolute configuration at C-6 of **1** was predicted to be the same as that of **7** based on a biogenetic consideration, and the absolute configuration of natural clavilactone was established by total synthesis [26–28]. The NOESY correlation (**Fig. 3**) between H-11 and CH_3-16 suggested that the configuration of the double bond between C-11 and C-12 should be *Z*. Thus, the structure of **1** was established as shown in **Fig. 1**, and the compound was named arnebinol A.

Compound **2** was isolated as a white powder and gave a pseudomolecular ion peak consistent with a molecular formula of $C_{16}H_{18}O_3$, 14 mass units less than that of **1**. The IR absorptions revealed the presence of hydroxyl and aromatic functionalities. In the ^{1}H NMR spectrum (**Table 1**), one methyl group signal, two *ortho*-coupled aromatic proton signals, together with two olefinic proton signals were observed. The ^{13}C NMR (**Table 1**), DEPT, and HSQC spectra exhibited 16 carbon signals. These data suggested that **2** was also a meroterpenoid. Comparative analyses of the ^{1}H and ^{13}C NMR data of **2** with those of **1** showed a very close structural similarity. The main differences of the 1D NMR spectra of **2** and **1** were the absence of the signal corresponding to the C-15 ester carbonyl and the presence of a methylene carbon atom [δ_C (77.4) and δ_H 4.75 (1H, dd, J = 11.9, 5.5 Hz, H-15β), 4.69 (1H, dt, J = 11.9, 3.0 Hz, H-15α)] in **2**. The HMBC correlations (**Fig. 2**) observed from H-9 to C-15, as well as from H_2-15 to C-6, C-7, and C-8, further confirmed the presence of the C-15 methylene in **2**. All signals of **2** were assigned from the 2D ^{1}H-^{1}H COSY, HSQC, and HMBC experiments. The absolute configuration of **2** was assigned to be identical with that of **1** by comparing their optical rotation data.

Compound **3** was isolated as a white amorphous powder, and its molecular formula was determined to be $C_{16}H_{16}O_4$ by means of HRESIMS, as well as ^{1}H and ^{13}C NMR data (**Table 2**), with nine degrees of unsaturation. In the IR spectrum, absorptions for hydroxyl, α-methylene-γ-lactone (1738 cm^{-1}), and aromatic functionalities were observed. In the ^{1}H NMR spectrum, three olefinic protons at δ_H 5.73 (1H, dd, J = 17.8, 10.7 Hz, H-10), 4.91–4.93 (2H, m, H_2-11) displayed the presence of a vinyl group. A pair of olefinic protons at δ_H 6.23 (1H, s, H-13α) and δ_H 5.81 (1H, s, H-13β) at C-13 (δ_C 123.4) indicated the presence of one terminal double bond, and a pair of *ortho*-coupled aromatic protons suggested the presence of one tetrasubstituted benzene ring. Combined analysis of ^{13}C NMR, DEPT, and HSQC data showed 16 carbon resonances attributed to one methyl, three methylenes, five methines, and seven quaternary carbons. From the above analysis of the ^{1}H and ^{13}C NMR spectroscopic data, it was implied that compound **3** is a meroterpenoid with a benzogeijerene skeleton and is similar to that of euchroquinol A, previously isolated from *A. euchroma* [14]. However, the main difference was that compound **3** displayed NMR data for an ester carbonyl (δ_C 173.1) at C-14 instead of the methylene group as in euchroquinol A. This

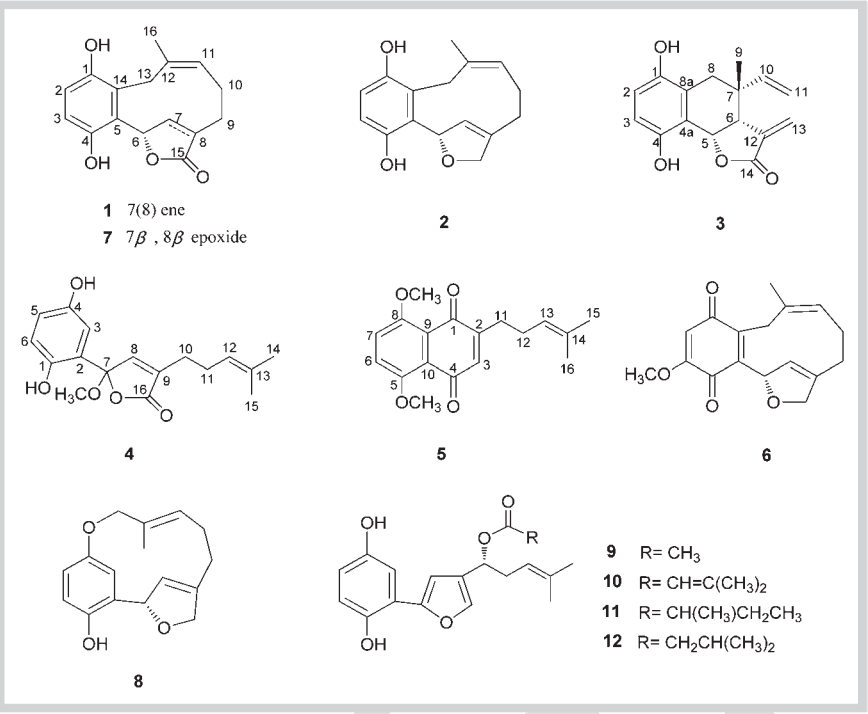

Fig. 1 Chemical structures of compounds **1–12**.

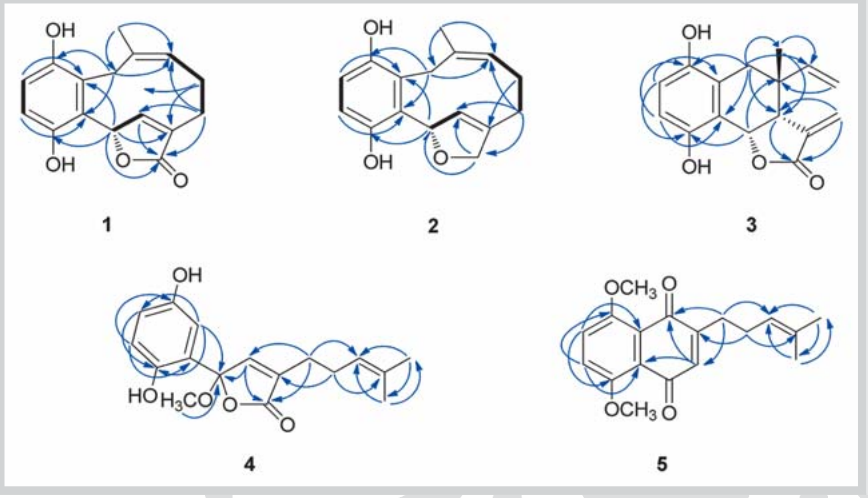

Fig. 2 COSY (bold lines) and key HMBC correlations (arrows) for compounds **1–5**. (Color figure available online only.)

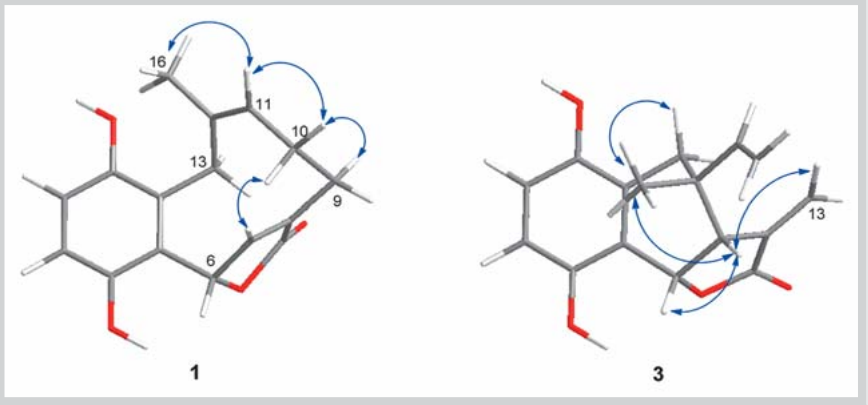

Fig. 3 Key NOESY correlations of compounds **1** and **3**. (Color figure available online only.)

Table 2 ^{1}H and ^{13}C NMR data for compounds **3**–**5**.

Position	3[a]		4[a]		5[b]	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1		148.4 (s)		149.3 (s)		185.3 (s)
2	6.69, d (8.6)	117.4 (d)		123.6 (s)		150.5 (s)
3	6.58, d (8.6)	114.1 (d)	6.91, d (1.3)	114.6 (d)	6.59, s	135.0 (d)
4		151.2 (s)		151.2 (s)		185.1 (s)
4a		119.7 (s)				
5	5.61, d (6.2)	75.7 (d)	6.67, d (8.1)	118.2 (d)		153.5 (s)
6	3.15, d (6.2)	50.1 (d)	6.69, d (8.1)	118.4 (d)	7.28, s[c]	120.1 (d)
7		38.5 (s)		108.6 (s)	7.28, s[c]	120.1 (d)
8α	2.97, d (16.6)	34.8 (t)	7.38, s	148.2 (d)		153.9 (s)
8β	2.44, d (16.6)					
8a		125.0 (s)				
9	1.21, s	24.7 (q)		135.9 (s)		121.8 (s)
10	4.75, dd (17.8, 10.7)	142.0 (d)	2.31, m	25.9 (t)		121.4 (s)
11	4.91–4.93, m[c]	115.1 (t)	2.24, m	26.9 (t)	2.51, t (7.7)	29.5 (t)
12		140.0 (s)	5.05, t (7.2)	123.8 (d)	2.24, q (7.3)	26.5 (t)
13	α 6.23, s	123.4 (t)		134.1 (s)	5.11, t (7.0)	122.9 (d)
	β 5.81, s					
14		173.1 (s)	1.59, s	17.7 (q)		133.2 (s)
15			1.49, s	25.7 (q)	1.66, s	17.8 (q)
16				173.4 (s)	1.57, s	25.7 (q)
5-OCH_3					3.94, s[c]	57.0 (q)
7-OCH_3			3.26, s	52.2 (q)		
8-OCH_3					3.94, s[c]	57.0 (q)

[a] Data were measured in CD_3OD at 600 MHz (^{1}H) and 150 MHz (^{13}C); [b] Data were measured in $CDCl_3$ at 600 MHz (^{1}H) and 150 MHz (^{13}C); [c] Overlapped signal

was further confirmed by the HMBC correlations (**Fig. 2**) between H-6, H_2-13 and C-14. The complete NMR assignments of **3** were determined by analysis of HSQC and HMBC spectra. The configurations of H-5 and H-6 were deduced by 2D NOESY experiment (**Fig. 3**) and proved to be both β-oriented due to correlations of Me-9/H-6, H-6/H-5 and the β-orientation of Me-9 in the benzogeijerene compounds [14,29–31]. The coupling constants (J = 6.2 Hz) between H-5 and H-6 also confirmed the *cis*-configuration at H-5 and H-6 [14]. Thus, the structure of **3** was proposed, and the compound was named arnebinol C.

Compound **4** was obtained as an optically inactive yellowish oil, characterized as a racemic mixture. The molecular formula was determined as $C_{17}H_{20}O_5$ by HRESIMS, suggesting eight degrees of unsaturation. The IR absorptions revealed the presence of hydroxyl, an α,β-unsaturated-γ-lactone, and aromatic functionalities. The ^{13}C NMR spectrum (**Table 2**) showed 17 carbons, of which three signals corresponded to methyls (including one methoxy group), two to methylenes, and five to methines. In addition, seven quaternary carbons were assigned. The ^{1}H NMR spectrum of **4** (**Table 2**) exhibited signals for a trisubstituted aromatic ring [δ 6.91 (1H, d, J = 1.3 Hz, H-3), 6.69 (1H, d, J = 8.1 Hz, H-6), and 6.67 (1H, d, J = 8.1 Hz, H-5)], a 4-methylpent-3-enyl unit [δ 2.31 (2H, m, H-10), 2.24 (2H, m, H-11), 5.05 (1H, t, J = 7.2 Hz, H-12), and 1.49 (3H, s, H-15), 1.59 (3H, s, H-14)], a trisubstituted double bond, and a methoxyl group. The aforementioned data suggested the presence of a 1,2,4-trisubstituted dihydroxybenzene structure connected with a 10-carbon side chain. Extensive analysis of the 1D and 2D NMR data indicated that **4** is similar to fornicin A, a prenylated phenolic isolated from *Ganoderma fornicatum* [32]. The main differences were the presence of a ketacetal carbon at δ_C 108.6 and an additional methoxyl group in **4**. In the HMBC spectrum of **4** (**Fig. 2**), two hydroxyl groups were assigned to C-1 (δ_C 149.3) and C-4 (δ_C 151.2) by the HMBC correlations of H-3/C-1, H-3/C-5, H-5/C-1, H-6/C-2, and H-6/C-4, respectively. The methoxyl group was located at C-7 (δ_C 108.6) due to the HMBC correlations between CH_3O-7, H-3, H-8, and C-7. Accordingly, the structure of **4** was proposed as shown, and the compound was named arnebinol D.

Compound **5** was obtained as reddish powder. Its HRESIMS provided the molecular formula $C_{18}H_{20}O_4$, requiring nine degrees of unsaturation. The IR spectrum exhibited absorption bands for carbonyl groups. The ^{1}H NMR spectrum of **5** (**Table 2**) showed a two-proton singlet at δ 7.28 (2H, s, H-6, 7), one singlet aromatic proton at δ 6.59 (1H, s, H-3), two methoxyl groups at δ 3.94 (6H, s, CH_3O-5, 8), and characteristic signals for a 4-methylpent-3-enyl unit at δ 2.51 (2H, t, J = 7.7 Hz, H-11), 2.24 (2H, q, J = 7.3 Hz, H-12), 5.11 (1H, t, J = 7.0 Hz, H-13), 1.57 (3H, s, H-16), and 1.66 (3H, s, H-15). The ^{1}H and ^{13}C NMR features of **5** (**Table 2**) suggested that this compound was a 1,4-naphthoquinone, especially due to the presence of the signals at δ_C 185.3 and 185.1. Comparison of the ^{1}H and ^{13}C NMR spectral data with those of naphthoquinones previously isolated from *A. euchroma* indicated that **5** was similar to the known compound 11-deoxyalkannin [33]. The significant differences between the two compounds were the presence of two methoxyl group signals in **5** instead of two hydroxyl group signals in 11-deoxyalkannin. The two methoxyl groups were placed at C-5 (δ_C 153.5) and C-8 (δ_C 153.9) by the HMBC (**Fig. 2**) correlations of H-6/C-8, H-7/C-5, CH_3O-5/C-5, and CH_3O-8/C-8, respectively. The full assignment of the proton and carbon signals of **5** was completed by HSQC and HMBC spectra. As a result, the structure of **5** was proposed as shown, and the compound was named 5,8-*O*-dimethyl-11-deoxyalkannin.

Compound **6** (arnebinone B) was isolated as red crystals. Its structure was established by extensive spectroscopic methods

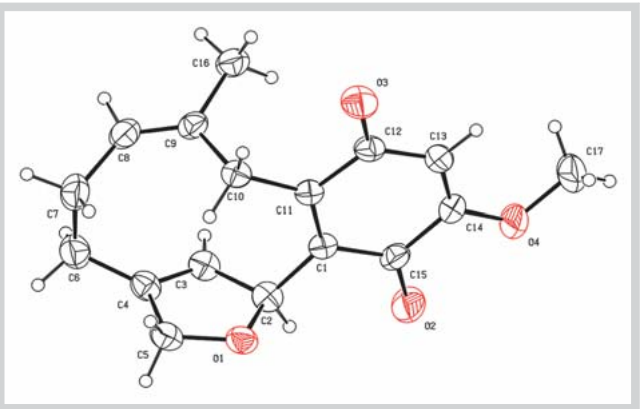

Fig. 4 X-ray structure of compound **6**. (Color figure available online only.)

Table 3 Cytotoxic activity of compounds **1**–**12**.

Compound	IC_{50} (μM)[a]	
	MG-63	SNU387
1	9.44	10.22
2	21.71	20.16
3	5.20	9.39
4	14.32	9.47
5	2.69	6.08
6	7.07	6.90
7	7.67	13.70
8	> 40	> 40
9	5.46	10.86
10	11.50	7.90
11	9.56	9.01
12	6.87	6.99
Cisplatin[b]	12.31	6.39

[a] Compounds with an IC_{50} > 40 μM were considered inactive; [b] Positive control

(UV, IR, HRESIMS, 1D and 2D NMR) and confirmed by X-ray crystal diffraction (**Fig. 4**). It possesses a 6/10/5 tricyclic ring system, featuring a benzoquinone unit linked to a ten-membered monoterpenoid moiety by two C–C bonds. The structure of **6** has been published in a patent [34], but its spectroscopic data has not been reported yet. In this paper, we report the spectroscopic data and crystallographic data of **6** for the first time.

Other known isolates were identified as clavilactone A (**7**) [23], 9,17-epoxyarnebinol (**8**) [14], shikonofuran A (**9**), shikonofuran E (**10**), shikonofuran B (**11**), and shikonofuran C (**12**) [35], based on NMR data and mass spectrometric analysis, as well as by comparison of the spectral data with those reported.

The isolated compounds **1**–**12** were tested for their cytotoxicity against MG-63 and SNU387 human cancer cell lines using the MTT assay, with cisplatin as the positive control. The results are summarized in **Table 3**. Compounds **1, 3, 5**–**7, 9, 11**, and **12** showed inhibitory effects against the MG-63 cell line with IC_{50} values ranging from 2.69 to 9.56 μM. Compounds **5, 6**, and **12** also exhibited activity against SNU387 cells, with IC_{50} values of 6.08, 6.90, and 6.99 μM, respectively. The structure-activity relationship analysis indicated that the basic structural requirement for the cytotoxic activity of these compounds was the quinone or hydroquinone moiety. Once the hydroquinone moiety was disrupted, as in **8**, it displayed no cytotoxicity. A comparison of the cytotoxic activity of the meroterpenoids (**1**–**3, 7**), which contain two C–C bonds between a benzene ring and the terpenoid moieties, established that the absence of a γ-lactone system reduced the activity significantly.

Materials and Methods

General experimental procedures

Optical rotations were measured by using a Perkin-Elmer 341 polarimeter at room temperature. UV spectra were run on a Shimadzu UV-2550 spectrophotometer. IR spectra were obtained on a Perkin-Elmer 1725X-FT spectrometer. NMR spectra were recorded on a Bruker Avance 600 spectrometer with TMS as the internal reference, and chemical shifts are expressed in δ (ppm). High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) was performed on a LTQ Orbitrap XL mass spectrometer. Analytical HPLC was carried out using LabAlliance Series III with a model 201 (SSI) detector and Ultimate C_{18} column (250 mm × 4.60 mm, 5 μm). Preparative HPLC was carried out on P3000 with a UV3000 detector (Beijing ChuangXinTongHeng Science and Technology Co., Ltd.) and Ultimate C_{18} column (250 mm × 21.2 mm, 5 μm). Sephadex LH-20 (Amersham Pharmacia Biotech), silica gel (100–200 mesh, Qingdao Marine Chemical Co.), and RP-C_{18} (150–200 mesh, Merck) were used for column chromatography (CC). TLC was carried out on precoated silica gel GF_{254} plates, and spots were visualized under UV light and by spraying with 5% H_2SO_4 in ethanol followed by heating.

Plant material

The roots of *A. euchroma* were purchased from Sichuan Neautus Traditional Chinese Medicine Co., Ltd., People's Republic of China, and were collected from the Xinjiang region, in October 2012, People's Republic of China. The plant identification was verified by Prof. W.K. Bao of the Chengdu Institute of Biology, Chinese Academy of Sciences. A voucher specimen (CIB-A-402) has been deposited at the Laboratory of Phytochemistry, Chengdu Institute of Biology, Chinese Academy of Sciences. Cisplatin (≥ 99.9%) was purchased from Sigma-Aldrich. The purity (> 96%) of compounds **1**–**12** used for biological assays was determined by HPLC.

Extraction and isolation

The dried and powdered roots of *A. euchroma* (10 kg) were extracted three times with 30 L of 95% EtOH at room temperature. The solvent was evaporated, and the residue (877 g) was suspended in water and then successively extracted with EtOAc and *n*-BuOH. The EtOAc extract (370 g) was subjected to a silica gel column (10 × 120 cm, 100–200 mesh, 2.5 kg), eluting with a gradient of petroleum ether–EtOAc (50 : 1, 20 : 1, 10 : 1, 20 : 3, 5 : 1, 4 : 1, 3 : 1, 2 : 1, 1 : 1, 0 : 1, each 15 L), providing 12 fractions. Fraction 6 (13 g) was separated on a Sephadex LH-20 column (5 × 55 cm, 160 g; solvent system: $CHCl_3$–MeOH, 1 : 1) to obtain three major fractions, 6a–6c. Fraction 6a (6.0 g) was applied to an RP-C_{18} column (4 × 45 cm, 120 g), eluting with MeOH–H_2O (65 : 35, 70 : 30, 75 : 25, 80 : 20, 100 : 0, each 600 mL) to afford fractions 6a1–6a5. Fraction 6a1 (420 mg) was purified by semipreparative HPLC using an isocratic solvent system of MeOH–H_2O (50 : 50) at 12 mL/min for 85 min to yield **8** (t_R = 74.6 min, 42 mg) and **4** (t_R = 78.4 min, 30 mg). Compound **6** (76 mg) was isolated from fraction 6a2 by crystallization from MeOH. Fraction 6a3 (280 mg) was further purified by semipreparative HPLC (MeOH–H_2O, 66 : 34) at 12 mL/min for 85 min to obtain **10** (t_R = 69.5 min,

22 mg), **11**(t_R = 79.2 min, 27 mg), and **12** (t_R = 81.0 min, 24 mg). Fraction 7 (11 g) was subjected on a Sephadex LH-20 column (5 × 55 cm, 160 g; solvent system: $CHCl_3$–MeOH, 1 : 1) to give five subfractions, 7a–7e. Subfraction 7 d (3.5 g) was further purified using semipreparative HPLC (MeOH–H_2O, 60 : 40) at 12 mL/min for 40 min to produce **9** (t_R = 27.5 min, 1.8 g). Fraction 8 (11 g) was submitted to separation over Sephadex LH-20 (5 × 55 cm, 160 g) with $CHCl_3$–MeOH (1 : 1) to afford five fractions (8a–8e). Fraction 8 d (1.5 g) was subjected to RP-C_{18} CC (3 × 30 cm, 45 g), eluting with MeOH–H_2O (40 : 60, 50 : 50, 60 : 40, 70 : 30, each 300 mL) to obtain fractions 8d1 to 8d4. Fraction 8d2 (150 mg) was further purified by preparative HPLC using a gradient solvent system of MeCN–H_2O (30 : 70 to 50 : 50) at 12 mL/min for 50 min to yield **1** (t_R = 33.2 min, 16 mg), **3** (t_R = 23.7 min, 22 mg), and **7** (t_R = 37.1 min, 26 mg). Fraction 8d3 (420 mg) was purified by semipreparative HPLC (MeOH–H_2O, 55 : 45) at 12 mL/min for 40 min to afford **2** (t_R = 30.4 min, 46 mg). Fraction 8b (4.2 g) was separated using an RP-C_{18} column (4 × 45 cm, 120 g) eluted with MeOH–H_2O (80 : 20, 90 : 10, 100 : 0, each 500 mL) to give three major fractions, 8b1–8b3. Compound **5** (t_R = 23.7 min, 28 mg) was obtained from fraction 8b1 by preparative HPLC with a gradient solvent system of MeCN–H_2O (50 : 50 to 70 : 30) at 12 mL/min for 40 min.

Isolates

Arnebinol A (**1**): amorphous powder; $[\alpha]_D^{20}$ + 34.5 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 292 (3.84) nm; IR (KBr) ν_{max} 3412, 2932, 1716, 1488, 1285 cm^{-1}; ^1H and ^{13}C NMR (CD_3OD) data, see **Table 1**; HRESIMS *m/z* 271.0969 [M – H]$^-$ (calcd. for $C_{16}H_{15}O_4$, 271.0976).

Arnebinol B (**2**): amorphous powder; $[\alpha]_D^{20}$ + 47.0 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 289 (3.96) nm; IR (KBr) ν_{max} 3302, 2928, 1612, 1486, 1291 cm^{-1}; ^1H and ^{13}C NMR (CD_3OD) data, see **Table 1**; HRESIMS *m/z* 257.1178 [M – H]$^-$ (calcd. for $C_{16}H_{17}O_3$, 257.1183).

Arnebinol C (**3**): amorphous powder; $[\alpha]_D^{20}$ + 106.5 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 288 (3.73) nm; IR (KBr) ν_{max} 3391, 1738, 1490, 1273, 1160 cm^{-1}; ^1H and ^{13}C NMR (CD_3OD) data, see **Table 2**; HRESIMS *m/z* 271.0970 [M – H]$^-$ (calcd. for $C_{16}H_{15}O_4$, 271.0976).

Arnebinol D (**4**): yellowish oil; $[\alpha]_D^{20}$ 0 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 293 (3.93) nm; IR (KBr) ν_{max} 3419, 2928, 1750, 1452, 1195 cm^{-1}; ^1H and ^{13}C NMR (CD_3OD) data, see **Table 2**; HRESIMS *m/z* 303.1231 [M – H]$^-$ (calcd. for $C_{17}H_{19}O_5$, 303.1238).

5,8-O-dimethyl-11-deoxyalkannin (**5**): reddish powder; UV (MeOH) λ_{max} (log ε) 440 (3.50), 240 (4.06) nm; IR (KBr) ν_{max} 2928, 1652, 1260, 1216, 1057 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$) data, see **Table 2**; HRESIMS *m/z* 301.1441 [M + H]$^+$ (calcd. for $C_{18}H_{21}O_4$, 301.1434).

Arnebinone B (**6**): red crystals (methanol); $[\alpha]_D^{20}$ + 159.1 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 258 (4.24) nm; IR (KBr) ν_{max} 2937, 2855, 1669, 1637, 1596, 1452, 1212, 1055 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$) data, see **Table 1**; HRESIMS *m/z* 285.1125 [M – H]$^-$ (calcd. for $C_{17}H_{17}O_4$, 285.1132).

X-ray crystallographic analysis of arnebinone B (**6**): Crystal data were collected from a red block (0.30 × 0.20 × 0.20 mm^3) at 293 (2) K: $C_{17}H_{18}O_4$, MW = 286.31, orthorhombic, space group $P2_12_12_1$, unit cell dimensions a = 8.1753(4) Å, b = 9.7373(5) Å, c = 18.6797(8) Å, V = 1487.01(13) $Å^3$; α = 90.00°, β = 90.00°, γ = 90.00°, *Z* = 4, ρ_{calc} = 1.279 mg/mm^3, *F*(000) = 608. Data collection was performed on a SuperNova Eos diffractometer with graphite monochromator, Mo Kα radiation. A total of 4634 reflections measured, 2704 unique (R_{int} = 0.0231), were used in all calculations. The structure was solved with the SHELXS (G.M. Sheldrick, Acta Cryst, 2008) and refined by a full-matrix least squares method on F^2 by means of SHELXL (G.M. Sheldrick, Acta Cryst, 2008). The final R indices [$I > 2\sigma(I)$], R_1 = 0.0481, wR_2 = 0.1009; R indices [all data], R_1 = 0.0662, wR_2 = 0.1118. Crystallographic data for the structure of **6** have been deposited with the Cambridge Crystallographic Data Centre (deposition number CCDC 1 001 388). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [Fax: + 44-(0)1223–336 033 or e-mail: deposit@ccdc.cam.ac.uk].

Cytotoxicity assay

The cytotoxicity of all isolated compounds against MG-63 (osteosarcoma) and SNU387 (hepatoma) cell lines (from the Shanghai Cell Bank, Chinese Academy of Sciences) were evaluated by means of the MTT assay [36]. Briefly, test cells were placed into 96-well plates at a density of 5000 cells per well and incubated at 37 °C for 24 h in 5% CO_2. The test compounds were added into triplicate wells at different concentrations and incubated for 48 h at 37 °C. After the incubation period, MTT solution (100 µL, 1 mg/mL) was added into each well. The plates were incubated for an additional 4 h, and then 100 µL of "triplex solution" (10% SDS/5% iBuOH/12 mM HCl) was added. The plates were incubated at 37 °C overnight and then measured using a plate reader at 490 nm. Cell viability (%) was measured, and the cell growth curve was plotted. IC_{50} values were calculated by the Reed and Muench method [37].

Supporting information

The 1D and 2D NMR spectra for compounds **1**–**6** are available as Supporting Information.

Acknowledgements

We are very grateful to the National Eleventh-Five Year Research Program of China (2011BAC09B04) and the Key Research Program of the Chinese Academy of Sciences, Grant No. KSZD-EW-Z-004 for financial support.

Conflict of Interest

The authors declare no competing financial interest.

Affiliations

[1] Key Laboratory of Mountain Ecological Restoration and Bioresource Utilization & Ecological Restoration Biodiversity Conservation Key Laboratory of Sichuan Province, Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, People's Republic of China
[2] University of Chinese Academy of Sciences, Beijing, People's Republic of China
[3] Beijing Entry-Exit Inspection and Quarantine Bureau, Beijing, People's Republic of China
[4] School of Life Science and Engineering, Southwest Jiaotong University, Chengdu, People's Republic of China

References

1 *Bhatia A, Arora S, Singh B, Kaur G, Nagpal A.* Anticancer potential of Himalayan plants. Phytochem Rev 2011; 10: 309–323
2 *State Pharmacopoeia Committee.* Chinese Pharmacopoeia, 2010 edition. Beijing: China Medical Pharmaceutical Science and Technology Publishing House; 2010: 320

3 *Huang ZS, Zhang M, Ma L, Gu LQ.* Chemical and pharmacological research progress of Chinese drug "Zicao". Nat Prod Res Dev 1999; 12: 73–82
4 *Papageorgiou VP, Assimopoulou AN, Couladouros EA, Hepworth D, Nicolaou KC.* The chemistry and biology of alkannin, shikonin, and related naphthazarin natural products. Angew Chem Int Ed 1999; 38: 270–300
5 *Shen CC, Syu WJ, Li SY, Lin CH, Lee GH, Sun CM.* Antimicrobial activities of naphthazarins from *Arnebia euchroma*. J Nat Prod 2002; 65: 1857–1862
6 *Damianakos H, Kretschmer N, Syklowska-Baranek, K, Pietrosiuk A, Bauer R, Chinou I.* Antimicrobial and cytotoxic isohexenylnaphthazarins from *Arnebia euchroma* (Royle) Jonst. (Boraginaceae) callus and cell suspension culture. Molecules 2012; 17: 14310–14322
7 *Kashiwada Y, Nishizawa M, Yamagishi T, Tanaka T, Nonaka G, Cosentino LM, Snider JV, Lee KH.* Anti-AIDS agents, 18. Sodium and potassium salts of caffeic acid tetramers from *Arnebia euchroma* as anti-HIV agents. J Nat Prod 1995; 58: 392–400
8 *Kashiwada Y, Bastow KF, Lee KH.* Novel lignan derivatives as selective inhibitors of DNA topoisomerase II. Bioorg Med Chem Lett 1995; 5: 905–908
9 *Roeder E, Rengel-Mayer B.* Pyrrolizidine alkaloids from *Lithospermum erythrorhizon*. Phytochemistry 1990; 29: 690–693
10 *Roeder E, Rengel-Mayer B.* Pyrrolizidine alkaloids from *Arnebia euchroma.* Planta Med 1993; 59: 192
11 *Yao XS, Ebizuka Y, Noguchi H, Kiuchi F, Sankawa U, Seto H.* Structure of arnebinone, a novel monoterpenylbenzoquinone with inhibitory effect to prostaglandin biosynthesis. Tetrahedron Lett 1983; 24: 3247–3250
12 *Yao XS, Ebizuka Y, Noguchi H, Kiuchi F, Iitaka Y, Sankawa U, Seto H.* Structure of arnebinol, a new ansa-type monoterpenylbenzenoid with inhibitory effect to prostaglandin biosynthesis. Tetrahedron Lett 1983; 24: 2407–2410
13 *Yao XS, Ebizuka Y, Noguchi H, Kiuchi F, Seto H, Sankawa U.* Structure of arnebifuranone, new monoterpenylbenzoquine from *Arnebia euchroma.* Tetrahedron Lett 1984; 25: 5541–5542
14 *Li HM, Tang YL, Zhang ZH, Liu CJ, Li HZ, Li RT, Xia XS.* Compounds from *Arnebia euchroma* and their related anti-HCV and antibacterial activities. Planta Med 2012; 78: 39–45
15 *Justicia J, Oltra JE, Cuerva JM.* General approach to polycyclic meroterpenoids based on stille couplings and titanocene catalysis. J Org Chem 2004; 69: 5803–5806
16 *Geris R, Simpson TJ.* Meroterpenoids produced by fungi. Nat Prod Rep 2009; 26: 1063–1094
17 *Yin X, Feng T, Li ZH, Dong ZJ, Li Y, Liu JK.* Highly oxygenated meroterpenoids from fruiting bodies of the mushroom *Tricholoma terreum*. J Nat Prod 2013; 76: 1365–1368
18 *Reyes CDL, Zbakh H, Motilva V, Zubia E.* Antioxidant and anti-inflammatory meroterpenoids from the brown alga *Cystoseira usneoides*. J Nat Prod 2013; 76: 621–629
19 *Fadli M, Aracil JM, Jeanty G, Banaigs B, Francisco C.* Novel meroterpenoids from *Cystoseira mediterranea*: use of the crown-gall bioassay as a primary screen for lipophilic antineoplastic agents. J Nat Prod 1991; 54: 261–264
20 *Fenical W, Sims JJ, Squatrit D, Wing RM, Radlick P.* Zonarol and isozonarol, fungitoxic hydroquinones from the brown seaweed *Dictyopteris aonarioides*. J Org Chem 1973; 38: 2383–2386
21 *Kaysser L, Bernhardt P, Nam SJ, Loesgen S, Ruby JG, Skewes-Cox P, Jensen PR, Fenical W, Moore BS.* Merochlorins A–D, cyclic meroterpenoid antibiotics biosynthesized in divergent pathways with vanadium-dependent chloroperoxidases. J Am Chem Soc 2012; 134: 11988–11991
22 *Sontag B, Arnold N, Steglich W, Anke T.* Montadial A, a cytotoxic metabolite from *Bondarzewia montana*. J Nat Prod 1999; 62: 1425–1426
23 *Arnone A, Cardillo R, Meille SV, Nasini G, Tolazzi M.* Secondary mould metabolites. 47. Isolation and structure elucidation of clavilactones A–C, new metabolites from the fungus *Clitocybe clavipes*. J Chem Soc Perkin Trans I 1994; 2165–2168
24 *Simon-Levert A, Arrault A, Bontemps-Subielos N, Canal C, Banaigs B.* Meroterpenes from the ascidian *Aplidium aff. densum*. J Nat Prod 2005; 68: 1412–1415
25 *Cassinelli G, Lanzi C, Pensa T, Gambetta RA, Nasini G, Cuccuru G, Cassinis M, Pratesi G, Polizzi D, Tortoreto M, Zunino F.* Clavilactones, a novel class of tyrosine kinase inhibitors of fungal origin. Biochem Pharmacol 2000; 59: 1539–1547
26 *Larrosa I, Da Silva MI, Gómez PM, Hannen P, Ko E, Lenger SR, Linke SR, White AJP, Wilton D, Barrett AGM.* Highly convergent three component benzyne coupling: the total synthesis of ent-clavilactone B. J Am Chem Soc 2006; 128: 14042–14043
27 *Takao K, Nanamiya R, Fukushima Y, Namba A, Yoshida K, Tadano K.* Total synthesis of (+)-clavilactone A and (−)-clavilactone B by ring-opening/ring-closing metathesis. Org Lett 2013; 15: 5582–5585
28 *Lv L, Shen B, Li Z.* Total synthesis of (±)-clavilactones A, B, and proposed D through iron-catalyzed carbonylation–peroxidation of olefin. Angew Chem Int Ed Engl 2014; 53: 4164–4167
29 *Moir M, Thomson RH.* Naturally occurring quinones. Part XXIII. Cordiachromes from *Patagonula americana* L. J Chem Soc Perkin I 1973; 1556–1561
30 *Moir M, Thomson RH.* Naturally occurring quinones. Part XXII. Terpenoid quinones in *Cordia* spp. J Chem Soc Perkin I 1973; 1352–1357
31 *Manners GD, Jurd L.* The hydroquinone terpenoids of *Cordia alliodora*. J Chem Soc Perkin I 1977; 405–410
32 *Niu XM, Li SH, Sun HD, Che CT.* Prenylated phenolics from *Ganoderma fornicatum*. J Nat Prod 2006; 69: 1364–1365
33 *Sevimli-Gur C, Akgun IH, Deliloglu-Gurhan I, Korkmaz KS, Bedir E.* Cytotoxic naphthoquinones from *Alkanna cappadocica*. J Nat Prod 2010; 73: 860–864
34 *Chen DF, Zhu HW, Li GX.* Benzoquinone compound and its application in preparing antitumor drug. CN Patent 103113335; 2013
35 *Yoshizaki F, Hisamichi S, Kondo Y, Sato Y, Nozoe S.* Studies on shikon. III. New furylhydroquinone derivatives, shikonofurans A, B, C, D and E, from *Lithospermum erythrorhizon* Sieb. Et Zucc. Chem Pharm Bull 1982; 30: 4407–4411
36 *Mosmann T.* Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65: 55–63
37 *Reed LJ, Muench H.* A simple method of estimating fifty percent endpoints. Am J Hyg 1938; 27: 493–497