Fitoterapia 116 (2017) 93–98

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Racemic alkaloids from the fungus *Ganoderma cochlear*

Xin-Long Wang [a,b,1], Man Dou [b,c,1], Qi Luo [b], Li-Zhi Cheng [b,d], Yong-Ming Yan [b], Rong-Tao Li [c], Yong-Xian Cheng [a,b,*]

[a] Henan University of Traditional Chinese Medicine, Zhengzhou, 450008, PR China
[b] State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, 650201, PR China
[c] Faculty of Life Science and Engineering, Kunming University of Science and Technology, Kunming, 650500, PR China
[d] Guangdong Pharmaceutical University, Guangzhou, 510006, PR China

ARTICLE INFO

Article history:
Received 6 September 2016
Received in revised form 11 November 2016
Accepted 19 November 2016
Available online 25 November 2016

Keywords:
Ganoderma cochlear
Alkaloids
ECD calculations
Racemates
Biosynthesis

ABSTRACT

Seven pairs of new alkaloid enantiomers, ganocochlearines C—I (1, 3–8), and three pairs of known alkaloids were isolated from the fruiting bodies of *Ganoderma cochlear*. The chemical structures of new compounds were elucidated on the basis of 1D and 2D NMR data. The absolute configurations of compounds 1, 3–10 were assigned by ECD calculations. Biological activities of these isolates against renal fibrosis were accessed in rat normal or diseased renal interstitial fibroblast cells. Importantly, the plausible biosynthetic pathway for this class of alkaloids was originally proposed.

1. Introduction

The genus *Ganoderma* embraces several medicinal mushrooms. Of them, *G. lucidum* and *G. sinense* are recorded in the Pharmacopoeia of PR China (2015 version), highlighting their significance in traditional Chinese medicine. Due to their medicinal values, modern investigations on *Ganoderma* species have been extensively conducted, which reveals the presence of triterpenoids, polysaccharides as major components. In addition, peptides, proteins and alkaloids were also reported from this genus [1–3]. Despite that mounting studies on *Ganoderma* were documented, new chemical findings are still expectable. For example, several structurally novel meroterpenoids have been characterized from *Ganoderma* in the recent years [4–7]. In contrast to triterpenoids and emerging meroterpenoids, alkaloids present in this genus are largely unknown. In 1990, ganoines I and II were isolated from *G. capense*, to our knowledge, representing the first alkaloid example in *Ganoderma* [8]. Thereafter, studies referring to *Ganoderma* alkaloids are relatively few. During our search for meroterpenoids from *Ganoderma* fungi, two alkaloids namely ganocochlearines A and B were isolated from *G. cochlear* [9]. As a follow-up investigation on this species, seven pairs of new alkaloid enantiomers, ganocochlearines C–I (**1**, **3**–**8**), together with three pairs of known alkaloids ganoapplanatumine B (**2**), sinensine E (**9**), and lucidimine C (**10**), were obtained. In this contribution, we describe their isolation, structure elucidation and biological evaluation.

2. Experimental

2.1. General

Column chromatography was performed by using silica gel (200–300 mesh; Qingdao Marine Chemical Inc., PR China), C-18 silica gel (40–60 μm; Daiso Co., Japan), MCI gel CHP 20P (75–150 μm, Mitsubishi Chemical Industries, Tokyo, Japan) and Sephadex LH-20 (Amersham Pharmacia, Sweden). Semi-preparative or analytic HPLC was carried out using an Agilent 1200 liquid chromatograph, the column used was a 250 mm × 9.4 mm, i.d., 5 μm, Zorbax SB-C18 or a 250 mm × 4.6 mm, i.d., 5 μm, Daicel Chiralpak (IC for **1**–**4**, **7**–**10** and AD-H for **5**–**6**). Optical rotations were recorded on a Horiba SEPA-300 polarimeter. UV spectra were recorded on a Shimadzu UV-2401PC spectrometer. CD spectra were measured on a Chirascan instrument. NMR spectra were recorded on a Bruker AV-600, with TMS as an internal standard. ESIMS and HRESIMS were collected by an Agilent G6230 TOF MS spectrometer.

2.2. Fungal material

The fruiting bodies of *G. cochlear* were purchased from Tongkang Pharmaceutical Co. Ltd. in Guangzhou Province, PR China, in September 2013. The material was identified by Prof. Zhu-Liang Yang at Kunming

* Corresponding author at: Henan University of Traditional Chinese Medicine, Zhengzhou, 450008, PR China.
E-mail address: yxcheng@mail.kib.ac.cn (Y.-X. Cheng).
[1] There authors contributed equally to this paper.

http://dx.doi.org/10.1016/j.fitote.2016.11.011
0367-326X/

Table 1
^{1}H (600 MHz) and ^{13}C NMR (150 MHz) data of compounds **1**, **3**–**8** (δ in ppm, J in Hz).

Position	**1**[a]		**3**[b]		**4**[c]		**5**[d]		**6**[c]		**7**[c]		**8**[e]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2		147.4		147.0		149.1		152.2		156.0		143.8	8.39, s	149.6
3		123.6		129.9		125.2		115.3	7.68, s	119.8	5.58, t, 7.8	78.9		134.5
4	2.62, m	28.9	3.21, m	30.3	3.02, m	28.6	3.57, m	32.1	3.07, overlap	27.4	2.73, m	34.1		147.3
	2.46, m		2.98, m		2.83, m		3.30, m		2.90, m		2.14, m			
5	2.29, m	32.8	2.81, m	34.7	2.43, m	33.5	2.57, m	35.9	2.05, m	31.1	2.97, m	28.7	5.58, t-like, 7.8	80.5
	1.55, m		2.30, m		2.16, m		2.04, overlap		1.86, m		2.88, m			
6	3.64, m	38.9	5.62, t-like, 7.8	79.3	5.04, dd, 6.5, 3.7	82.5	5.43, t-like, 6.4	74.1	4.15, m	66.6		129.4	2.82, m	35.6
													2.26, m	
7	8.56, s	145.1	8.54, s	148.9	8.48, s	146.1	8.96, s	152.5	3.07, overlap	35.5	8.28, s	150.3	3.17, m	29.9
									2.74, m				3.03. m	
8		149.2		148.6		151.3		158.0	8.24, s	147.8		132.2		135.1
9		142.8		133.4		140.4		144.5		149.0		148.3		150.8
10	5.22, dd, 13.8, 4.2	66.2	5.47, s	101.8	5.15, s	66.3		160.9		130.9	2.26, s	15.4	4.68, s	60.9
1′		125.5		123.5		124.7		121.3		120.6		123.6		124.3
2′		150.6		151.4		151.0		147.1		150.8		149.0		151.6
3′	7.16, d, 8.6	118.7	6.91, d, 8.7	118.9	6.81, d, 8.9	118.8	7.24, d, 8.8	118.7	6.73, overlap	119.4	6.83, d, 8.7	117.6	6.86, d, 8.5	119.7
4′	7.22 overlap	119.2	6.84, dd, 8.7, 3.0	118.8	6.77, dd, 8.9, 2.9	119.4	7.12, dd, 8.8, 3.0	120.5	6.73, overlap	119.4	6.71, dd, 8.7, 3.0	118.3	6.78, dd, 8.5, 2.9	119.5
5′		154.5		152.8		153.6		155.0		153.4		152.4		153.8
6′	8.45, d, 2.6	112.0	7.60, d, 3.0	110.1	7.52, d, 2.9	111.4	8.03, brs	110.3	7.24, brs	112.9	7.37, d, 3.0	109.0	7.44, d, 2.9	110.6
6-OCH_3			3.34, s	52.8										
6-$O\underline{CH_2}CH_3$					3.67, t-like, 7.0	65.5								
6-$O\overline{CH_2}\underline{CH_3}$					1.23, t, 7.0	15.7								
6-$\underline{CH_2}\overline{C}O\overline{CH_3}$	2.87, m	49.2												
	2.53, m													
6-$CH_2\overline{C}OCH_3$		207.3												
6-$CH_2\overline{CO}\underline{CH_3}$	2.09, s	30.4												
10-$\underline{OCH_3}$				3.37, s	53.3									

[a] ^{1}H and ^{13}C NMR in pyridine-d_6. [b] ^{1}H and ^{13}C NMR in $CDCl_3$. [c] ^{1}H and ^{13}C NMR in methanol-d_4. [d] ^{1}H and ^{13}C NMR in acetone-d_6. [e] ^{1}H and ^{13}C NMR in DMSO-d_6.

Institute of Botany, Chinese Academy of Sciences, and a voucher specimen (CHYX0570) was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

2.3. Extraction and isolation

The powders of fruiting bodies of *G. cochlear* (100 kg) were extracted by reflux with 70% EtOH (300 L. × 48 h × 6) at room temperature and concentrated under reduced pressure to give a crude extract (10 kg), which was suspended in water followed by partition with ethyl acetate to afford an ethyl acetate soluble extract (2 kg). This extract was divided into seven parts (Fr.1–Fr.7) by using a silica gel column eluted with increasing MeOH in $CHCl_3$ (100:1–1:1). Fr.5 (440 g) was further separated by a MCI gel CHP 20P column washed with aqueous MeOH (30%–100%) to yield 7 portions (Fr.5.1–Fr.5.7). Fr.5.3 (34 g) was submitted to gel filtration on Sephadex LH-20 (MeOH) column followed by RP-18 column chromatography eluted with gradient aqueous MeOH (30%–60%) to yield 8 fractions (Fr.5.3.1–Fr.5.3.8). Of which, Fr.5.3.3 (970 mg) was separated by Sephadex LH-20 (MeOH) followed by preparative TLC ($CHCl_3$/MeOH, 12:1) to give Fr.5.3.3.1 and compound **5** (2.6 mg). Fr. 5.3.3.1 (16 mg) was purified by semi-preparative HPLC (MeOH/H_2O, 60%–90%) to afford compounds **2** (2.3 mg, t_R = 16.2 min) and **3** (1.3 mg, t_R = 21.8 min). Fr.5.3.4 (560 mg) was submitted to preparative TLC ($CHCl_3$/MeOH, 12:1) followed by semi-preparative HPLC (MeOH/H_2O, 65%) to produce compounds **1** (3.4 mg, t_R = 8.2 min) and **4** (2.1 mg, t_R = 12.4 min). Fr.5.4 (32 g) was fractionated by a MCI gel CHP 20P column eluted with gradient aqueous MeOH (40%–100%) to provide 6 portions (Fr.5.4.1–Fr.5.4.6). Fr.5.4.3 (1.2 g) was separated by a RP-18 column eluted with gradient aqueous MeOH (30%–60%) followed by preparative TLC ($CHCl_3$/Me_2CO, 6:1) to afford compound **6** (5.1 mg). Fr.5.4.4 (5.2 g) was fractionated by a combination of Sephadex LH-20 (MeOH), RP-18 (MeOH/H_2O, 40%–60%), and preparative TLC ($CHCl_3$/Me_2CO, 6:1) to yield compounds **8** (16.3 mg) and **9** (5.9 mg). Fr.5.4.5 (12 g) was passed through a MCI gel CHP 20P column eluted with gradient aqueous MeOH (40%–80%) followed by a combination of Sephadex LH-20 filtration (MeOH), RP-18 (50%–65%) and semi-preparative HPLC (MeOH/H_2O, 55%) to yield compounds **7** (1.9 mg, t_R = 15.7 min) and **10** (4.9 mg, t_R = 19.1 min).

Racemic or partially racemic compounds were submitted to chiral phase on Daicel Chiralpak IC (**1**–**4**, **7**–**10**) or AD-H (**5**.**6**) to afforded their enantiomers (Table 2).

Table 2
Chiral separation of racemic mixtures of **1**–**10**.

Compd		Chiral phase	Mobile phase	Flow rate (mL/min)	Retention time (min)	Amount (mg)	Peak area ratio
1	**1a**	IC	*n*-hexane/ethanol,	1	15.0	0.83	0.65:1
	1b		94:6		20.4	1.16	
2	**2a**	IC	*n*-hexane/ethanol,	1	15.0	0.80	1.08:1
	2b		80:20		21.7	0.60	
3	**3a**	IC	*n*-hexane/ethanol,	1	7.8	0.34	0.97:1
	3b		85:15		8.5	0.33	
4	**4a**	IC	*n*-hexane/ethanol,	1	13.9	0.62	1.38:1
	4b		90:10		15.9	0.55	
5	**5a**	AD-H	*n*-hexane/ethanol,	1	15.7	0.60	1.77:1
	5b		70:30		17.2	0.55	
6	**6a**	AD-H	*n*-hexane/ethanol,	1	18.0	2.33	1.08:1
	6b		70:30		29.0	2.15	
7	**7a**	IC	*n*-hexane/ethanol,	1	7.6	0.38	1.33:1
	7b		75:25		9.4	0.32	
8	**8a**	IC	*n*-hexane/ethanol,	1	12.3	1.86	1.88:1
	8b		75:25		15.8	1.78	
9	**9a**	IC	*n*-hexane/ethanol,	1	22.9	2.02	0.94:1
	9b		75:25		31.9	1.86	
10	**10a**	IC	*n*-hexane/ethanol,	1	10.9	1.04	0.93:1
	10b		85:15		14.8	1.26	

Ganocochlearine C (**1**): white solid; {$[\alpha]^{19}{}_D$ unstable; CD (MeOH) $\Delta\varepsilon_{203}$ + 1.25, $\Delta\varepsilon_{235}$–1.31, $\Delta\varepsilon_{257}$ + 0.70, $\Delta\varepsilon_{286}$–0.94, $\Delta\varepsilon_{340}$ + 0.33; **1a**}; {$[\alpha]^{19}{}_D$ unstable; CD (MeOH) $\Delta\varepsilon_{198}$–2.29, $\Delta\varepsilon_{237}$ + 0.82, $\Delta\varepsilon_{254}$–0.58, $\Delta\varepsilon_{287}$ + 0.75, $\Delta\varepsilon_{347}$–0.37; **1b**}; UV (MeOH) λ_{max} (log ε): 342 (3.52), 276 (3.63), 235 (3.81), 214 (4.09), 203 (4.11); ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS (positive) *m/z* 296.1282 $[M + H]^+$ (calcd for $C_{18}H_{18}NO_3$, 296.1281).

Ganocochlearine D (**3**): white solid; {$[\alpha]^{20}{}_D$ = +58.6 (*c* 0.70, MeOH); CD (MeOH) $\Delta\varepsilon_{219}$ + 16.48, $\Delta\varepsilon_{262}$–0.58, $\Delta\varepsilon_{295}$ + 6.06, $\Delta\varepsilon_{355}$–2.17; **3a**}; {$[\alpha]^{19}{}_D$ = −194.8 (*c* 0.55, MeOH); CD (MeOH) $\Delta\varepsilon_{218}$–19.31, $\Delta\varepsilon_{267}$ + 0.66, $\Delta\varepsilon_{294}$–6.54, $\Delta\varepsilon_{352}$ + 2.30; **3b**}; UV (MeOH) λ_{max} (log ε): 411 (1.91), 349 (3.66), 286 (3.74), 236 (3.94), 203 (4.19); ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS (positive) *m/z* 300.1234 $[M + H]^+$ (calcd for $C_{17}H_{18}NO_4$, 300.1230).

Ganocochlearine E (**4**): white solid; {$[\alpha]^{20}{}_D$ = +45.1 (*c* 1.24, MeOH); CD (MeOH) $\Delta\varepsilon_{202}$ + 5.76, $\Delta\varepsilon_{232}$–3.49, $\Delta\varepsilon_{259}$ + 1.75, $\Delta\varepsilon_{292}$–2.14, $\Delta\varepsilon_{346}$ + 0.98; **4a**}; {$[\alpha]^{20}{}_D$ = −16.0 (*c* 2.75, MeOH); CD (MeOH) $\Delta\varepsilon_{198}$–8.91, $\Delta\varepsilon_{232}$ + 2.34, $\Delta\varepsilon_{258}$–1.66, $\Delta\varepsilon_{294}$ + 1.63, $\Delta\varepsilon_{346}$–0.77; **4b**}; UV (MeOH) λ_{max} (log ε): 343 (3.87), 275 (3.99), 236 (4.15), 204 (4.42); ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS (positive) *m/z* 284.1283 $[M + H]^+$ (calcd for $C_{17}H_{18}NO_3$, 284.1281).

Ganocochlearine F (**5**): white solid; {$[\alpha]^{20}{}_D$ unstable; CD (MeOH) $\Delta\varepsilon_{203}$ + 18.45, $\Delta\varepsilon_{241}$–1.66, $\Delta\varepsilon_{254}$ + 1.30, $\Delta\varepsilon_{278}$–7.12; **5a**}; {$[\alpha]^{20}{}_D$ unstable; CD (MeOH) $\Delta\varepsilon_{205}$–5.52, $\Delta\varepsilon_{238}$ + 0.53, $\Delta\varepsilon_{256}$–0.25, $\Delta\varepsilon_{276}$ + 2.32; **5b**}; UV (MeOH) λ_{max} (log ε): 325 (3.77), 270 (4.13), 225 (4.69), 207 (4.64); ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS (positive) *m/z* 270.0761 $[M + H]^+$ (calcd for $C_{15}H_{12}NO_4$, 270.0761).

Ganocochlearine G (**6**): white solid; {$[\alpha]^{16}{}_D$ = +33.9 (*c* 1.45, MeOH); CD (MeOH) $\Delta\varepsilon_{222}$ + 1.15, $\Delta\varepsilon_{240}$ + 0.19, $\Delta\varepsilon_{266}$ + 1.04; **6a**}; {$[\alpha]^{16}{}_D$ = −88.1 (*c* 1.25, MeOH); CD (MeOH) $\Delta\varepsilon_{218}$–2.18, $\Delta\varepsilon_{235}$–0.42, $\Delta\varepsilon_{266}$–1.95; **6b**}; UV (MeOH) λ_{max} (log ε): 343 (3.89), 264 (4.19), 213 (4.46), 202 (4.45); ^{1}H and ^{13}C NMR data, see Table 2; HRESIMS (positive) *m/z* 258.1126 $[M + H]^+$ (calcd for $C_{15}H_{16}NO_3$, 258.1125).

Ganocochlearine H (**7**): white solid; {$[\alpha]^{20}{}_D$ = +181.9 (*c* 1.40, MeOH); CD (MeOH) $\Delta\varepsilon_{219}$ + 24.20, $\Delta\varepsilon_{263}$–1.22, $\Delta\varepsilon_{294}$ + 9.53, $\Delta\varepsilon_{341}$–3.03; **7a**}; {$[\alpha]^{19}{}_D$ = −212.3 (*c* 1.10, MeOH); CD (MeOH) $\Delta\varepsilon_{219}$–19.25, $\Delta\varepsilon_{263}$ + 1.02, $\Delta\varepsilon_{295}$–7.59, $\Delta\varepsilon_{342}$ + 2.41; **7b**}; UV (MeOH) λ_{max} (log ε): 344 (3.84), 277 (3.85), 229 (4.58), 213 (4.62); ^{1}H and ^{13}C NMR data, see Table 2; HRESIMS (positive) *m/z* 240.1022 $[M + H]^+$ (calcd for $C_{15}H_{14}NO_2$, 240.1019).

Ganocochlearine I (**8**): white solid; {$[\alpha]^{20}{}_D$ = +100.9 (*c* 1.86, MeOH); CD (MeOH) $\Delta\varepsilon_{220}$ + 15.21, $\Delta\varepsilon_{263}$–0.95, $\Delta\varepsilon_{295}$ + 6.01, $\Delta\varepsilon_{349}$–2.18; **8a**}; {$[\alpha]^{19}{}_D$ = −172.3 (*c* 1.78, MeOH); CD (MeOH) $\Delta\varepsilon_{219}$–19.43, $\Delta\varepsilon_{265}$ + 0.71, $\Delta\varepsilon_{295}$–7.61, $\Delta\varepsilon_{352}$ + 2.15; **8b**}; UV (MeOH) λ_{max} (log ε): 347 (3.73), 277 (3.77), 233 (4.14), 204 (4.34); ^{1}H and ^{13}C NMR data, see Table 2; HRESIMS (positive) *m/z* 256.0974 $[M + H]^+$ (calcd for $C_{15}H_{14}NO_3$, 256.0968).

Sinensine E (**9**): white solid; {$[\alpha]^{19}{}_D$ = +1.3 (*c* 1.84, MeOH); CD (MeOH) $\Delta\varepsilon_{200}$–4.62, $\Delta\varepsilon_{233}$ + 2.13, $\Delta\varepsilon_{260}$–1.66, $\Delta\varepsilon_{286}$ + 1.80, $\Delta\varepsilon_{345}$–0.72; **9a**}; {$[\alpha]^{18}{}_D$ = −8.2 (*c* 1.82, MeOH); CD (MeOH) $\Delta\varepsilon_{197}$ + 6.47, $\Delta\varepsilon_{226}$–3.10, $\Delta\varepsilon_{258}$ + 1.38, $\Delta\varepsilon_{289}$–2.12, $\Delta\varepsilon_{345}$ + 0.35; **9b**}; UV (MeOH) λ_{max} (log ε): 343 (3.85), 274 (3.98), 233 (4.18), 203 (4.44); HRESIMS (positive) *m/z* 256.0972 $[M + H]^+$ (calcd for $C_{15}H_{14}NO_3$, 256.0968)

Lucidimine C (**10**): white solid; {$[\alpha]^{20}{}_D$ unstable; CD (MeOH) $\Delta\varepsilon_{217}$ + 19.24, $\Delta\varepsilon_{254}$–5.24, $\Delta\varepsilon_{290}$ + 5.45, $\Delta\varepsilon_{328}$–3.26; **10a**}; {$[\alpha]^{20}{}_D$ unstable; CD (MeOH) $\Delta\varepsilon_{218}$–13.39, $\Delta\varepsilon_{251}$ + 2.52, $\Delta\varepsilon_{292}$–4.89, $\Delta\varepsilon_{332}$ + 0.71; **10b**}; UV (MeOH) λ_{max} (log ε): 331 (3.90), 268 (4.16), 213 (4.53); HRESIMS (positive) *m/z* 270.1129 $[M + H]^+$ (calcd for $C_{16}H_{16}NO_3$, 270.1130)

2.4. Cell viability assay

Rat normal renal interstitial fibroblast cells (NRK-49F) were purchased from ATCC (American Type Culture Collection) and routinely grown and maintained in DMEM with 10% FBS and with 100 units/mL penicillin and 100 μg/mL streptomycin (all obtained from Gibco BRL,

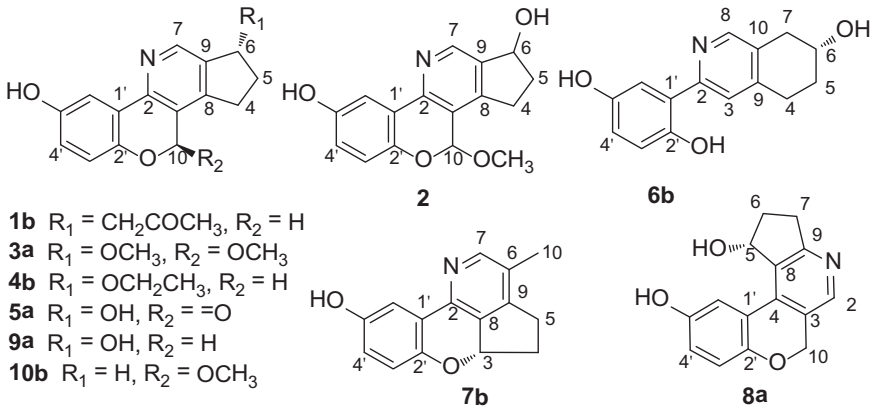

Fig. 1. Chemical structures of compounds **1**–**10**.

Grand Island, NY). Cells were incubated in an incubator with 5% CO_2 in air at 37 °C. Cell viability was determined by the MTT assay. Cells in log phase were seeded at a density of 5000 cells/well of 96-well flat-bottomed microtiter plates and treated with compounds (20 μM) or solvent control. After 24 h of incubation, 20 μL of 5 mg/mL MTT were introduced to each well and incubated for 4 h of exposure. The plates were centrifuged and medium was decanted. Cells were subsequently dissolved in 150 μL DMSO with gentle shaking for 15 min at room temperature followed by measurement of absorbance at 490 nm.

2.5. TGF-β1-stimulated proliferation inhibitory assay

NRK-49F cells in log phase were growing by 5000 cells/well of 96-well flat-bottomed microtiter plates. 24 h before compounds (20 μM) treatment, cells were treated with TGF-β1 (5 ng/mL). After 24 h of incubation, 20 μL of 5 mg/mL MTT were added to each well and incubated for 4 h of exposure. The plates were centrifuged and medium was decanted. Cells were subsequently dissolved in 150 μL DMSO with gentle shaking for 15 min at room temperature followed by measurement of absorbance at 490 nm.

3. Results and discussion

Compound **1** has a molecular formula $C_{18}H_{17}NO_3$, as deduced by analysis of its positive HRESIMS at *m/z* 296.1282 $[M + H]^+$ (calcd for 296.1281, $C_{18}H_{18}NO_3$), ^{13}C NMR and DEPT spectra, having eleven degrees of unsaturation. The ^{13}C NMR and DEPT spectra (Table 1) show 18 carbon resonances ascribe to one methyl, four methylene (including one oxygenated), five methine (four sp^2 and one sp^3), and eight quaternary carbons (one carbonyl and seven olefinic). These data resemble those of known compound **9**, differing in that an OH group attached at C-6′ is replaced by a CH_3COCH_2 moiety. The ^1H—^1H COSY correlations of H-4/H-5/H-6/6-CH_2COCH_3 and HMBC correlations of 6-CH_2COCH_3/6-CH_2COCH_3, 6-CH_2COCH_3, 6-CH_2COCH_3/C-5, C-6, C-9, H-6/6-CH_2COCH_3 unambiguously clarify the substituted group at C-6. In addition, the ^1H NMR signals [δ_H 8.45 (1H, d, $J = 2.6$ Hz, H-6′), δ_H 7.16 (1H, d, $J = 8.6$ Hz, H3′), δ_H 7.22, (1H, overlap, H-4′)] support the presence of an ABX spin system in the structure. There is one chiral center in the structure of **1**, therefore, it makes sense to determine its absolute configuration. **1** was isolated as a partially racemic mixture. Separation by using chiral HPLC afforded **1a** and **1b**, the absolute configuration of **1a** was assigned as 6*R* by comparison of its calculated electronic circular dichroism (ECD) with that of experimental data (Supplementary Information). As a result, the structure of **1** was deduced and named as ganocochlearine C.

The molecular formula of compound **3** was assigned as $C_{17}H_{17}NO_4$ by the positive HRESIMS at *m/z* 300.1234 $[M + H]^+$ (calcd for 300.1230), having ten degrees of unsaturation. The ^1H and ^{13}C NMR data (Table 1) of **3** are very similar to those of known compound **2**. The difference between **2** and **3** is that an OH at C-6 in **2** is replaced by an OCH_3 in **3**, which is supported by the HMBC correlation of OCH_3/C-6. **3** was also isolated as a racemic mixture, subsequent chiral HPLC separation afforded two enantiomers. Their absolute configurations were determined by the mentioned computational methods. It was found that the ECD spectrum of 6*R*,10*S* instead of 6*S*,10*S* agrees well with the experimental data of **3b**, naturally clarifying the absolute configuration of **3b** as 6*R*,10*S* (Supplementary Information). Therefore, the structure of **3** was established and named as ganocochlearine D.

The molecular formula of **4** was deduced as $C_{17}H_{17}NO_3$ by the combination of its HRESIMS and NMR data. Comparison of NMR data (Table 1) of **4** with those of **9** indicated their structures were similar, except for the presence of an OCH_2CH_3 group in **4**. The ^1H—^1H COSY correlations of

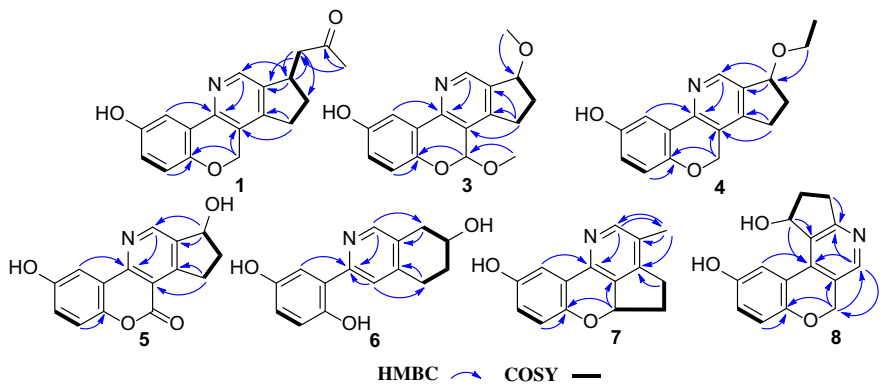

Fig. 2. Key HMBC and COSY correlations of compounds **1**, **3**–**8**.

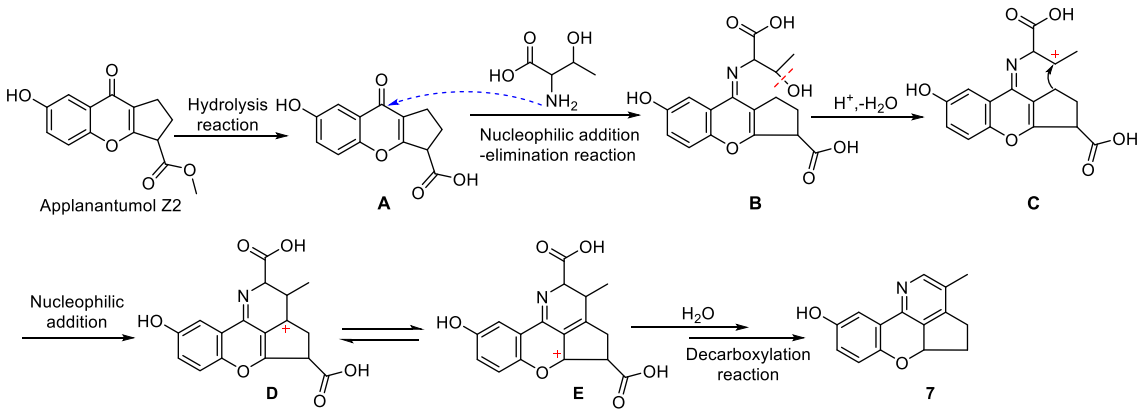

Fig. 3. A plausible biosynthetic pathway for **7**.

6-OCH$_2$CH$_3$/6-OCH$_2$CH$_3$ and the HMBC correlation of 6-OCH$_2$CH$_3$/C-6 support that 6-OCH$_2$CH$_3$ is attached to C-6. Compound **4** is not enantiomercally pure, chiral HPLC separation afforded two enantiomers (**4a** and **4b**). The absolute configuration of **4a** was assigned to be 6*S* by using ECD calculations (Supplementary Information). As a result, the structure of **4** was identified as named as ganocochlearine E.

Compound **5** has the molecular formula $C_{15}H_{11}NO_4$ as determined by its HRESIMS and NMR data. The ^{1}H NMR spectrum of **5** (Table 1) contains resonances for a typical ABX spin system [δ_H 8.03 (1H, brs, H-6′), δ_H 7.24 (1H, d, J = 8.8 Hz, H3′), δ_H 7.12, (1H, d, J = 8.8, 3.0 Hz, H-4′)]. The ^{13}C NMR and DEPT spectra (Table 1) show 15 carbons, including two methylene, five methane (four sp^2 and one sp^3), and eight quaternary carbons (one carbonyl and seven olefinic). The ^{1}H and ^{13}C NMR data of **5** are similar to those of **9**, indicating they are analogues. The difference is that C-10 in **9** is oxidized into a ketone in **5** evident from the chemical shift of C-10 (δ_C 160.9). Chiral HPLC analysis indicated partially racemic nature of **5**, successive separation on chiral phase by HPLC afforded a pair of enantiomers. Likewise, the absolute configuration of **5b** was determined to be 6*S* by utilizing computational methods (Supplementary Information). Consequently, the structure of **5** was identified and named as ganocochlearine F.

Analysis of HREIMS, ^{13}C NMR and DEPT spectra of compound **6** reveals that it has the molecular composition $C_{15}H_{15}NO_3$, suggesting nine degrees of unsaturation. The ^{1}H NMR spectrum of **6** (Table 1) exhibits signals [δ_H 7.24 (1H, brs, H-6′), δ_H 6.73 (2H, overlap, H3′, 4′)], suggesting the presence of an ABX spin system in **6**, and signals [δ_H 7.68 (1H, s, H3), δ_H 8.24 (1H, s, H-8)], indicating the possibility of a 2,4,5-pyridyl ring. The ^{13}C NMR and DEPT spectra of **6** contains resonances for fifteen carbons, including three methylene, six methine, six quaternary carbons. These data imply that **6** and **9** have similar structures, differing in two places. On the one hand, a six-membered ring in **6** is replaced by a five-membered ring in **9**; On the other hand, an oxygenated methylene attaching to C-3 in **9** is absent in **6**. The ^{1}H—^{1}H COSY correlations of H-4/H-5/H-6/H-7 and HMBC correlations of H-4/C-3, C-9, C-10, H-5/C-9, H-7/C-8, C-9, C-10 indicate the presence of a six-membered ring as shown (Fig. 1). Compound **6** was isolated as a racemic mixture. Chiral HPLC separation afforded **6a** and **6b**. The absolute configuration of **6b** was determined to be 6*R* by using computational methods (Supplementary Information). Thus far, the structure of **6** was identified and named as ganocochlearine G (See Figs. 2 and 3).

Compound **7** has a molecular formula $C_{15}H_{13}NO_2$ (ten degrees of unsaturation), as deduced by analysis of its positive HRESIMS at *m/z* 240.1022 $[M + H]^+$ (calcd for 240.1019, $C_{15}H_{14}NO_2$), ^{13}C NMR and DEPT spectra. The ^{1}H NMR spectrum of **8** contains a typical ABX coupling system [δ_H 6.83 (1H, d, J = 8.7 Hz, H3′), δ_H 6.71 (1H, d, J = 8.7, 3.0 Hz, H-4′), δ_H 7.37 (1H, d, J = 3.0 Hz, H-6′)], and one proton signal at δ_H 8.28 (1H, s, H-7). The ^{13}C NMR and DEPT spectra give 15 carbons ascribe to one methyl, two methylene, five methine (four olefinic and one oxygenated aliphatic), and seven olefinic quaternary carbons. These data disclose that compound **7** is an analogue of lucidimine D [10], differing in that a CH(OCH$_3$)$_2$ group at C-6 of lucidimine D is replaced by a methyl of **7**. HMBC correlations of H$_3$–10/C-6, C-7, C-9 and H-7/C-10 strongly support the above conclusion. Like compound **6**, **7** is partially racemic. Successive chiral HPLC provided enantiomers **7a** and **7b**. Of which, the absolute configuration of **7a** was assigned as 6*R* by ECD calculations (Supplementary Information). Taken together, the structure of **7** was identified and named as ganocochlearine H.

The molecular composition of **8** was determined to be $C_{15}H_{13}NO_3$ (ten degrees of unsaturation) by analysis of its positive HRESIMS, ^{13}C NMR and DEPT spectra. Similar NMR data between **8** and **9** reveal that they are analogues. The difference is mainly at rings C and D. The ^{1}H—^{1}H COSY spectrum displays interactions between H-5 (δ_H 5.58)/H-6/H-7. HMBC correlations of H-6′/*C*4, H-5/*C*4, C-8 (δ_C 135.1), C-9 (δ_C 150.8), H-7/C-8, C-9 indicate that ring D is connected with *C*4 via *C*4-C-8. In addition, HMBC correlations of H-10/C-2 (δ_C 149.6), C-3 and H-2 (δ_H 8.39)/C-10 indicate that C-3 is attached to C-2. In addition to rings A, B, and D, two double bonds between C-3 and *C*4, C-8 and C-9, accounting for 8 degrees of unsaturation, the olefinic nature of C-2 and the requirement of remaining 2 degrees of unsaturation suggests the presence of a C═N group and an additional ring as shown, in accordance with biogenetic origin of such type of alkaloids. Compound **8** is partially racemic, which was subjected to chiral HPLC to afford a pair of enantiomers (**8a** and **8b**). The absolute configuration of **8a** was assigned as 4*R* by utilizing computational methods (Supplementary Information). Therefore, the structure of compound **8**, named as ganocochlearine I, was determined.

Known compounds were respectively identified as ganoapplanatumine B (**2**) [6], sinensine E (**9**) [11], and lucidimine C (**10**) [10] by comparison of their NMR data with literature data. However, it was found that these three compounds are all racemic or partially racemic. Successive chiral HPLC separation afforded their respective enantiomers (Table 2). For compounds **9** and **10**, computational methods were used to clarify their stereochemistry at the chiral center. The absolute configurations of **9b** and **10a** were therefore assigned as 6*S* and 6*R*, respectively (Supplementary Information). For compound **2**, there exist two chiral carbons in the structure, theoretically, there should have two pairs of diastereoisomers. Fortunately, we obtained two enantiomers from racemic **2**. As previously described [6], ECD calculations were not helpful for differentiating the absolute configurations of diastereoisomers due to ECD curves of ganoapplanatumine B and *epi*-ganoapplanatumine B were quite similar.

Alkaloids similar to the present findings have been characterized from *Ganoderma* [6,9–11]. Investigations on the biosynthetic pathway of this class of alkaloids are of interest. To be associated with meroterpenoids isolated from this genus, we proposed that these alkaloids are probably biosynthesized via meroterpenoidal intermediates

and threonine. In detail, hydrolysis of applanantumol Z2 forms monomer A [6], where the meroterpenoid A reacts with threonine to give intermediate B. Compound **7** can be formed by nucleophilic addition and decarboxylation of B. The biosynthetic pathway for the other compounds are given in Supplementary Information.

Notably, all the isolates in this study are racemic or partially racemic mixtures. As mentioned above, similar structures were also characterized from *Ganoderma* species by the other investigators [6,9–13]. Unfortunately, racemic nature of such alkaloids was not pointed out. Based on our observations, we highly suspect that the isolated alkaloids in the previous reports are also racemic or partially racemic, which could be concluded from their small values of optical rotations. As such, our present results might subvert the previous reports on this class of alkaloids. In general, natural compounds as an enzyme-catalyzed consequence should be optically pure, the reason for racemic nature of such alkaloids might be associated with their minor abundance in Nature. Although these alkaloids are from similar or same biogenetic origins, the acetonyl residue is incorporated into the backbone of **1**, which makes the structure of **1** unusual. In contrast to compounds **1**–**5** and **7**–**10**, migration of a methyl followed by ring extension in **6** makes this structure novel. Increasing meroterpenoids present in the genus *Ganoderma* with a 1.4-dihydroxyl hybridized with a terpenoid have been characterized by us [3–7,14–17]. Without exception, all the alkaloids isolated in the present study bear a common 1.4-dihydroxyl motif, which is indeed an intermediate of shikimic acid pathway, prompting us to speculate that meroterpenoids and alkaloids in *Ganoderma* fungi might partly share a common biogenetic pathway.

Multiple factors are involved in renal fibrosis. Whereas, activation and proliferation of interstitial fibroblasts and consequent accumulation of extracellular matrix components is implicated in renal fibrogenesis. Therefore, interstitial fibroblasts are considered as target cells for the treatment of renal fibrosis. Inhibiting proliferation of fibroblasts might be beneficial for the prevention and treatment of renal fibrosis [18–20]. In this study, the selected compounds were evaluated for their inhibition on proliferation of normal (with exception of **7a**/**7b**) or TGF-β1-induced (with exception of **3a**/**3b** and **7a**/**7b**) fibroblasts NRK-49F. Unfortunately, it was found that all the tested compounds are inactive towards these assays even at the concentration of 20 μM (data not shown), indicating that these alkaloids are neither effective against diseased cells nor toxic towards normal cells.

Acknowledgments

We thank National Science Fund for Distinguished Young Scholars (81525026), NSFC-Joint Foundation of Yunnan Province (U1202222), National Natural Science Foundation of China (21472199), and a project from State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany (P2016-ZZ02) for their financial supports.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2016.11.011.

References

[1] A. Trigos, J.S. Medellín, Biologically active metabolites of the genus *Ganoderma*: three decades of myco-chemisty research, Rev. Mex. Micol. 34 (2011) 63–83.
[2] S. Baby, A.J. Johnson, B. Govindan, Secondary metabolites from *Ganoderma*, Phytochemistry 114 (2015) 66–101.
[3] R.R.M. Paterson, *Ganoderma* — a therapeutic fungal biofactory, Phytochemistry 67 (2006) 1985–2001.
[4] F.J. Zhou, Y. Nian, Y.M. Yan, Y. Gong, Q. Luo, Y. Zhang, B. Hou, Z.L. Zuo, S.M. Wang, H.H. Jiang, J. Yang, Y.X. Cheng, Two new classes of T-type calcium channel inhibitors with new chemical scaffolds from *Ganoderma cochlear*, Org. Lett. 17 (2015) 3082–3085.
[5] Y.M. Yan, X.L. Wang, Q. Luo, L.P. Jiang, C.P. Yang, B. Hou, Z.L. Zuo, Y.B. Chen, Y.X. Cheng, Metabolites from the mushroon *Ganoderma* lingzhi as stimulators of neural stem cell proliferation, Phytochemistry 114 (2015) 155–1162.
[6] Q. Luo, X.H. Yang, Z.L. Yang, Z.C. Tu, Y.X. Cheng, Miscellaneous meroterpenoids from *Ganoderma applanatum*, Tetrahedron 72 (2016) 1–11.
[7] Q. Luo, X.L. Wang, L. Di, Y.M. Yan, Q. Lu, X.H. Yang, D.B. Hu, Y.X. Cheng, Isolation and identification of renoprotective substances from the mushroom *Ganoderma lucidum*, Tetrahedron 71 (2015) 840–845.
[8] J.J. Yang, D.Q. Yu, Synthesis of ganoderma alkaloid a and B, Acta Pharm. Sin. 25 (1990) 555–559.
[9] L. Tian, X.L. Wang, Y.Z. Wang, Y.X. Cheng, Two new alkaloids from *Ganoderma cochlear*, Nat. Prod. Res. Dev. 27 (2015) 1325–1328.
[10] Z.Z. Zhao, H.P. Chen, T. Feng, Z.H. Li, Z.J. Dong, J.K. Liu, Lucidimine A– D, four new alkaloids from the fruiting bodies of *Ganoderma lucidum*, J. Asian Nat. Prod. Res. 17 (2015) 1160–1165.
[11] J.Q. Liu, C.F. Wang, X.R. Peng, M.H. Qiu, New alkaloids from the fruiting bodies of *Ganoderma sinense*, Nat. Prod. Bioprospect. 1 (2011) 93–96.
[12] S.Z. Huang, B.H. Cheng, Q.Y. Ma, Q. Wang, F.D. Kong, H.F. Dai, S.Q. Qiu, P.Y. Zheng, Z.Q. Liu, Y.X. Zhao, Anti-allergic prenylated hydroquinones and alkaloids from the fruiting body of *Ganoderma calidophilum*, RSC Adv. 6 (2016) 21139–21147.
[13] C. Liu, F. Zhao, R.Y. Chen, A novel alkaloid from the fruiting bodies of *Ganoderma sinense* Zhao, Xu *et* Zhang, Chin. Chem. Lett. 21 (2010) 197–199.
[14] M. Dou, L. Di, L.L. Zhou, Y.M. Yan, X.L. Wang, F.J. Zhou, Z.L. Yang, R.T. Li, F.F. Hou, Y.X. Cheng, Cochlearols A and B, polycyclic meroterpenoids from the fungus *Ganoderma cochlear* that have renoprotective activities, Org. Lett. 16 (2014) 6064–6067.
[15] Q. Luo, L. Di, W.F. Dai, Q. Lu, Y.M. Yan, Z.L. Yang, R.T. Li, Y.X. Cheng, Applanatumin A, a new dimeric meroterpenoid from *Ganderma applanatum* that displays potent antifibrotic activity, Org. Lett. 17 (2015) 1110–1113.
[16] Y.M. Yan, J. Ai, L.L. Zhou, A.C.K. Chung, R. Li, J. Nie, P. Fang, X.L. Wang, J. Luo, Q. Hu, F.F. Hou, Y.X. Cheng, Lingzhiols, unprecedented rotary door-shaped meroterpenoids as potent and selective inhibitor of p-Smad3 from *Ganoderma lucidum*, Org. Lett. 15 (2013) 5488–5491.
[17] Q. Luo, L. Tian, L. Di, Y.M. Yan, X.Y. Wei, X.F. Wang, Y.X. Cheng, (±)-Sinensilactam A, a pair of rare hybrid metabolites with Smad3 phosphorylation inhibition from *Ganoderma sinensis*, Org. Lett. 17 (2015) 1565–1568.
[18] J.S. Duffield, I. Grgic, B.D. Humphreys, The origin of interstitial myofibroblasts in chronic kidney disease, Pediatr. Nephrol. 27 (2012) 183–193.
[19] S. Meran, R. Steadman, Fibroblasts and myofibroblasts in renal fibrosis, Int. J. Exp. Pathol. 92 (2011) 158–167.
[20] M. Ponnusamy, X.X. Zhou, Y.L. Yan, J.H. Tang, E. Tolbert, T.C. Zhao, R.J. Gong, S.G. Zhuang, Blocking Sirtuin 1 and 2 inhibits renal interstitial fibroblast activation and attenuates renal interstitial fibrosis in obstructive nephropathy, J. Pharmacol. Exp. Ther. 350 (2014) 243–256.