Isolation and Bioactivity Evaluation of Terpenoids from the Medicinal Fungus *Ganoderma sinense*

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Key words
- *Ganoderma sinense*
- Ganodermataceae
- triterpenoids
- sesquiterpene
- cytotoxicity
- CYP3A4 induction

Abstract

A new pentanorlanostane, ganosineniol A (**1**), eight new lanostane triterpenoids, ganosinoside A (**2**), ganoderic acid Jc (**3**), ganoderic acid Jd (**4**), ganodermatetraol (**5**), ganolucidic acid γa (**6**), ganolucidate F (**7**), ganoderiol J (**8**), and methyl lucidenate Ha (**9**), and a new sesquiterpenoid, ganosinensine (**10**), together with eleven known triterpenoids (**11**–**21**), were isolated from the fruiting bodies of the fungus *Ganoderma sinense*. Chemical structures were determined based on spectroscopic evidence, including 1D, 2D NMR, and mass spectral data. Furthermore, all isolates were tested for cytotoxic activity and induction ability of hPXR-mediated CYP3A4 expression. Among them, ganoderic acid Jc (**3**) displayed selective inhibitory activity against HL-60 cells (IC_{50} = 8.30 µM), and ganoderiol E (**11**) exhibited selective cytotoxic activity against MCF-7 cells (IC_{50} = 6.35 µM). Meanwhile, compounds **5**, **7**, and ganolucidic acids B and C (**19**, **20**) showed induction ability of hPXR-mediated CYP3A4 expression.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

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Introduction

The members of the genus *Ganoderma* (Ganodermataceae) are widely used as valuable crude drugs in China, Japan, and Korea. *G. lucidum* (lingzhi), one of the most well-known species, has been used as a medicine for more than 2000 years in China for treatment of chronic hepatitis, coronary heart disease, hyperlipemia, neurasthenia, bronchitis, and insomnia [1]. Because of their potential medicinal value, much attention has been paid to the search for significant pharmacological constituents from this species. For the past 30 years, over one hundred triterpenoids have been isolated and characterized from *G. lucidum* [2, 3], some of which reveal anti-HIV-1 protease activity [4], antiviral effects [5], potential antitumor activity [6], inhibition of aldose reductase [7], and anti-inflammatory effect [8].

G. sinense, a well-known species, is widely distributed in Yunnan Province, in the southwest of China. Many chemical studies have been carried out on *G. lucidum*, but studies on *G. sinense* have rarely been reported [9–12]. To find out the differences of chemical composition between *G. sinense* and *G. lucidum* and to test whether the constituents of *G. sinense* also have multiple pharmacological activities, we explored the fruiting bodies of *G. sinense* hoping the results could contribute to the fair use of the resources. In the context of our research work on the cytotoxic constituents of *G. sinense*, three new cytotoxic triterpenoids containing a four-membered ring have been reported [13]. Further work led to the isolation of ten new (**1**–**10**) and eleven known compounds (**11**–**21**) (**Fig. 1**), including a rare pentanorlanostane triterpenoid (**1**) and a C_{15}-trichothecene with a modified structure (**10**). Moreover, *in vitro* screenings for cytotoxic activity and induction ability towards CYP3A4 expression were made. In this paper, the isolation, structural elucidation, and cytotoxic activity of these compounds are described herein.

Materials and Methods

General experimental procedures

Optical rotations were measured on a SEPA-300 polarimeter. UV spectra were carried out on a Shimadzu double-beam 22210A spectrophotometer in MeOH. IR spectra were obtained on a Bio-Rad

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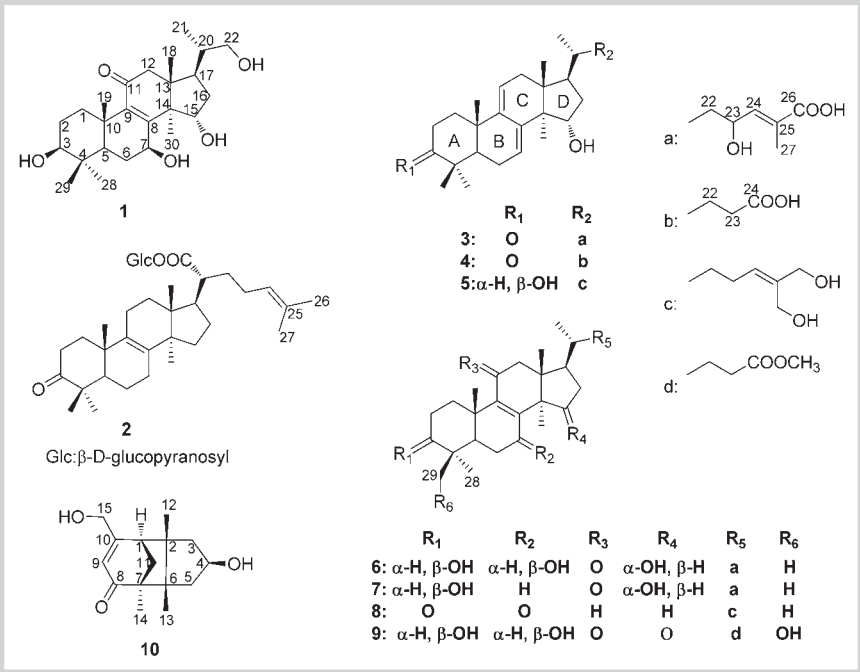

Fig. 1 Structures of new compounds **1**–**10**.

FTS-135 infrared spectrometer with KBr pellets. NMR spectra were recorded on Bruker AV-400 and DRX-500 spectrometers with TMS as the internal standard. Electrospray-ionization (ESIMS) and high-resolution electrospray-ionization mass spectra (HRESIMS), and fast atom bombardment mass spectra (FABMS) were acquired on an APIQSTAR time-of-flight mass spectrometer and a VG Autospec-3000 mass spectrometer, respectively. Column chromatography was performed on silica gel (200–300 mesh; Qingdao Marine Chemical Factory) and Lichroprep RP-18 gel. Fractions were monitored by TLC, and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4. OPTI-MEM (Gibco), cationic liposome (Lipofectamin TM 2000; Invitrogen), double-luciferase reporter gene assay (Promega), hepatocarcinoma cell line HuH-7 (Kunming Institute of Zoology, CAS), luminescence microplate readers (Thermo Fisher), rifampicin (Sigma), and DMSO (Amresco) were used for establishing an *in vitro* screening system for the compounds' inductions of hPXR-mediated CYP3A4 expression in HuH7 cells.

Plant material

The fruiting bodies of *Ganoderma sinense* were bought in Nan Hua Chinese Medicine Market in Nanhua County, Yunnan Province, P.R. China, in November 2008. A specimen (No. 08112806) was deposited in the Herbarium of the Department of Taxonomy, Kunming Institute of Botany, Chinese Academy of Sciences, and was identified by Prof. Pei-Gui Liu.

Extraction and isolation

Air-dried and powered fruiting bodies of *G. sinense* (50 kg) were extracted with 95% methanol under reflux (3 × 100 L, 3 h each) and filtered. The combined extracts were concentrated to give a dark brown residue which was suspended in H_2O (5 L), and then the residue was extracted with petroleum ether (3 × 5 L) and ethyl acetate (3 × 5 L), sequentially. The EtOAc extract (1.5 kg) was chromatographed on silica gel (6 kg, 20 × 150 cm) with $CHCl_3$-MeOH mixtures (99 : 1 → 1 : 1). Fractions (2 L each) were collected, and their homogeneity was monitored by TLC with solvent systems of increasing polarity [$CHCl_3$-MeOH, 99 : 1 (20 L), 98 : 2 (30 L), 95 : 5 (32 L), 9 : 1 (10 L), 5 : 1 (10 L) and 1 : 1 (10 L)]. Those showing similar TLC Rf values were combined to give four fractions (I–IV). Fraction I (110 g) was repeatedly chromatographed on silica gel [a, 8 × 120 cm, $CHCl_3$-MeOH, 110 : 1 (15 L); b, 5 × 100 cm, petroleum ether-acetone 20 : 1 (8 L)] and Sephadex LH-20 (1.5 × 200 cm, MeOH, 500 mL, each time) to yield **12** (30 mg) and **13** (45 mg). Fraction II (250 g) was further chromatographed on a silica gel column (2.5 kg, 8 × 150 cm) eluting with petroleum ether-acetone [6 : 1 (18 L), 3 : 1 (15 L), and 1 : 1 (18 L)] to afford subfractions A1–A3. Subfraction A2 (50 g) was repeatedly chromatographed over a silica gel column [a, 5 × 100 cm, $CHCl_3$-MeOH, 50 : 1 (15 L); b, 3 × 60 cm, $CHCl_3$-acetone 10 : 1 (5 L)], further over an RP-18 column [300 g, MeOH-H_2O, 65% (5 L)], followed by Sephadex LH-20 to yield **4** (18 mg), **8** (16 mg), **11** (25 mg), **14** (40 mg), **15** (37 mg), and **16** (50 mg). Fraction III (200 g) was further chromatographed on a silica gel column (2.0 kg, 8 × 150 cm) eluting with $CHCl_3$-MeOH [40 : 1 (15 L), 20 : 1 (18 L), and 10 : 1 (17 L)] to afford subfractions B1 (45 g), B2 (52 g), B3 (55 g). Subfraction B2 was chromatographed over an RP-18 column (450 g, 50% → 100% MeOH-H_2O) to provide B21 (10 g, 50%, 6 L), B22 (20 g, 65%, 8 L), and B23 (15 g, 80%, 6 L). Compound **3** (15 mg) and **17** (32 mg) were purified from B22 by silica gel column chromatography (600 g, 5 × 100 cm) eluting with petroleum ether-acetone (3 : 1, 15 L). Subfraction B3 was chromatographed over an RP-18 column (450 g, 50% → 100% MeOH-H_2O) to afford B31 (20 g, 45%, 8 L), B32 (10 g, 60%, 9 L), and B33 (15 g, 75%, 7 L). Subsequently, compounds **1** (12 mg), **2** (6 mg), **5** (2 mg), **9** (10 mg), **10** (1.5 mg), **18** (4 g), and **19** (2 g) were purified from B31 by silica gel column chromatography, eluted with petroleum ether-acetone (300 g, 4 × 80 cm, 1 : 1, 10 L) and $CHCl_3$-MeOH (100 g, 3 × 60 cm, 20 : 1, 10 L), successively. Fraction IV (50 g) was chromatographed over an RP-18 column (300 g, 50% → 100% MeOH-H_2O) to afford subfraction C1 (10 g, 50%, 4 L), C2 (22 g, 65%, 5 L), C3 (13 g, 80%, 5 L). Subfraction C2 was further chromatographed over an RP-18 column (65% MeOH-H_2O) to afford subfraction C21 (4 g, 2 L), C22 (8 g, 3 L), and C23 (5 g, 2 L). Compounds **6**

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(26 mg), **7** (20 mg), **20** (30 mg), and **21** (15 mg) were purified from C22 by silica gel column chromatography (200 g, 3 × 100 cm), eluted with $CHCl_3$-MeOH (15 : 1, 16 L), and further purified by Sephadex LH-20 (1.5 × 200 cm, MeOH, 500 mL/each time).

Ganosineniol A (**1**): white, amorphous powder; $[\alpha]_D^{25}$ +97.0 (*c* 0.90, MeOH); UV (MeOH) λ_{max} (log ε): 232 (4.25) nm; IR (KBr) ν_{max}: 3453, 1689, 1658 cm^{-1}; ^1H and ^{13}C NMR: in ▶ **Tables 1** and **2**; positive ESIMS *m/z* 421 $[M + H]^+$; positive HRESIMS *m/z* 421.2947 $[M + H]^+$ (calcd. for $C_{25}H_{41}O_5$ [M + H], *m/z* 421.2953).

Ganosinoside A (**2**): white, amorphous powder; $[\alpha]_D^{25}$ +32.0 (*c* 0.73, MeOH); UV (MeOH) λ_{max} (log ε) 213 (1.50) nm; IR (KBr) ν_{max}: 3430, 1721, 1662 cm^{-1}; ^1H and ^{13}C NMR: in ▶ **Tables 1** and **2**; negative ESIMS *m/z* 651 $[M + Cl]^-$; negative HRESIMS *m/z* 651.3646 $[M + Cl]^-$ (calcd. for $C_{36}H_{56}O_8Cl$ $[M + Cl]^-$, *m/z* 651.3663).

Ganoderic acid Jc (**3**): white, amorphous powder, $[\alpha]_D^{25}$ +40.8 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε): 226 (4.10) nm; IR (KBr) ν_{max}: 3440, 1725, 1660 cm-^{1}; ^1H and ^{13}C NMR: in ▶ **Tables 1** and **2**; negative FABMS *m/z* 483 $[M]^-$; positive HRESIMS *m/z* 485.3251 $[M + H]^+$ (calcd. for $C_{30}H_{45}O_5$ $[M + H]^+$, 485.3267).

Ganoderic acid Jd (**4**): white, amorphous powder, $[\alpha]_D^{25}$ +45.1 (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ε): 225 (4.05) nm; IR (KBr) ν_{max}: 3400, 1720, 1705 cm^{-1}; ^1H and ^{13}C NMR ▶ **Tables 1** and **2**; negative FABMS *m/z* 427 $[M]^-$; positive HRESIMS $[M + Na]^+$ *m/z* 451.2832 (calcd. for $C_{27}H_{40}O_4Na$ $[M + Na]^+$, 451.2824).

Ganodermatetraol (**5**): white, amorphous powder; $[\alpha]_D^{25}$ +46.3 (*c* 0.36, MeOH); UV (MeOH) λ_{max} (log ε): 225 (4.08) nm; IR (KBr) ν_{max}: 3420, 1624 cm^{-1}; ^1H and ^{13}C NMR: in ▶ **Tables 1** and **2**; positive ESIMS *m/z* 495 $[M + Na]^+$; positive HRESIMS $[M + Na]^+$ *m/z* 495.3445 (calcd. for $C_{30}H_{48}O_4Na$ $[M + Na]^+$, 495.3450).

Ganolucidic acid γa (**6**):white, amorphous powder; $[\alpha]_D^{25}$ +93.9 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ε): 232 (3.80) nm; IR (KBr) ν_{max}: 3434, 1670, 1660 cm^{-1}; ^1H and ^{13}C NMR: in ▶ **Tables 1** and **2**; positive ESIMS *m/z* 519 $[M]^+$; positive HRESIMS *m/z* 541.3180 $[M + Na]^+$ (calcd. for $C_{30}H_{46}O_7Na$ $[M + Na]^+$, 541.3141).

Ganolucidate F (**7**): white, amorphous powder; $[\alpha]_D^{25}$ +132.8 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε): 220 (4.14) nm; IR (KBr) ν_{max}: 3400, 1675, 1655 cm^{-1}; ^1H and ^{13}C NMR: in ▶ **Tables 1** and **2**; positive FABMS *m/z* 503 $[M + H]^+$; positive HRESIMS *m/z* 503.3415 $[M + H]^+$ (calcd. for $C_{30}H_{47}O_6$ $[M + H]^+$, 503.3372).

Ganoderiol J (**8**): white, amorphous powder, $[\alpha]_D^{25}$ −17.0 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε): 223 (3.92) nm; IR (KBr) ν_{max}: 3440, 1730, 1680 cm^{-1}; ^1H NMR, see ▶ **Table 1**; ^{13}C NMR, see ▶ **Table 1** and **Table 2**; negative FABMS *m/z* 427 $[M]^-$; positive HRESIMS $[M + H]^+$ *m/z* 471.3476 (calcd. for $C_{30}H_{47}O_4$ $[M + H]^+$, 471.3474).

Methyl lucidenate Ha (**9**): white, amorphous powder; $[\alpha]_D^{25}$ +82.0 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 232 (4.02) nm; IR (KBr) ν_{max}: 3450, 1740, 1725, 1680 cm^{-1}; ^1H and ^{13}C NMR ▶ **Tables 1** and **2**; positive FABMS *m/z* 490 $[M + H]^+$; positive HRESIMS *m/z* 513.2886 $[M + Na]^+$ (calcd. for $C_{28}H_{42}O_7Na$ $[M + Na]^+$, 513.2930).

Ganosinensine (**10**): white, amorphous powder; $[\alpha]_D^{25}$ +104.5 (*c* 0.45, MeOH); UV (MeOH) λ_{max} (log ε): 229 (4.26) nm; IR (KBr) ν_{max}: 3435, 1687, 1620 cm^{-1}; ^1H NMR (C_5D_5N, 600 MHz) δ_H: 2.13 (1H, d, *J* = 4.3 Hz, H-1), 2.36 (1H, m, H-3a), 1.72 (1H, dd, *J* = 13.6, 6.5 Hz, H-3b), 4.49 (1H, m, H-4), 2.23 (1H, m, H-5a), 1.59 (1H, dd, *J* = 13.7, 6.5 Hz, H-5b), 6.64 (1H, s, H-9), 1.95 (1H, dd, *J* = 11.4, 4.5 Hz, H-11a), 1.80 (1H, d, *J* = 11.4 Hz, H-11b), 1.30 (3H, s, H-12), 1.16 (3H, s, H-13), 1.20 (3H, s, H-14), 4.59 (dd, *J* = 18.5, 3.7 Hz, H-15a) 4.51 (1H, dd, *J* = 18.5, 3.7 Hz, H-15b), 6.10 (d, *J* = 3.0 Hz, 4-OH), 7.29 (t, *J* = 5.1 Hz, 16-OH); ^{13}C NMR (C_5D_5N, 150 MHz) δc: 50.1 (C-1, CH), 57.3 (C-2, C), 48.6 (C-3, CH_2), 73.7 (C-4, CH), 47.4 (C-5, CH_2), 54.5 (C-6, C), 61.2 (C-7, C), 205.3 (C-8, C), 122.5 (C-9, CH), 173.9 (C-10, C), 46.4 (C-11, CH_2), 29.2 (C-12, CH_3), 24.1 (C-13, CH_3), 18.4 (C-14, CH_3), 65.5 (C-15, CH_2); positive ESIMS *m/z* 273 $[M + Na]^+$; positive HRESIMS *m/z* 273.1459 $[M + Na]^+$ (calcd. for $C_{15}H_{22}O_3Na$ $[M + Na]^+$, 273.1466).

Acid hydrolysis of 2

Compound **2** (4 mg) was refluxed with 2 N HCl/1,4-dioxane 1 : 1 (2 mL) at 100 °C for 2 h. After neutralizing with Ag_2CO_3 (300 mg), $CHCl_3$ (2 mL × 3) was used for extraction. The filtrate of the aqueous layer was concentrated to dryness under reduced pressure and then dissolved in pyridine (100 mL). After that, L-cysteine methyl ester hydrochloride (1.1 mg) was added, and the mixture was kept at 60 °C for 1 h. Then the trimethylsilylation reagents HMDS (100 mL) and TMCS (50 mL) were added successively, and the mixture was kept at 4–8 °C for 8 h. The filtrate was subjected to GC analysis under the following conditions: column temperature, 180 °C → 250 °C, 8 °C/min; column pressure, 80 kPa; injector and detector temperature, 250 °C and 280 °C, respectively; injection volume, 8 mL; and split ratio, 1/30. GC analysis showed the presence of D-glucose when compared with the authentic D-glucose (t_R 9.93 min).

Induction of hPXR-mediated CYP3A4 expression in HuH7 cells

Cotransfecting the tk-(CYP3A4) 3-Luc reporter plasmid, pCMX-hPXR expression plasmid, and pRL-TK internal control plasmid via liposome into HuH7 cells was performed to establish a dual-luciferase reporter gene system. The dual luciferases were firefly luciferase and renilla luciferase (reference luciferase). After 12 h cotransfection, the HuH7 cells were cultured by the complete medium mix with 50 µM tested compounds (all of the purities > 90%), rifampicin (RIF; Sigma, > 97%) or 0.05% DMSO for 48 h. After that, the HuH7 cells were lysed, and the dual-luciferase activities were detected and analyzed [14, 15].

Cytotoxicity assay

A panel of human tumor cell lines was used: breast cancer MCF-7, hepatocellular carcinoma SMMC7721, human myeloid leukemia HL-60, colon cancer SW480, and lung cancer A549. The cell lines were obtained from Shanghai Cell Bank in China. All cells were cultured in RPMI-1640 or DMEM medium (Hyclone), supplemented with 10% fetal bovine serum (Hyclone) at 37 °C in a humidified atmosphere with 5% CO_2. The cytotoxicity assay was performed according to the MTT [2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method in 96-well microplates [16]. After subsequent treatment, cell viability was detected, and a cell growth curve was graphed. IC_{50} values were calculated by Reed and Muench's method [17]. Cisplatin (DDP; Sigma, > 98%) was used as a positive control.

Supporting information

1D and 2D NMR spectra of compounds **1**–**10** (**Figs. 1S**–**47S**) are available as Supporting Information.

Results

▼

Ganosineniol A (**1**), obtained as an amorphous powder, possessed the molecular formula $C_{25}H_{40}O_5$ on the basis of the HRESIMS molecular ion peak at *m/z* 421.2947 $[M + H]^+$ (calcd. for $C_{25}H_{41}O_5$ at *m/z* 421.2953). The ^1H NMR spectrum of **1** (▶ **Table 1**) showed signals for six methyls, including a doublet methyl (δ_H 1.15,

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Table 1 ^{1}H NMR data of compounds **1–9**[a].

No.	1	2	3	4	5	6	7	8	9
1	3.13 d (13.5)	1.62[b]	2.21[b]	2.22[b]	2.17[b]	3.15 d (13.4)	3.46[b]	2.09[b]	3.21 m
	1.15[b]	1.31[b]	1.70 m	1.72 m	1.60 m	1.16 m	1.30 m	1.76[b]	1.18 m
2	1.92 m	2.48 m	2.78 m	2.78 dt (14.4, 5.6)	1.99 m	1.92[b]	2.02 [b]	2.68 m	2.15[b]
		2.35 m	2.37[b]	2.39[b]			1.96[b]	2.43 m	1.27[b]
3	3.48 dd (11.0, 4.7)				3.51 m	3.49 dd (11.4, 4.4)	3.51[b]		3.66 m
5	1.18 m	1.51[b]	1.64 m	1.65 m	1.37[b]	1.20 d (12.9)	1.14 d (11.5)	2.11[b]	1.24 d (13.6)
6	2.39 m	1.50[b]	2.11[b]	2.13 [b]	2.22[b]	2.40 dd (12.6, 7.4)	2.89 m	2.52 t (15.2)	2.55 m
	1.98 m	1.45[b]	1.99[b]	2.00 m	2.17[b]	1.98[b]	2.71 m	2.30[b]	1.99[b]
7	4.94 dd (9.7, 7.5)	1.98[b]	6.40 d (6.0)	6.43 d (6.2)	6.52 d (6.24)	4.95[b]	1.79 m		5.14 t (8.6)
							1.47[b]		
11		2.03[b]	5.40 d (5.3)	5.39 d (5.0)	5.45 d (6.18)			2.28[b]	
		1.95[b]						1.77[b]	
12	3.00 d (15.1)	2.16 m	2.40[b]	2.37[b]	2.41[b]	3.00 d (15.0)	2.96 d (16.4)	1.76[b]	2.95 m
	2.71 d (14.9)	1.89[b]	2.13 m	2.09[b]	2.12[b]	2.72 d (15.0)	2.66 d (16.4)	1.22, m	2.81[b]
15	5.22 dd (9.2, 6.9)	1.61[b]	4.60 dd (9.2, 6.1)	4.65 t (7.5)	4.68 m	5.19 t (7.9)	4.60 dd (9.2, 5.9)	2.04 m	
		1.22 m						1.66 m	
16	2.21 m	1.39[b]	2.33[b]	2.23[b]	2.20[b]	2.28 m	2.26 m	1.93[b]	2.81[b]
	2.07 m		2.23[b]		2.09[b]	2.10[b]	2.17[b]	1.29[b]	2.23 m
17	2.15[b]	2.39[b]	1.96 m	1.82 m	1.79 dd (18.5, 10.2)	2.05[b]	2.07 m	1.42[b]	2.01[b]
18	1.11 s	1.06 s	0.77 s	0.78 s	0.80 s	1.08 s	0.93 s	0.64 s	1.10 s
19	1.52 s	0.94 s	1.16 s	1.17 s	1.16 s	1.50 s	1.36 s	1.31 s	1.44 s
20	1.65 m	2.73 dt (11.1, 2.7)	1.70[b]	1.54[b]	1.45 m	1.70 m	1.65 m	1.38[b]	1.50[b]
21	1.15 d (6.5)		1.19 d (6.2)	0.96 d (5.0)	0.95 d (6.47)	1.10 d (6.3)	1.10 d (6.2)	0.91 d (5.8)	0.83 d (6.2)
22	3.85 dd (10.3, 2.6)	1.89[b]	2.02[b]	2.12[b]	1.19[b]	1.96[b]	1.96 m	1.48[b]	1.80 m
	3.58 m	1.73 m	1.85 m	1.54[b]		1.81 m	1.81 m	1.09[b]	1.35[b]
23		2.39[b]	5.00 dd (14.4, 8.7)	2.63 m	2.41[b]	4.99[b]	4.99 dt (8.1, 6.0)	2.11[b]	2.42 m
		2.22[b]		2.51 m	2.22[b]			1.96[b]	2.31 m
24		5.20 t (6.5)	7.40 d (8.7)		5.92 t (7.29)	7.39 d (8.8)	7.40 dd (9.0, 1.4)	5.50 t (7.2)	
26		1.61 s			4.75 s			4.16 s	
27		1.63 s	2.18 s		4.75 s	2.13 s	2.15 s	4.27 s	
28	1.25 s	1.11 s	1.09 s	1.10 s	1.21 s	1.26 s	1.24 s	1.08 s	1.55 s
29	1.08 s	1.02 s	1.03 s	1.04 s	1.16 s	1.08 s	1.08 s	1.06 s	4.55 d (11.0)
									3.83 d (11.0)
30	1.50 s	0.91 s	1.32 s	1.31 s	1.38 s	1.52 s	1.47 s	0.89 s	1.38 s
OCH_3									3.62 s
Glc-1′		6.42 d (8.1)							
2′		4.22 t (7.6)							
3′		4.07 m							
4′		4.33[b]							
5′		4.29[b]							
6′		4.47 d (11.3)							
		4.39 m							

[a] ^{1}H NMR data were measured at 400 MHz in C_5D_5N for **1, 2, 3, 4, 6, 7**, and **9**; at 400 MHZ in $CDCl_3$ for **8**, and at 600 MHz in C_5D_5N for **5** (*J* are in Hz). The assignments are based on DEPT, ^{1}H-^{1}H COSY, HSQC, and HMBC spectra. [b] Overlapped

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Table 2 ^{13}C NMR and DEPT data of compounds **1–9**[a].

Position	1	2	3	4	5	6	7	8	9
1	35.5, CH_2	36.1, CH_2	36.8, CH_2	36.8, CH_2	36.8, CH_2	35.5, CH_2	35.2, CH_2	35.2, CH_2	35.3, CH_2
2	28.8, CH_2	34.7, CH_2	35.0, CH_2	35.0, CH_2	29.2, CH_2	29.0, CH_2	28.9, CH_2	34.3, CH_2	28.9, CH_2
3	77.6, CH	216.3, C	215.3, C	215.3, C	78.4, CH	77.6, CH	77.9, CH	214.9, C	79.3, CH
4	39.4, C	47.3, C	47.5, C	47.4, C	39.8, C	39.3, C	39.7, C	47.1, C	43.1, C
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6	29.0, CH_2	19.6, CH_2	23.8, CH_2	23.8, CH_2	23.9, CH_2	28.8, CH_2	30.9, CH_2	37.0, CH_2	28.5, CH_2
7	69.5, CH	26.5, CH_2	121.6, CH	121.6, CH	122.5, CH	69.4, CH	17.9, CH_2	198.2, C	67.0, CH
8	160.5, C	135.0, C	142.0, C	142.0, C	142.4, C	160.5, C	164.8, C	139.4, C	158.0, C
9	141.9, C	133.9, C	145.3, C	145.3, C	147.4, C	141.3, C	139.8, C	163.0, C	142.6, C
10	39.1, C	37.1, C	37.5, C	37.5, C	38.3, C	39.1, C	38.3, C	39.3, C	39.1, C
11	200.3, C	21.4, CH_2	117.2, CH	117.2, CH	116.6, CH	200.1, C	198.6, C	23.8, CH_2	198.4, C
12	52.8, CH_2	29.0, CH_2	38.8, CH_2	38.7, CH_2	39.2, CH_2	52.9, CH_2	52.9, CH_2	30.0, CH_2	51.1, CH_2
13	47.7, C	45.0, C	44.5, C	44.5, C	45.0, C	47.6, C	47.4, C	44.8, C	45.6, C
14	54.5, C	49.9, C	52.6, C	52.6, C	53.0, C	54.7, C	54.0, C	47.7, C	59.0, C
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17	45.4, CH	47.6, CH	50.1, CH	49.3, CH	49.8, CH	49.5, CH	49.9, CH	48.8, CH	46.3, CH
18	17.7, CH_3	16.5, CH_3	16.3, CH_3	16.4, CH_3	16.8, CH_3	17.4, CH_3	17.0, CH_3	15.8, CH_3	17.9, CH_3
19	20.0, CH_3	18.6, CH_3	22.1, CH_3	22.1, CH_3	23.6, CH_3	19.9, CH_3	19.4, CH_3	17.8, CH_3	19.2, CH_3
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22	66.9, CH_2	33.4, CH_2	44.7, CH_2	32.0, CH_2	37.3, CH_2	44.5, CH_2	44.4, CH_2	36.0, CH_2	31.0, CH_2
23		26.4, CH_2	67.1, CH	31.9, CH_2	25.1, CH_2	67.0, CH	66.9, CH	24.2, CH_2	30.9, CH_2
24		124.7, CH	145.5, CH	176.4, C	127.9, CH	145.4, CH	145.4, CH	131.4, CH	174.1, C
25		131.9, C	128.7, C		141.3, C	128.7, C	128.7, C	136.7, C	
26		25.8, CH_3	170.3, C		65.8, CH_2	170.8, C	170.8, C	67.4, CH_2	
27		17.8, CH_3	13.5, CH_3		58.8, CH_2	13.5, CH_3	13.5, CH_3	59.8, CH_2	
28	28.8, CH_3	26.4, CH_3	25.6, CH_3	25.6, CH_3	29.3, CH_3	28.8, CH_3	28.9, CH_3	25.2, CH_3	23.7, CH_3
29	16.7, CH_3	21.3, CH_3	22.3, CH_3	22.3, CH_3	17.1, CH_3	16.7, CH_3	16.7, CH_3	21.3, CH_3	64.2, CH_2
30	20.2, CH_3	24.4, CH_3	18.0, CH_3	17.9, CH_3	18.6, CH_3	20.2, CH_3	19.8, CH_3	24.8, CH_3	24.9, CH_3
									51.4, CH_3
Glc-1′		95.8, CH							
2′		74.0, CH							
3′		79.2, CH							
4′		71.4, CH							
5′		79.0, CH							
6′		62.5, CH_2							

[a] ^{13}C NMR data were measured at 100 MHz in C_5D_5N for **1, 2, 3, 4, 6, 7**, and **9**; at 100 MHZ in $CDCl_3$ for **8**, and at 150 MHz in C_5D_5N for **5**. The assignments are based on DEPT, ^1H-^1H COSY, HSQC, and HMBC spectra

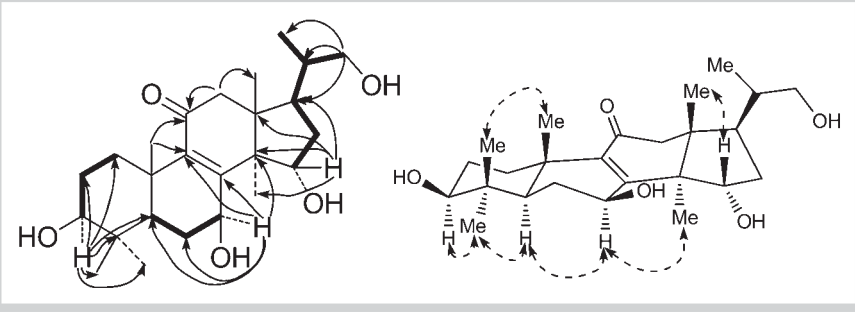

Fig. 2 Key HMBC (→), ^1H-^1H COSY (–), and NOESY (↔) correlations of **1**.

J = 6.5 Hz), three oxymethines (δ_H 3.48, 4.94, and 5.22), and an oxymethylene (δ_H 3.58 and 3.85). The ^{13}C NMR spectroscopic data analysis in combination with DEPT and HSQC spectra showed twenty-five carbon signals due to six methyls, six methylenes, six methines, and seven quaternary carbons (including an α,β-unsaturated ketone group) (**Table 2**). Detailed comparison of the ^{13}C NMR spectroscopic data showed similarity between **1** and ganoderic acid C [18], especially the signals of A–D ring carbons. Thus, we consider **1** to be a pentanorlanostane derivative. This hypothesis could be further confirmed by 2D NMR spectral analysis (**Fig. 2**). The HMBC spectrum revealed a clear correlation between H-22 (δ_H 3.85, 3.58) and C-20, C-21, C-17 indicating the attachment of the hydroxyl group to be at C-22. In addition, there were no other correlations with H-22, suggesting that C(23)-C (27) could be degraded.

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HMBC correlations observed from H-3 (δ_H 3.48) to C-1, C-2, C-4, C-5, C-28, and C-29 indicated a hydroxyl group at C-3. The similarity of ^{13}C NMR, HMBC, and ^1H-^1H COSY resonances between ganoderic acid C [18] and **1** implied an α,β-unsaturated ketone group at C-8, C-9, and C-11 as well as the other two hydroxyl groups at C-7 and C-15 (**Fig. 2**). The β orientation of the hydroxyl group at C-3 was deduced from the multiplicity of H-3 (δ_H 3.48, dd, J = 11.0, 4.7 Hz), supporting the existence of 3β,15α,22β-trihydroxylanosta-7,9(11),24-trien-26-oic acid [19]. This was further confirmed by NOE correlations observed between H-3 and H-5 in the NOESY spectrum. The β orientation of the hydroxyl group at C-7 and the α orientation of the hydroxyl group at C-15 were also supported by a NOESY experiment, which showed clear NOE correlations between H-7/H-5, H-7/H_3-30, and H-15/H_3-18 (**Fig. 2**). Thus, **1** was elucidated as a pentanorlanostane and named ganosineniol A.

Ganosinoside A (**2**) had a quasimolecular ion peak at *m/z* 651.3646 $[M + Cl]^-$ (calcd. 651.3663) in the HRESIMS, which matched a formula of $C_{36}H_{56}O_8$. The NMR spectra of **2** indicated a lanostane glycoside. The ^1H NMR spectrum of **2** exhibited one anomeric proton at δ_H 6.42 (d, J = 8.1 Hz). Acid hydrolysis of **2** liberated D-glucose by GC analysis. D-glucose was positioned at C-21 based on the HMBC correlations of H-1′ to C-21. Comparison of the ^{13}C NMR data beween **2** and 3α-acetoxy-5α-lanosta-8,24-dien-21-oic acid ester β-D-glucoside (**17**) [20] revealed that **2** had a carbonyl function at C-3 (δ_C 216.3) rather than the acetyl function in **17**, which was confirmed by the HMBC correlations of H-28 and H-29 to C-3, C-4, and C-5. Consequently, the structure of ganosinoside A was assigned as 3-oxo-5α-lanosta-8,24-dien-21-oic acid ester β-D-glucoside.

Ganoderic acid Jc (**3**) was assigned the molecular formula $C_{30}H_{44}O_5$ by HRESIMS at *m/z* 485.3251 $[M + H]^+$ (calcd. 485.3267). The ^{13}C NMR and DEPT spectra showed similarities to those of 3β,15α,22β-trihydroxylanosta-7,9(11),24-trien-26-oic acid [19]. The hydroxyl group at C-3 in the above acid was replaced by a carbonyl group in **3**, supported by HMBC correlations from H-28 (δ_H 1.09) and H-29 (δ_H 1.03) to C-3 (δ_C 215.3). A hydroxyl moiety at C-23 (δ_H 5.00, δ_C 67.1) was deduced from HMBC correlations of H-23 to C-24 and C-25 and from the ^1H-^1H COSY cross-peak of H-23 with H-24 (δ_H 7.40). The configuration of the hydroxyl group at C-15 was confirmed to have α orientation by the ^1H NMR coupling constant of H-15 (δ_H 4.60, dd, J = 9.2, 6.1 Hz), which resembled that of **1**. The configuration at C-23 was not assigned. Hence, the structure of **3** was determined as 15α,23-dihydroxy-3-oxo-5α-lanosta-7,9(11),24-trien-26-oic acid.

Ganoderic acid Jd (**4**) was obtained as a white powder. The molecular formula was $C_{27}H_{40}O_4$, based on HRESIMS data at *m/z* 451.2832 $[M + Na]^+$ (calcd. 451.2824). The ^1H and ^{13}C NMR spectra showed that **4** had the same A–D ring system as **3** except for the side chain. HMBC correlations established the structure of the side chain in **4**: the methyl signal at δ_H 0.96 (Me-21) showed a cross-peak with a methine at δ_C 36.1 (C-20) and a methylene at δ_C 32.0 (C-22); moreover, the proton signals of H-22 and H-23 exhibited correlations with C-24 (δ_C 176.4). The above observations suggested that compound **4** is a trinorlanostane with a carboxylic acid group at C-24. The α orientation of the hydroxyl group at C-15 was determined to be the same as in **3** according to the coupling constant and chemical signal of H-15. Consequently, the structure of **4** was determined as 15α-hydroxy-3-oxo-5α-lanosta-7,9(11)-dien-24-oic acid.

The molecular formula of ganodermatetraol (**5**) gave a quasimolecular ion at *m/z* 495.3445 $[M + Na]^+$ (calcd. 495.3450), corresponding to the molecular formula of $C_{30}H_{48}O_4$. ^1H and ^{13}C NMR spectroscopic data showed a close similarity between **5** and anodermatriol (**14**) [21]. In the 1D NMR spectra, the only difference between the above two compounds was the existence of an oxygenated methine signal at C-15 [δ_C 74.2 (CH)] in **5** rather than a signal of methylene in ganodermatriol. The HMBC correlations from H-15 (δ_H 4.68) to C-14, C-17, and C-30 also substantiated the above proposal. The α orientation of the hydroxyl group at C-15 was determined to be the same as that of **3**. Therefore, the structure of **5** was assigned as 3β,15α,26,27-tetrahydroxy-5α-lanosta-7,9(11),24-trien.

The molecular formula of ganolucidic acid γa (**6**) gave a quasimolecular ion at *m/z* 541.3180 $[M + Na]^+$, corresponding to the molecular formula of $C_{30}H_{46}O_7$, indicating 2 mass units less than the known compound ganoderic acid γ [22]. 1D NMR data reveals the only difference between **6** and ganoderic acid γ was that the carboxyl group at C-3 in ganoderic acid γ was replaced by a hydroxyl group in **6**. Further HMBC correlations observed from H-3 to C-4, C-28, and C-29 also demonstrated that the hydroxyl group linked to C-3. The β orientation of the hydroxyl group at C-3 and C-7, and the α orientation of the hydroxyl group at C-15 were assigned in the same way by analyzing the NOE correlations as for **1**. The configuration at C-23 was not assigned. Thus, the structure of **6** was established as 3β,7β,15α,23-tetrahydroxy-11-oxo-5α-lanosta-8,24-dien-26-oic acid.

Ganolucidate F (**7**) was isolated as a white powder. Its molecular formula was established as $C_{30}H_{47}O_6$ by HRESIMS at *m/z* 503.3415 $[M + H]^+$ (calcd. 503.3372). Comparison of the ^1H and ^{13}C NMR data between **7** and **6** indicated that they were analogous (**Table 1**). However, a methylene signal at δ_C 17.9 in **7** instead of a methine signal at δ_C 69.4 in **6** was observed. These data indicated that **7** could be a dehydroxy analogous of **6**, which was further proved by the HMBC correlations from H-7 (δ_H 1.79 m) to C-5, C-8, C-9, and C-14. Therefore, the structure of **7** was established as 3β,15α,23-trihydroxy-11-oxo-5α-lanosta-8,24-dien-26-oic acid.

Ganoderiol J (**8**) had the molecular formula of $C_{30}H_{46}O_4$ established by HRESIMS at *m/z* 471.3476 $[M + H]^+$ (calcd. for $C_{30}H_{47}O_4$, 471.3474), corresponding to seven degrees of unsaturation. The NMR spectroscopic data were compatible with those of ganoderiol E (**11**) [23] except that the hydroxyl group at C-3 in **11** was replaced by a carbonyl carbon (δ_C 214.9) in **8**, which was validated by HMBC correlations from H-28 and H-29 to C-3 (δ_C 214.9), C-4, and C-5. On the basis of these data, compound **8** was identified as 26,27-dihydroxy-5α-lanosta-8,24-dien-3,7-dione.

Methyl lucidenate Ha (**9**), obtained as a white amorphous solid, was designated the molecular formula $C_{28}H_{42}O_7$ by its HRESIMS data at *m/z* 513.2886 $[M + Na]^+$ (calcd. 513.2930). 1D NMR spectra indicated that it was a trinorcucurbitane derivative. A careful comparison of the ^1H and ^{13}C NMR spectra of **9** with those of methyl lucidenate H [24] indicated that they had the identical signals of the B, C, and D rings and the side chain. However, the ^{13}C NMR signals of C-3, C-5, C-28, and C-29 in **9** were different from those of methyl lucidenate H. In methyl lucidenate H, CH_3-28 (α-methyl) was oxidized to -CH_2OH, while CH_3-29 (β-methyl) was oxidized to -CH_2OH in **9**. This deduction was confirmed by NOE correlations between H_3-28 and H-3, H-3 and H-5α, H_2-29 and H_3-19β. The β orientation of the hydroxyl group at C-7 was proved by the NOE correlation observed between H-7 and H-5 in the NOESY spectrum. Thus, the structure of **9** was determined.

Ganosinensine (**10**) was isolated as a white powder. Its molecular formula, $C_{15}H_{22}O_3$, was deduced by HRESIMS (calcd. *m/z*

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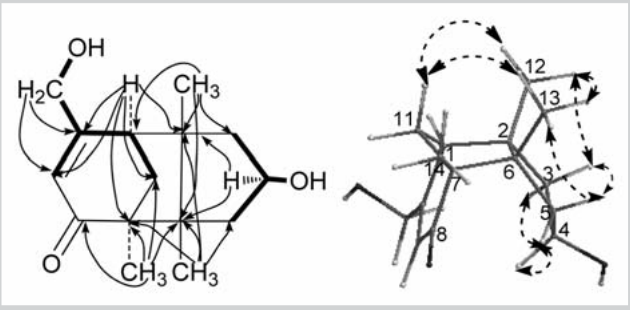

Fig. 3 Key HMBC (→), ^{1}H-^{1}H COSY (–), and NOESY (↔) correlations of **10**.

273.1466; found 273.1459, [M + Na]$^+$). The ^{1}H NMR spectrum showed signals for three methyls, one oxymethylene [δ_H 4.59 (dd, J = 18.5, 3.7 Hz) and 4.51 (dd, J = 18.5, 3.7 Hz)], one oxymethine (δ_H 4.49), and an olefinic methine (δ_H 6.64). The ^{13}C NMR spectrum together with the DEPT spectrum, showed signals for three quaternary methyls, four methylenes (one oxygenated), three methines (one olefinic, one oxygenated), and five quaternary carbons (one olefinic, one carbonyl). Five unsaturation degrees of $C_{15}H_{22}O_3$ (one belonging to a double bond and one belonging to a carbonyl group) suggested that **10** was a tricyclic sesquiterpene. 1D NMR spectroscopic data revealed that **10** and sambucinic acid [25] had the same skeleton.

The locations of hydroxyl groups and the ketone group in **10** were confirmed by the HMBC and ^{1}H-^{1}H COSY correlations (**Fig. 3**). The HMBC correlations between H-15 to C-9 (δ_C 122.5) and C-10 (δ_C 173.8), and ^{1}H-^{1}H COSY correlations between H-15 (δ_H 4.59 and 4.51) and H-9 (δ_H 6.64), enabled the oxymethylene to be placed at C-10. A conjugated ketone located at C-8 was confirmed by HMBC correlations of H-9 (δ_H 6.64) with C-8, and H-14 (δ_H 1.20) with C-8. HMBC correlations from H-4 (δ_H 4.49) to C-2 (δ_C 57.3), C-3 (δ_C 48.6), C-5 (δ_C 47.4), and C-6 (δ_C 54.5), and ^{1}H-^{1}H COSY correlations of H-4/H-3 and H-4/H-5, enabled the hydroxyl group to be placed at C-4. Further HMBC correlations of H-12 (δ_H 1.30) with C-1 (δ_C 50.1), C-2, C-3, and C-6 proved a methyl attaching to C-2.

The relative configuration was assigned by a NOESY experiment (**Fig. 3**). The NOESY correlations of H-4/H-3 (δ_H 2.25) and H-4/H-5 (δ_H 2.23), of H-3 (δ_H 1.72)/H-12 (δ_H 1.30) and H-3/H-5 (δ_H 1.59), of H-5 (δ_H 1.59)/H-13 (δ_H 1.16) and H-5/H-3 (δ_H 1.72) established the configurations of H-4α, H-12β, and H-13β. The NOESY correlations of H-11 (δ_H 1.95) to H-12 (δ_H 1.30) and H-13 (δ_H 1.59) suggested that the bridge-ring and the two methyls were in the same direction, which further indicated the configurations of H-1α and H-14α. Therefore, the structure of **10** was fully established and named ganosinensine.

The known compounds were identified as ganoderiol E (**11**) [23], ganoderiol F (**12**) [23], lucidumol B (**13**) [26], ganodermatriol (**14**) [21], ganodermanontriol (**15**) [27], ganoderate E (**16**) [28], 3α-acetoxy-5α-lanosta-8,24-dien-21-oic acid ester β-D-glucoside (**17**) [20], ganoderic acid A (**18**) [18], ganolucidic acid B (**19**) [29], ganolucidic acid C (**20**) [30], and lucidadiol (**21**) [31], compatible with spectroscopic data in the literature.

All of the isolates (purities > 90%) were evaluated for their cytotoxicity against the human HL-60, SMMC-7721, A-549, MCF-7, and SW480 cell lines using the MTT assay. Compared with positive control cisplatin, ganoderic acid Jc (**3**) displayed selective inhibitory activity against MCF-7 cells with an IC_{50} value of 8.30 µM, and ganoderiol E (**11**) exhibited selective cytotoxic activity against HL-60 cells with an IC_{50} value of 6.35 µM (**Table 3**). Others were inactive with IC_{50} values of > 40 µM (data not shown).

The abilities to induce hPXR-mediated CYP3A4 expression were assessed for compounds **1–21** by an *in vitro* screening model. As shown in **Fig. 4**, compounds **5, 7, 19**, and **20** exhibited more potent ability to activate hPXR-mediated CYP3A4 expression than 0.05% DMSO but a lower ability than RIF, whereas other compounds could not activate CYP3A4 expression as their induction abilities were lower than 0.05% DMSO.

Compounds	HL-60	SMMC-7721	A-549	MCF-7	SW480
3	8.30	> 40	> 40	> 40	> 40
11	21.70	> 40	> 40	6.35	> 40
Cisplatin (MW300)	3.17	11.27	16.46	18.70	8.00

Table 3 Cytotoxic activity of compounds **3** and **11** (IC_{50} values: µM).

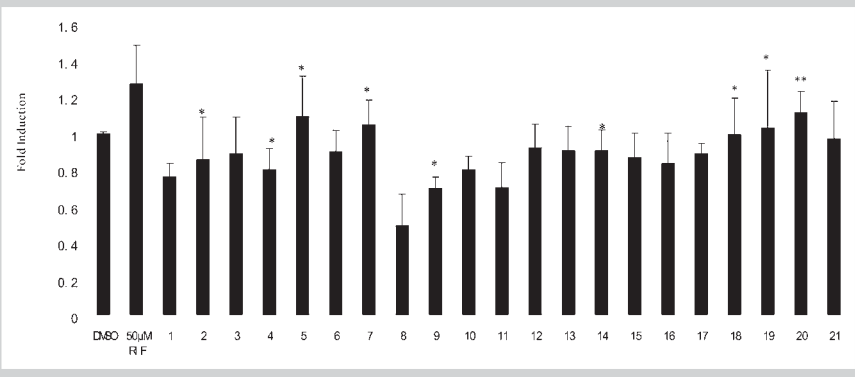

Fig. 4 The induction of PXR-mediated CYP3A4 expression by compounds **1–21**. Compounds (50 µM) **1–21** induce the expression of CYP3A4 in Huh7 cells. RIF was included as a positive control for CYP3A4 induction. Results are shown as fold induction over vehicle control and represent the average from triplicate assays. Each value represents the mean ± SD (n = 3).* $P < 0.05$, ** $p < 0.01$, compared to 50 µM RIF.

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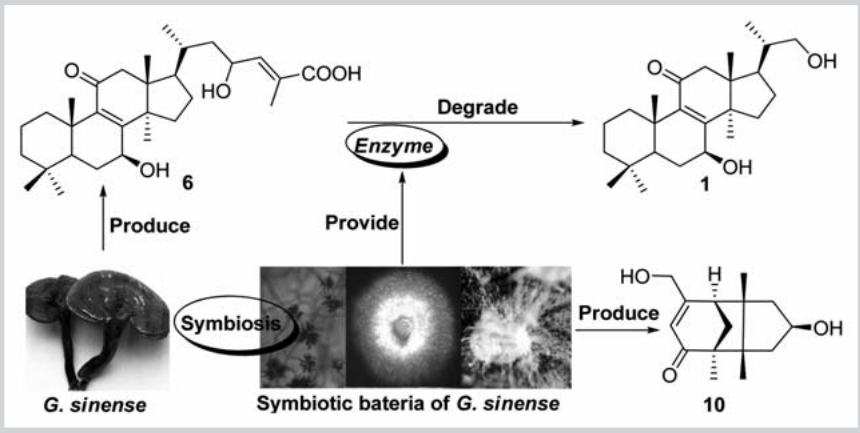

Fig. 5 The possible producing pathway of **1** and **10** in *G. sinense*.

Discussion

Ganosineniol A (**1**) was the first pentanorlanostane triterpenoid isolated from the genus *Ganoderma*, while its analogues appears to be 23,24,25,26,27-pentanorlanost-8-en-3,22-diol [32], previously isolated from the bacteria of *Verticillium lecanii*. Sambucinic acid [25], the closest analogue of ganosinensine (**10**), was also isolated from the bacteria of *Fusarium sambucinum*. Previous literature seemed to tell us that **1** and **10** would be produced or coproduced by the symbiotic bacteria of *G. sinense*. In other words, the symbiotic bacteria may provide the related enzyme which could degrade compound **6** into **1**, and may produce **10** automatically due to the absence of sesquiterpenes in the genus *Ganoderma* (**Fig. 5**). Thus, it seems that it was worth researching the symbiotic bacteria of *G. sinense* to discover more bioactive constituents.

Many adverse drug reactions or poor therapeutic responses to drugs can be traced back to CYP3A4 (one of the cytochrome P450s) activity [33]. The bioassay results showed that compounds **5, 7, 19**, and **20** can, to some extent, activate hPXR-mediated CYP3A4 expression for more fold inductions than 0.05% DMSO. So, compounds **5, 7, 19**, and **20** may have some effect on the coadministered drugs metabolism.

Acknowledgements

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Conflict of Interest

All authors declare that there are no conflicts of interests. Isolation and structural elucidation were accomplished by Jie-Qing Liu, Cui-Fang Wang, and Ming-Hua Qiu; cell culture and cytotoxicity assays were performed by Yan Li, and the induction of CYP3A4 assay was performed by Huai-Rong Luo. All the authors knew about this manuscript and had no objection to submitting it.

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