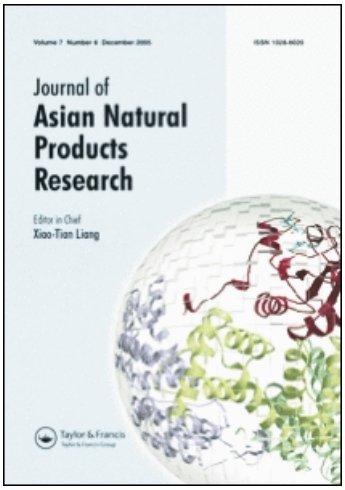

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

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To cite this Article Guan, Shu-Hong , Xia, Jia-Meng , Yang, Min , Wang, Xiao-Ming , Liu, Xuan and Guo, De-An(2008) 'Cytotoxic lanostanoid triterpenes from *Ganoderma lucidum*', Journal of Asian Natural Products Research, 10: 8, 695 — 700
To link to this Article: DOI: 10.1080/10286020802016297
URL: http://dx.doi.org/10.1080/10286020802016297

Journal of Asian Natural Products Research
Vol. 10, No. 8, August 2008, 695–700

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(*Received 28 April 2007; final version received 25 July 2007*)

Two new lanostanoid triterpenes, 23*S*-hydroxy-3,7,11,15-tetraoxo-lanost-8,24*E*-diene-26-oic acid (**1**), and 12β-acetoxy-3β-hydroxy-7,11,15,23-tetraoxo-lanost-8,20*E*-diene-26-oic acid (**16**), together with 17 known compounds, were isolated from the fruit bodies of *Ganoderma lucidum*. Their structures were established by spectroscopic methods, especially 2D-NMR and MS analyses and by comparison with literature data. The cytotoxic assay of the above compounds against p388, Hela, BEL-7402, and SGC-7901 human cancer cell lines showed their cytotoxicity with the IC_{50} values in the range of 8–25 μm.

Keywords: lanostanoid triterpenes; *Ganoderma lucidum*; fruit bodies; cytotoxicity

1. Introduction

Lingzhi (*Ganoderma lucidum*), a well-known traditional Chinese medicine, has been used clinically in China and other Asian countries for thousands of years. Ancient Chinese believed that it could cure various diseases and worshipped it as an 'immortal herb'. Modern research revealed the bioactivity components of *G. lucidum* to be triterpenes and polysaccharides, which were reported to possess anti-virus [1], anti-inflammation [2], anti-tumor (including cytotoxic and antimutagenic activities) [3,4], immunity-promoting [5], anti-diabetic [6] effects, etc. In order to elucidate the cytotoxic activity and evaluate the quality of *G. lucidum*, the guided isolation of triterpenes from the fruit bodies of *G. lucidum* were carried out. In our previous papers, we reported the isolation of two new lanostanoid triterpenes [7] and the quality control of *G. lucidum* [8,9]. Our further study led to the isolation of two new lanostanoid triterpenes together with other 17 known compounds with three types of structural skeleton. They all showed cytotoxicity *in vitro* against p388, Hela, BEL-7402, and SGC-7901 cell lines.

2. Results and discussion

Compound **1** was isolated as white powder, with $[\alpha]_D^{25} = +163.0$ ($c = 0.13$, MeOH). The determination of its molecular formula ($C_{30}H_{40}O_7$) was based on HREIMS analysis. In ^{1}H NMR spectrum, there were six quaternary methyls (one of which appeared at δ 1.74) and one tertiary methyl at δ 0.95. The lowest-field proton signal at δ 6.44 (1H, d, $J = 8.4$ Hz) suggested a methyl-substituted double bond considering the downfield quaternary methyl signal at δ 1.74. Proton signal at δ 4.40 (1H, m) combining its corresponding carbon signal at δ 65.4 observed in the HSQC spectrum did not show any characteristics of the usual hydroxy substitution in the rings of the molecule

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ISSN 1028-6020 print/ISSN 1477-2213 online

DOI: 10.1080/10286020802016297
http://www.informaworld.com

(at C-3, C-7, C-12, and C-15 positions), hence the hydroxyl group was presumed to attach to the side chain. This was confirmed by $^1H-^1H$ COSY and HMBC spectra. In the $^1H-^1H$ COSY spectrum, the proton signal at δ 4.40 was coupled with proton signals at δ 1.39 (H-22) and 6.44 (H-24), and the proton signal at δ 6.44 was coupled with proton signals at δ 4.40 and 1.74 (H-27), respectively. In the HMBC spectrum, correlations between the proton at δ 1.74 (H-27) and carbons at δ 169.0 (C-26, downfield), 144.4, and 126.9 implied that the methyl-substituted double bond was located at the terminal carboxylic carbon in the side chain. The structure of compound **1** was similar to that of known compounds ganolucidic acid D [10], ganoderic acids δ, ε, γ, ξ [11], and ganoderic acid LM_2 [12], except for its highly oxygenated four rings. Four carbonyl groups were positioned at C-3, C-7, C-11, and C-15, which were readily assigned by its HSQC and HMBC spectra. The configurations of the quaternary methyls in the rings were accordant with those of the above known compounds, which were confirmed by the ROESY spectrum. *S*-Configuration of C-23 was determined by comparison of the coupling constant of $J_{\text{H-23/H-24}}$ (8.4 Hz) with the known compounds [10–12] and confirmed by a modification of Mosher's method (In the ^{1}H-NMR spectrum of (*S*)-(−)-MTPA ester, proton signals assigned for H-20, H-21, and H-22 were observed at a field higher than those in the (*R*)-(+)-MTPA ester, while proton signals of H-24 and H-27 in the former ester were shifted to a field lower than those in the latter ester). *E*-configuration of the double bond in the side chain was clearly followed due to the carbon chemical shift (δ 12.7) of its substituted methyl [13], which was also in accordance with the above known compounds. Therefore, compound **1** was assigned as 23*S*-hydroxy-3,7,11,15-tetraoxo-lanost-8,24*E*-diene-26-oic acid.

Compound **16** was isolated as white needles, with $[\alpha]_D^{25} = +64.0$ ($c = 0.12$, MeOH). Its molecular formula was determined to be $C_{32}H_{42}O_9$ (*m/z* 570.2812, calcd 570.2829) on the basis of HR-EI-MS analysis, which indicated a molecule with 12 degrees of unsaturation. In the ^{13}C NMR and DEPT spectra, four carbonyl groups at δ 204.6, 198.5, 197.8, and 193.5, four olefinic carbon signals at δ 151.9 (C), 145.8 (C), 154.7 (C), and 126.0 (CH, s; implying two double bonds with three substituted and one unsubstituted olefinic carbons), one carboxyl group at δ 180.2, and one carboxyl ester group at δ 170.3 were observed, requiring eight degrees of unsaturation. The left four degrees of unsaturation suggested the presence of four rings in the molecule. In the ^{1}H NMR spectrum, eight methyl groups were discerned, seven of which were quaternary methyl groups [including two methyl groups that appeared in the low field (δ 2.12, 2.10), implying acetyl methyl or olefinic methyl], except for one tertiary methyl group at δ 1.21 (d, $J = 7.2$ Hz). Above all, observations indicated that **16** was also a lanostanoid triterpene. In the HMBC spectrum, the correlations between the proton at δ 5.71 and the ester carbon at δ 170.3, the carbonyl carbon at δ 193.5, and the quaternary carbon at δ 57.8, and between the methyl proton at δ 2.10 and the ester carbon at δ 170.3, indicated an acetyl group attached to C-12 position. The correlation between the methyl proton at δ 2.12 and olefinic carbons at δ 154.7 and 126.0 (CH), and between the proton at δ 3.32 (H-17) and the substituted olefinic carbon at δ 154.7, suggested a methyl-substituted double bond in the side chain attached to C-17 position as that of ganoderenic acid F [14]. The proton signals due to an olefinic methyl group at δ 2.12 (3H, d, $J = 0.8$ Hz) and an olefinic proton at δ 6.11 (1H, d, $J = 0.8$ Hz), together with the NOE between H-22 and H-17, indicated the *E*-configuration[20,22] of Δ double bond by analogy with ganoderenic acids A–E [15]. β-Configuration hydroxyl group in C-3 position was determined by the typical chemical shifts and coupling constants of α-configuration of H-3 [observed at δ_H 3.25 (dd, $J = 4.8$, 11.2 Hz)], which was also supported by the $^1H-^1H$ COSY, HMBC, and NOESY spectral data. In the NOESY spectrum, the correlation between

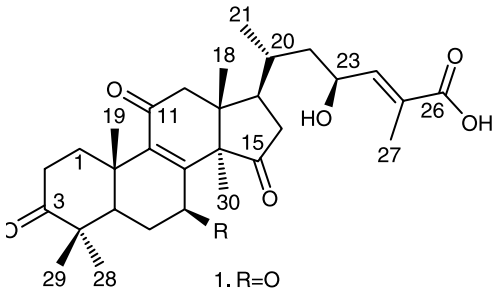
the protons at δ 5.71 (H-12) and 0.82 (H-18) confirmed the β-configuration of the acetyl group in the molecule (Figure 1). From the above all deduction, compound **16** was finally established as 12β-acetoxy-3β-hydroxy-7,11,15,23-tetraoxo-lanost-8,20*E*-diene-26-oic acid.

The 17 known compounds **2**–**15** and **17**–**19** were identified by the spectroscopic and literature data as ganoderic acid LM_2 (**2**) [12], ganoderic acid B (**3**) [16], ganoderic acid G (**4**) [17], ganoderic acid B methyl ester (**5**) [16], ganoderic acid K (**6**) [18], ganoderic acid D (**7**) [16], ganoderic acid D methyl ester (**8**) [16], ganoderic acid A (**9**) [19], ganoderic acid AM_1 (**10**) [20], ganoderic acid H (**11**) [17], ganoderic acid J (**12**) [21], ganoderic acid F (**13**) [22], ganoderic acid C_6 (**14**) [23], ganoderic acid C_2 (**15**) [16], ganoderenic acid A (**17**) [22], ganoderenic acid B (**18**) [22], and ganoderenic acid H (**19**) [24], respectively.

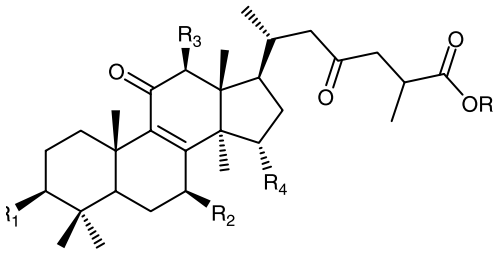
3. Experimental

3.1 General experimental procedures

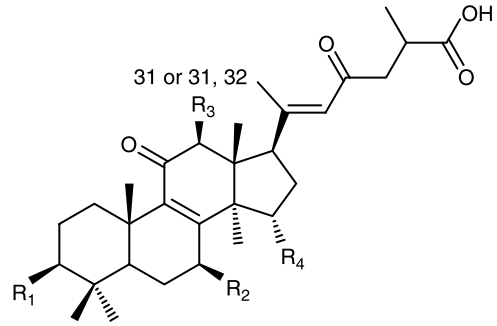
Optical rotations were measured on a PE-341 polarimeter. CD spectra were recorded on a JASCO J-810 spectropolarimeter. IR spectra were obtained using a Nicolet-Magna-FT-IR 750 spectrometer. UV spectra were recorded on a Shimadzu UV-2450 spectrophotometer. NMR spectra were recorded on a Varian Mercury-plus 400 (1D) and Varian Inova-600 (2D) spectrometer, with chemical shifts given as δ values with reference to tetramethylsilane as an internal standard. EI-MS and HR-EI-MS spectra were recorded on a MAT 95 XL Thermo Finnigan mass spectrometer. Sephadax LH-20 (Amersham Biosciences, Piscataway, NJ, USA) and silica gel (Qing Dao Marine Chemical Group Co., Qingdao, China; 200–300 and 400–600 mesh) were used for column chromatography. Precoated silica gel plates (Yan Tai Zi Fu Chemical Group Co, Yan Tai China; G60 F-254) were used for TLC analysis. HPLC semi-preparation was fulfilled on an Agilent 1100 series



1. R=O
2. R=OH



3. R_1=OH,R_2=OH,R_3=H,R_4=O,R_5=H
4. R_1=OH,R_2=OH,R_3=OH,R_4=O,R_5=H
5. R_1=OH,R_2=OH,R_3=H,R_4=O,R_5=CH_3
6. R_1=OH,R_2=OH,R_3=OAc,R_4=O,R_5=H
7. R_1=O,R_2=OH,R_3=H,R_4=O,R_5=H
8. R_1=O,R_2=OH,R_3=H,R_4=O,R_5=CH_3
9. R_1=O,R_2=OH,R_3=H,R_4=OH,R_5=H
10. R_1=OH,R_2=O,R_3=H,R_4=O,R_5=H
11. R_1=OH,R_2=O,R_3=OAc,R_4=O,R_5=H
12. R_1=O,R_2=O,R_3=H,R_4=OH,R_5=H
13. R_1=O,R_2=O,R_3=OAc,R_4=O,R_5=H
14. R_1=OH,R_2=O,R_3=OH,R_4=O,R_5=H
15. R_1=OH,R_2=OH,R_3=H,R_4=OH,R_5=H



16. R_1=OH,R_2=O,R_3=OAc,R_4=O
17. R_1=O,R_2=OH,R_3=H,R_4=OH
18. R_1=OH,R_2=OH,R_3=H,R_4=O
19. R_1=OH,R_2=O,R_3=H,R_4=O

Figure 1. Chemical structures of compounds **1**–**19**.

Table 1. NMR spectral data of compounds **1** and **16** (^{1}H at 600 MHz; ^{13}C at 100 MHz).

	^{1}H NMR		^{13}C NMR	
	1[a]	**16**[b]	**1**[a]	**16**[b]
1α 1β	1.76 (1H, overlapped) 2.60 (1H, overlapped)	1.16 (1H, overlapped) 2.72 (1H, overlapped)	34.0	33.3
2α 2β	2.64 (1H, overlapped) 2.28 (1H, overlapped)	1.70 (2H, overlapped)	33.5	27.3
3	/	3.25 (1H, dd, J = 4.8, 11.2 Hz)	215.5	77.4
4	/	/	38.7	39.1
5	2.31 (1H, overlapped)	1.54 (1H, dd, J = 3.2, 14.0 Hz)	49.7	51.2
6α 6β	2.30 (1H, overlapped) 2.72 (1H, overlapped)	2.60 (1H, overlapped) 2.66 (1H, overlapped)	36.8	36.5
7	/	/	199.6	198.5
8	/	/	146.1	145.8
9	/	/	149.0	151.9
10	/	/	46.4	40.5
11	/	/	199.8	193.5
12α 12β	2.54 (1H, overlapped) 3.06 (1H, d, J = 16.4 Hz)	5.71 (1H, s)	48.5	78.5
13	/	/	56.8	57.8
14	/	/	43.5	48.7
15	/	/	207.8	204.6
16α 16β	2.92 (1H, dd, J = 9.6, 18.4 Hz) 1.64 (1H, dd, J = 8.0, 18.4 Hz)	2.74 (1H, dd, J = 9.6, 18.4 Hz) 2.38 (1H, dd, J = 8.8, 18.4 Hz)	39.9	37.6
17	2.20 (1H, m)	3.32 (1H, dd, J = 8.8, 9.6 Hz)	44.4	48.9
18	0.70 (3H, s)	0.82 (3H, s)	15.4	13.3
19	1.14 (3H, s)	1.30 (3H, s)	18.4	17.8
20	1.36 (1H, overlapped)	/	32.9	154.7
21	0.95 (3H, overlapped)	2.12 (3H, d, J = 0.8 Hz)	19.7	21.1
22	1.39 (2H, overlapped)	6.11 (1H, d, J = 0.8 Hz)	42.8	126.0
23	4.40 (1H, m, H-23)	/	65.4	197.8
24	6.44 (1H, d, J = 8.4 Hz)	2.52 (1H, dd, J = 4.0, 17.2 Hz) 3.88 (1H, overlapped)	144.4	47.5
25	/	2.95 (1H, overlapped)	126.9	34.4
26	/	/	169.0	180.2
27	1.74 (3H, s)	1.21 (1H, d, J = 7.2 Hz)	12.7	17.0
28	1.02 (3H, s)	1.01 (3H, s)	27.0	27.9
29	0.97 (3H, s)	0.87 (3H, s)	19.9	15.5
30	1.56 (3H, s)	1.74 (3H, s)	20.0	21.3
31		/		170.3
32		2.10 (3H, s)		20.5

[a] Measured in DMSO-d_6.
[b] Measured in $CDCl_3$.

and Eclipse XDB-C_{18} reversed-phase column (5 μm, 250 × 9.4 mm) with an Eclipse XDB-C_{18} guard column (both from Agilent).

3.2 Plant material

The fruit bodies of *G. lucidum* were collected in July 2006 from Wu Yi Mountain (Fujian Province) GAP cultivation base of Green Valley Pharmaceutical Co. Ltd, China. The voucher specimen (No. 200608008) has been deposited in Shanghai Research Center for TCM Modernization.

3.3 Extraction and isolation

The fruit bodies of *G. lucidum* (1 kg) were extracted as our previous report [7]. The $CHCl_3$ layer after dryness (20 g) under

Table 2. Cytotoxicity of compounds **1**–**19** (IC_{50} μm).

Compounds	Cell lines			
	p388	Hela	BEL-7402	SGC-7901
1	15.7	9.72	25.6	23.1
2	13.4	10.7	20.6	21.5
3	12.9	10.5	21.1	22.8
4	16.9	9.41	23.5	30.7
5	11.7	10.0	25.0	20.1
6	13.8	8.23	16.5	21.0
7	14.3	9.34	23.2	21.2
8	12.4	7.95	21.2	27.0
9	13.6	9.47	20.8	26.5
10	13.2	9.75	20.9	23.0
11	14.9	8.39	20.4	20.7
12	15.8	12.2	25.2	20.2
13	13.8	9.62	19.1	18.4
14	11.9	8.97	17.6	21.2
15	15.0	11.5	22.0	18.2
16	12.7	8.72	24.2	18.7
17	11.2	10.6	23.5	22.3
18	13.6	10.0	18.6	20.4
19	16.5	9.75	17.6	19.8
VP-16	0.08	4.50	2.92	4.80

vacuum was chromatographed on a silica gel column (4 × 45 cm) eluted with a gradient $CHCl_3$/MeOH (30:1-5:1) and further chromatographed on a Sephadex LH-20 column (3 × 100 cm) eluted with MeOH, and then rechromatographed on a silica gel column (2 × 40 cm) eluted with hexane:EtOAc:HOAc (2:1:0.03-1:2:0.03). Finally, all compounds were purified by semi-preparative HPLC. New compound **1** (7.0 mg) was obtained by HPLC using MeOH:0.05% HOAc/H_2O = 40:60 (270 nm, 25°C). Compound **16** (4.5 mg) was purified by HPLC using MeOH:0.05%HOAc/H_2O:MeCN = 52:43:5 (270 nm, 25°C).

3.3.1 *Compound **1***

White powder (MeOH); $[\alpha]_D^{25}$ + 163.0 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.75), 251 nm (4.39); IR (KBr) ν_{max} 3433 (br.), 2974, 2929, 1747, 1703, 1678, 1460, 1425, 1387, 1228, 1176, 1126, 1049 cm^{-1}. For ^1H NMR and ^{13}C NMR spectral data see Table 1. EIMS (*m*/*z*, %): 512 $[M]^+$ (100), 494 (52), 149 (40), 121 (32); HREIMS *m*/*z* 512.2775 $[M]^+$ (calcd for $C_{30}H_{40}O_7$, 512.2774).

3.3.2 *Compound **16***

White powder (MeOH); $[\alpha]_D^{25}$ + 64.0 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 244 nm (4.59); IR (KBr) ν_{max} 3437 (br.), 2976, 2937, 1751, 1693, 1612, 1462, 1375, 1228, 1194, 1123, and 1040 cm^{-1}. For ^1H NMR and ^{13}C NMR spectral data see Table 1. EIMS (*m*/*z*, %): 570 $[M]^+$ (12), 552 (16), 529 (40), 528 (100), 510 (80), 495 (20), 331 (16), and 304 (36); HREIMS *m*/*z* 570.2812 $[M]^+$ (calcd for $C_{32}H_{42}O_9$, 570.2829).

3.4 *Cytotoxicity assay*

Growth inhibitory effect of all compounds on tumor cell lines (p388, Hela, BEL-7402 and SGC-7901) was measured by microculture tetrazolium assay [25]. Etoposide (VP-16)

was used as reference substance. The cytotoxic effects of 19 compounds against p388, BEL-7402, SGC-7901, and Hela cells were assayed as in our previous method [7]. Results were expressed as IC_{50} that was calculated by the Logit method. Finally, the mean IC_{50} was calculated using the data from three replicate tests (Table 2).

Acknowledgements

This work was supported by the National Natural Science Foundation of China (30701077).

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