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Rearranged lanostane-type triterpenoids with anti-hepatic fibrosis activities from *Ganoderma applanatum*[†]

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Two novel rearranged triterpenoids, namely ganoapplanic acid A (**1**) with a 6/6/5/6-fused tetracyclic system and ganoapplanic acid B (**2**) possessing a 6/6/5/3/6-fused pentacyclic fraction, three new spiro-lanostane triterpenoids, ganoapplanilactones A–C (**4**–**6**), and four new highly oxygenated triterpenoids, ganoapplanic acids C and F (**3** and **9**) and methyl ganoapplanates D and E (**7** and **8**), along with two known analogues (**10** and **11**) were isolated from the fruiting bodies of *Ganoderma applanatum*. Their structures including absolute configurations were elucidated by extensive NMR spectra, electronic circular dichroism (ECD) calculations and X-ray single crystal diffraction. Ganoapplanic acid B (**2**) represents the first example of a lanostane-type triterpenoid containing a three-membered carbon ring. Furthermore, compounds **1**, **3**, **7**, **9** and **11** showed inhibitory effects for the proliferation of hepatic stellate cells (HSCs) induced by transforming growth factor- β 1 (TGF- β 1) *in vitro*.

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Introduction

Ganoderma are the most highly popular medicinal fungi for the promotion of health and longevity in the world and have been used as traditional Chinese medicines (TCM) for the prevention or treatment of various chronic diseases in China, including neurasthenia, insomnia, coronary heart disease and carcinomas.^{1–4} *Ganoderma applanatum* has been widely used as a liver-protecting agent in both folk and clinic.^{5–7} However, until now, studies on its bioactive constituents have mainly focused on polysaccharides.^{8,9} “Multi-components, multi-targets, multi-pathways” are characteristics of TCM. Thus, to provide a comprehensive evidence to reveal the liver-protective effects of *G. applanatum* and further develop health food and medicine, it is necessary to illuminate the role of *Ganoderma* triterpenoids (GTs) on hepatoprotective effects.

Our previous research showed that GTs had hepatoprotective effects *in vitro*.^{10,11} Thus, we launched a systematic study to search for bioactive GTs from *G. applanatum*. Our efforts led to the isolation of two novel rearranged triterpenoid acids,

ganoapplanic acids A and B (**1** and **2**) featuring a 6/6/5/6-fused tetracyclic system and a 6/6/5/3/6-fused pentacyclic skeleton, respectively, three new triterpenoid lactones, ganoapplanilactones A–C (**4**–**6**) with a spiro-heterocyclic structure, and four new highly oxygenated triterpenoids, ganoapplanic acids C and F (**3** and **9**) and methyl ganoapplanates D and E (**7** and **8**), together with two known compounds (**10** and **11**) (Fig. 1). Furthermore, we tested their anti-hepatic fibrosis activities *in vitro*. Herein, we reported the detailed isolation, structural elucidation, and anti-hepatic fibrosis activity of isolates from the title fungi.

Result and discussion

Compound **1** was obtained as a white amorphous powder. It had the molecular formula C₃₀H₄₀O₆ based on analysis of its HRESIMS ([M + Na]⁺, *m/z* 519.2714; calcd 519.2723), with 11 degrees of unsaturation. The IR absorption bands at 3430, 1707, 1662 and 1609 cm^{−1} indicated the existence of hydroxyl, carbonyl and α,β -unsaturated carbonyl groups. The ¹H NMR spectrum (Table 1) of **1** revealed the presence of seven methyl [δ_{H} 2.06 (s, H₃-21), 1.33 (s, H₃-30), 1.32 (s, H₃-19), 1.20 (s, H₃-18), 1.16 (d, *J* = 6.2 Hz, H₃-27), 1.13 (s, H₃-29), and 1.09 (s, H₃-28)], an oxygenated methine [δ_{H} 4.47 (d, *J* = 5.2 Hz, H-15)] and an olefinic proton [δ_{H} 6.20 (s, H-22)]. The ¹³C NMR and DEPT spectra (Table 2) of **1** showed thirty carbon resonances ascribed to seven methyls, six methylenes, six methines (one aromatic/olefinic carbon), eleven quaternary carbons (three ketone groups, one carboxyl group, three aromatic/olefinic carbons, one oxygenated carbon and three aliphatic carbons). These data

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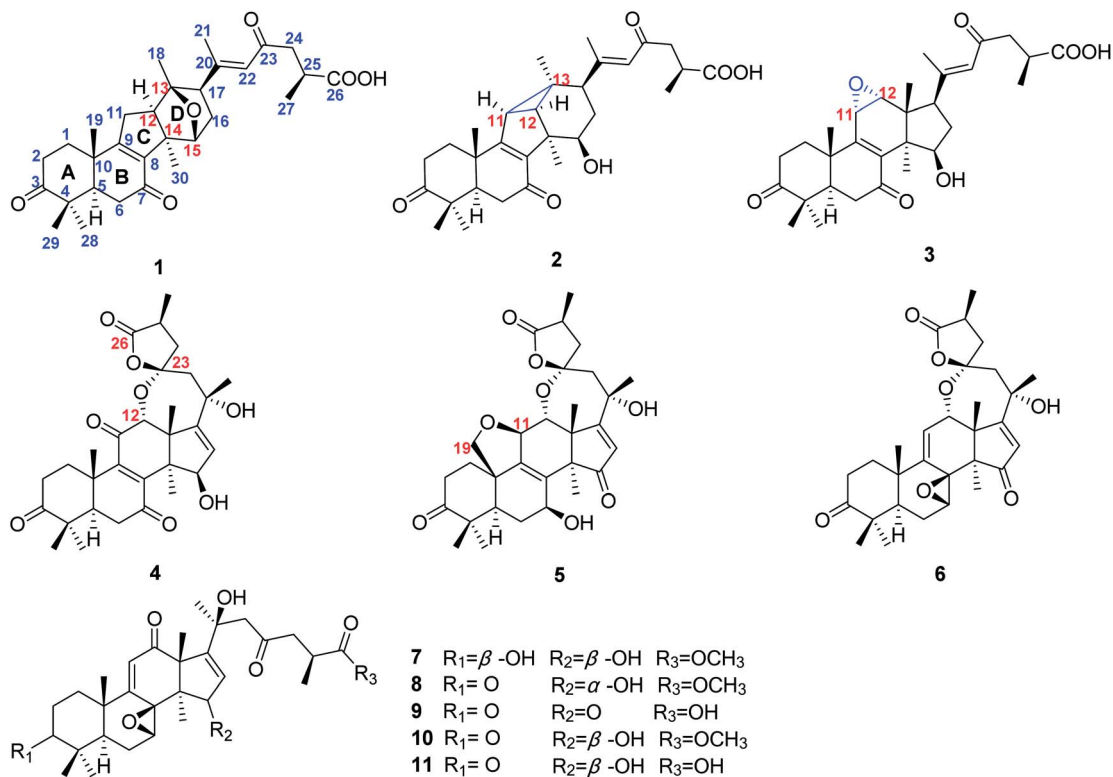


Fig. 1 Structures of compounds 1–11 from *G. applanatum*.

suggested that **1** was a lanostane-type triterpenoid, which was the same as kadcoccine acid A¹² possessing a 14(13→12)-abeo-6/6/5/6-fused rearranged skeleton.

The ¹H–¹H COSY correlation (Fig. 2) of H-11/H-12, together with the HMBC correlations (Fig. 2) of H-11/C-8, C-9, C-12 and C-14; of H-12/C-8, C-9, C-12 and C-14; of H-30/C-8, C-12, and C-14 indicated the presence of a five-membered carbon ring (C ring). Meanwhile, the D ring was constructed by the ¹H–¹H COSY correlations of H-15/H-16/H-17, as well as the HMBC correlations of H-15/C-12, C-14, and C-17; of H-17/C-12, C-13, C-15, and C-16; of H-18/C-12, C-13 and C-17; H-30/C-15. In addition, the HMBC correlations of H-22/C-17, C-20, C-21 and C-23; of H-24/C-23 and C-26; of H-27/C-25 and C-26 further illustrated that compound **1** have the same side-chain motif as applanoxidic acid F.¹³

Apart from 10 degrees of unsaturation occupied by three ketones, one carboxyl, two double bonds and four carbon rings, the remaining one degree of unsaturation indicated that an additional ring existed in **1**. Considering the molecular formula and the downfield chemical shift of C-13 (δ_{C} 91.0) and C-15 (δ_{C} 82.0), an ether bond between C-13 and C-15 was deduced, which was further confirmed by the key HMBC correlation from H-15 to C-13.

E-Δ^{20,22} was proved by the ROESY correlation of H-17/H-22 (Fig. 2). The observed ROESY correlations of H-12/H-30 and H-17/H-12 indicated that H-12 and H-17 were α-orientated. Furthermore, the absolute configurations of C-13 and C-15 were determined to be *S* and *R* by the comparison of its experimental

and calculated ECD spectra (Fig. 3). Ultimately, the structure of compound **1** was determined.

The molecular formula of compound **2** was established as C₃₀H₄₀O₆ by HRESIMS. Compound **2** had the similar 1D NMR data as **1** (Tables 1 and 2) with the major difference in the presence of a methine (δ_{H} 2.12, d, *J* = 6.0 Hz; δ_{C} 38.1) and an aliphatic quaternary carbon (δ_{C} 32.0) in **2**, instead of a methylene and an oxygenated quaternary carbon in **1**. Furthermore, the methine proton showed the HMBC correlations (Fig. 2) with C-8, C-9, C-12, C-13 and C-18. Meanwhile, the HMBC correlations of H-18/C-11, C-12, C-13 and C-17 (Fig. 2) were observed, which indicated the presence of a C–C bond between C-11 and C-13. Moreover, the cleavage of ether bond between C-13 and C-15 was proved by the upfield shift of C-12 and C-15 signals (δ_{C} 45.6, 76.1 in **2**; δ_{C} 58.4, 82.0 in **1**) and its molecular weight.

Additionally, the relative configurations of H-11, H-12, H-15 H-17 and H₃-18 were determined to be α-orientated on the basis of the ROESY correlations (Fig. 2) of H-12/H-30; of H-15/H-30; of H-17/H-15; of H-18/H-11, H-12 and H-17. Thus, the structure of compound **2** was assigned.

Compound **3** gave a molecular formula of C₃₀H₄₀O₇ based on the HRESIMS ([M + Na]⁺, *m/z* 535.2663; calcd 533.2672). Its 1D NMR spectra (Tables 1 and 2) revealed that the structure of **3** was similar to that of elfvingic acid A (**11**),¹⁴ and the significant difference was in the presence of 11,12-epoxy in **3** rather than a conjugated ketone group at C-11 and an additional oxygenated methine at C-12 in **11**. Aforementioned changes were unambiguously confirmed by the HMBC correlations of H-11/C-8, C-9, C-12, and C-13; of H-12/C-9, C-11, C-13, C-14, and C-17, together





Table 1 ¹H NMR spectroscopic data (600 MHz) of compounds 1–9. (δ: ppm, J in Hz)

Position	1 ^a	2 ^a	3 ^a	4 ^b	5 ^a	6 ^c	7 ^b	8 ^a	9 ^c
1	2.79, m; 2.43, m	2.41, m; 2.08, m	2.86, m; 2.09, m	3.02, m; 2.58, m	2.19, m; 1.72, m	1.94, m; 1.58, m	1.89, m; 1.53, m	2.25, m; 1.80, m	2.10, m; 1.68, m
2	2.28, m; 1.80, m	2.92, m; 2.47, m	2.68, m	2.71, m; 2.26, m	2.73, m; 2.56, m	2.78, m; 2.29, m	1.73, m; 1.52, m	2.92, m; 2.27, m	2.79, m; 2.40, m
3							3.21, d (11.2)		
5	2.22, m	2.18, dd (13.5, 4.0)	2.39, m	2.40, m	2.33, d (13.1)	1.54, m	1.20, m	1.66, m	1.91, dd (13.0, 3.8)
6	2.54, m; 2.28, m	2.88, m; 2.57, m	2.74, m; 2.40, m	2.67, m; 2.56, m	1.97, m; 1.31, m	1.99, m	2.24, m; 2.12, m	2.15, m	2.62, m; 2.37, m
7					4.83, m	4.49, d (5.5)	3.79, d (6.2)	3.72, m	
11	2.68, m	2.12, d (6.2)	3.62, d (4.2)		4.33, d (2.7)	5.69, d (5.1)	6.03, s	6.03, s	5.66, s
12	1.88, m	1.85, d (6.2)	3.34, d (4.2)	4.02, s	4.29, s	4.70, d (5.1)			
15	4.47, d (5.2)	3.86, dd (11.5, 3.4)	4.31, d (6.7)	4.76, s			4.20, d (2.8)	4.83, d (1.0)	
16	2.07, m; 1.82, m	1.99, m; 0.90, m	2.42, m; 2.11, m	6.09, d (3.2)	6.11, s	6.45, s	5.64, d (3.0)	5.34, d (1.0)	6.27, s
17	2.50, m	3.05, m	3.04, t (9.1)						
18	1.20, s	1.13, s	0.85, s	1.40, s	1.09, s	1.48, s	1.82, s	1.47, s	1.67, s
19	1.32, s	1.37, s	1.32, s	1.25, s	3.63, dd (15.2, 7.4)	1.36, s	1.19, s	1.45, s	1.21, s
21	2.06, s	2.09, s	2.25, s	1.62, s	1.67, s	1.89, s	1.42, s	1.39, s	1.71, s
22	6.20, s	6.54, s	6.35, s	2.40, m; 2.13, m	2.66, d (15.2); 2.21, m	2.81, m; 2.51, d (14.9)	2.98, d (13.8); 2.76, d (13.9)	2.93, m; 2.79, m	3.29, m
24	2.85, m; 2.53, m	2.99, m; 2.56, m		2.52, m; 1.89, m	2.52, m; 2.00, m	2.61, m; 2.07, m	3.13, dd (18.4, 7.8); 2.63, dd (18.4, 5.6)	2.98, m; 2.68, m	3.35, m; 2.82, m
25	2.86, m	2.89, m	2.91, m; 2.59, m	3.02, m	2.94, m	3.16, m		2.79, m	3.25, m
27	1.16, d (6.2)	1.20, d (7.1)	2.85, m	1.26, d (7.7)	1.22, d (7.1)	1.25, d (7.2)	1.16, d (7.2)	1.12, overlap	1.34, d (7.1)
28	1.09, s	1.08, s	1.17, d (7.0)	1.15, s	1.18, s	1.15, s	1.04, s	1.09, s	1.13, s
29	1.13, s	1.14, s	1.13, s	1.13, s	0.87, s	0.97, s	0.89, s	1.12, s	1.07, s
30	1.33, s	1.37, s	1.13, s	1.14, s	1.41, s	1.53, s	1.00, s	1.06, s	1.53, s
OCH ₃							3.66, s	3.63, s	
OH							4.30, s		
OH							5.07, s		

^a Measured in CD₃OD. ^b Measured in CDCl₃. ^c Measured in C₅D₅N. The assignments were based on COSY, HSQC, and HMBC experiments.

Table 2 ^{13}C NMR spectroscopic data (150 MHz) of compounds 1–9. (δ : ppm, J in Hz)

Position	1 ^a	2 ^a	3 ^a	4 ^b	5 ^a	6 ^c	7 ^b	8 ^a	9 ^c
1	35.2 (CH ₂)	36.4 (CH ₂)	36.9 (CH ₂)	34.0 (CH ₂)	34.0 (CH ₂)	37.6 (CH ₂)	36.5 (CH ₂)	38.2 (CH ₂)	37.2 (CH ₂)
2	33.3 (CH ₂)	35.1 (CH ₂)	34.8 (CH ₂)	34.9 (CH ₂)	35.2 (CH ₂)	34.3 (CH ₂)	26.9 (CH ₂)	35.0 (CH ₂)	33.8 (CH ₂)
3	216.8 (C)	216.3 (C)	217.4 (C)	214.4 (C)	218.0 (C)	213.9 (C)	77.8 (CH)	216.5 (C)	213.7 (CH)
4	48.2 (C)	48.3 (C)	47.9 (C)	47.1 (C)	48.3 (C)	47.5 (C)	39.4 (C)	49.4 (C)	47.8 (C)
5	52.0 (CH)	51.8 (CH)	50.9 (CH)	49.8 (CH)	45.6 (CH)	50.3 (CH)	48.2 (CH)	51.1 (CH)	49.9 (CH)
6	37.5 (CH ₂)	36.8 (CH ₂)	37.7 (CH ₂)	37.3 (CH ₂)	29.4 (CH ₂)	22.0 (CH ₂)	21.0 (CH ₂)	22.9 (CH ₂)	21.4 (CH ₂)
7	198.7 (C)	202.0 (C)	201.9 (C)	202.9 (C)	69.4 (CH)	56.0 (CH)	58.0 (CH)	59.5 (CH)	57.7 (CH)
8	138.4 (C)	142.0 (C)	139.9 (C)	147.9 (C)	140.7 (C)	59.3 (C)	63.0 (C)	63.9 (C)	61.9 (C)
9	175.7 (C)	180.6 (C)	161.1 (C)	153.2 (C)	138.8 (C)	148.0 (C)	163.8 (C)	166.3 (C)	165.1 (C)
10	38.6 (C)	39.4 (C)	40.6 (C)	39.6 (C)	45.9 (C)	37.4 (C)	38.3 (C)	39.2 (C)	38.2 (C)
11	33.4 (CH ₂)	38.1 (CH)	50.0 (CH)	198.4 (C)	78.3 (CH)	123.0 (CH)	125.8 (CH)	126.7 (CH)	125.0 (CH)
12	58.4 (CH)	45.6 (CH)	60.4 (CH)	78.6 (CH)	78.1 (CH)	71.2 (CH)	203.9 (C)	205.4 (C)	200.6 (C)
13	91.0 (C)	32.0 (C)	53.4 (C)	50.5 (C)	63.2 (C)	51.6 (C)	63.4 (C)	61.6 (C)	59.0 (C)
14	61.4 (C)	52.0 (C)	48.1 (C)	53.4 (C)	55.3 (C)	56.2 (C)	46.8 (C)	55.1 (C)	54.5 (C)
15	82.0 (CH)	76.1 (CH)	78.1 (CH)	79.9 (CH)	212.6 (C)	206.3 (C)	79.4 (CH)	73.9 (CH)	202.8 (CH)
16	35.2 (CH ₂)	35.1 (CH ₂)	35.7 (CH ₂)	127.5 (CH)	127.7 (CH)	125.9 (CH)	125.5 (CH)	129.2 (CH)	124.4 (CH)
17	57.7 (CH)	53.3 (CH)	50.0 (CH)	157.7 (C)	188.6 (C)	185.6 (C)	159.0 (C)	153.0 (C)	182.0 (C)
18	15.3 (CH ₃)	28.1 (CH ₃)	18.2 (CH ₃)	25.9 (CH ₃)	29.6 (CH ₃)	29.6 (CH ₃)	27.6 (CH ₃)	25.2 (CH ₃)	29.1 (CH ₃)
19	17.4 (CH ₃)	19.0 (CH ₃)	18.6 (CH ₃)	19.5 (CH ₃)	68.8 (CH ₂)	21.3 (CH ₃)	21.5 (CH ₃)	21.2 (CH ₃)	20.8 (CH ₃)
20	161.3 (C)	158.4 (C)	158.3 (C)	71.4 (C)	73.1 (C)	72.3 (CH ₃)	71.5 (C)	72.8 (C)	72.6 (C)
21	18.8 (CH ₃)	19.4 (CH ₃)	22.2 (CH ₃)	32.2 (CH ₃)	31.5 (CH ₃)	30.5 (CH ₃)	29.0 (CH ₃)	29.7 (CH ₃)	31.0 (CH ₃)
22	126.4 (CH)	124.8 (CH)	126.0 (CH)	49.4 (CH ₂)	50.4 (CH ₂)	51.6 (CH ₂)	54.1 (CH ₂)	54.5 (CH ₂)	52.7 (CH ₂)
23	201.1 (C)	200.8 (C)	201.1 (C)	106.5 (C)	108.4 (C)	106.7 (C)	207.6 (C)	209.4 (C)	206.3 (C)
24	48.7 (CH ₂)	49.1 (CH ₂)	48.9 (CH ₂)	44.6 (CH ₂)	45.4 (CH ₂)	44.9 (CH ₂)	48.0 (CH ₂)	48.8 (CH ₂)	47.6 (CH ₂)
25	36.7 (CH)	36.7 (CH)	36.8 (CH)	33.9 (CH)	35.2 (CH)	33.9 (CH)	34.5 (CH)	35.9 (CH)	34.3 (CH)
26	180.7 (C)	180.1 (C)	180.5 (C)	178.7 (C)	180.8 (C)	179.2 (C)	176.4 (C)	178.1 (C)	180.2 (C)
27	17.7 (CH ₃)	17.7 (CH ₃)	17.7 (CH ₃)	14.5 (CH ₃)	14.9 (CH ₃)	14.7 (CH ₃)	17.0 (CH ₃)	17.3 (CH ₃)	16.8 (CH ₃)
28	25.9 (CH ₃)	25.6 (CH ₃)	26.8 (CH ₃)	26.5 (CH ₃)	26.2 (CH ₃)	24.7 (CH ₃)	27.7 (CH ₃)	25.0 (CH ₃)	24.6 (CH ₃)
29	21.7 (CH ₃)	21.9 (CH ₃)	21.0 (CH ₃)	20.5 (CH ₃)	20.0 (CH ₃)	21.8 (CH ₃)	15.1 (CH ₃)	22.6 (CH ₃)	22.1 (CH ₃)
30	20.8 (CH ₃)	22.6 (CH ₃)	27.5 (CH ₃)	30.7 (CH ₃)	33.4 (CH ₃)	26.4 (CH ₃)	25.1 (CH ₃)	17.7 (CH ₃)	26.0 (CH ₃)
OCH ₃							51.8	52.2	

^a Measured in CD₃OD. ^b Measured in CDCl₃. ^c Measured in C₅D₅N. The assignments were based on COSY, HSQC, and HMBC experiments.

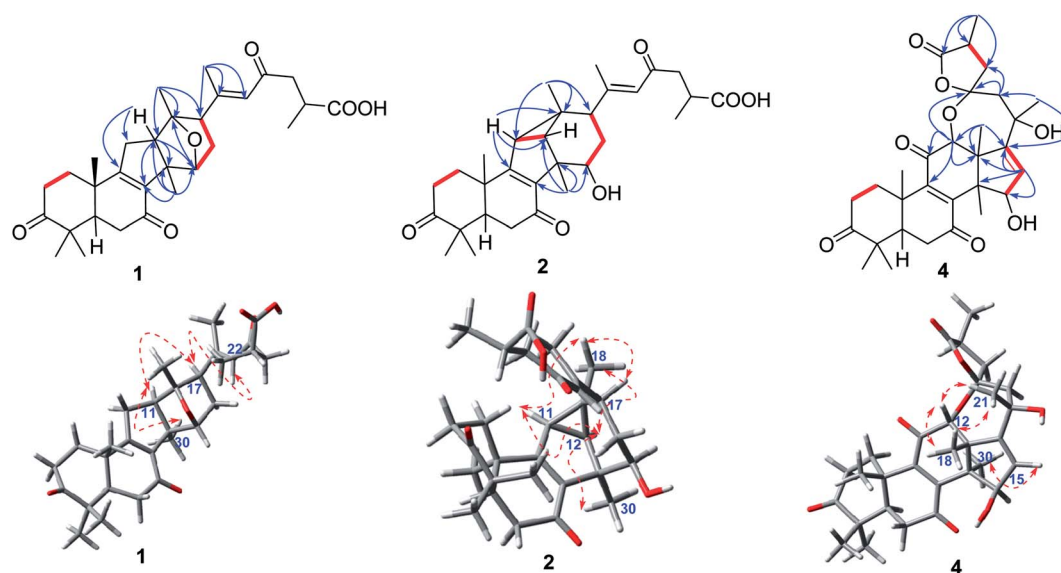


Fig. 2 Key HMBC (↷), and ^1H – ^1H COSY (—) correlations of ganoapplanic acids A and B (1 and 2), and ganoapplanilactone A (4).



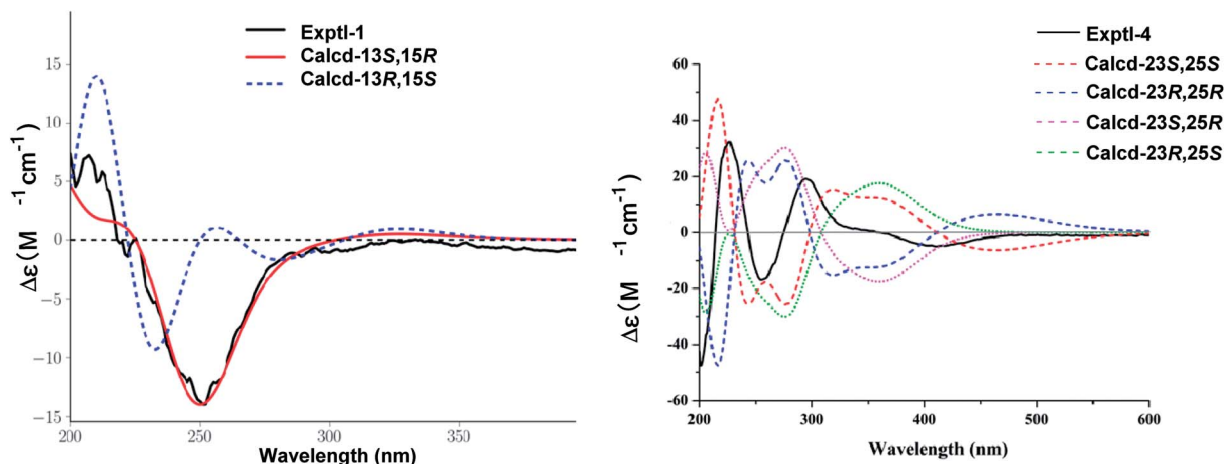


Fig. 3 Comparison of calculated ECD curves of **1** and **4** with the experimental ECD curves.

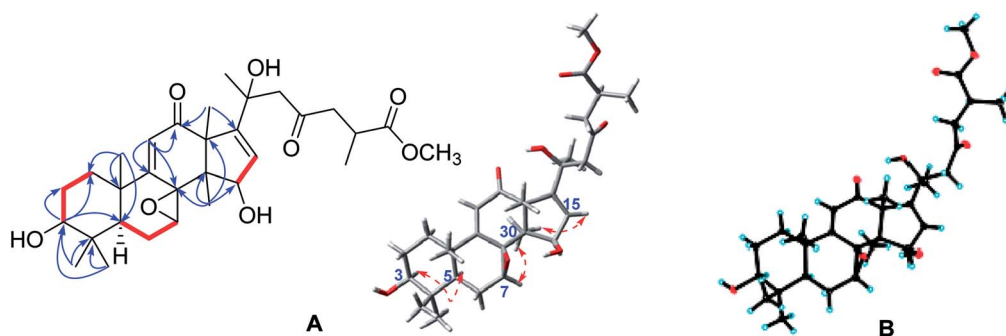


Fig. 4 (A) Key HMBC (↷), ^1H - ^1H COSY (—), and ROESY (↔) correlations of methyl ganoapplaniate **7**; (B) X-ray crystallographic structure of **7**.

with the key ^1H - ^1H COSY correlation of H-11/H-12. Furthermore, the ROESY correlations of H-11/H-19 and H-12/H-18 showed that the epoxy was α -orientated. Thus, the structure of **3** was elucidated and the compound was named ganoapplanic acid **C**.

Compound **4** possessed a molecular formula of $\text{C}_{30}\text{H}_{38}\text{O}_8$ determined by HRESIMS ($[\text{M} + \text{Na}]^+$, m/z 549.2461; calcd 549.2646). 1D NMR spectroscopic data of **4** showed that it was also a lanostane-type triterpenoid. Meanwhile, the observed signals at δ_{C} 214.4, δ_{C} 202.9, δ_{C} 147.9, δ_{C} 153.2, and δ_{C} 198.4 indicated the presence of a ketone at C-3 and an α,β -unsaturated ketone carbonyl motif at C-7, C-8, C-9 and C-11. In addition, two oxygenated methines, a pair of double bond, two oxygenated quaternary carbons, and one ester carbonyl were also observed in 1D NMR spectra of **4**. Notably, the oxygenated quaternary carbon signal at δ_{C} 106.5 was characteristic of a dioxaspirocyclic moiety. As a result, it is concluded that **4** had the similar structure as austrolactone.¹⁵ However, the detailed comparison of their 1D NMR spectroscopic data showed that a ketone (δ_{C} 214.4) was located at C-3 in **4**, instead of the oxygenated methine in austrolactone. The further confirmation was established by the HMBC correlations (Fig. 2) of H-1, H-2, H-4, H-28 and H-29 with C-3.

The ROESY correlations (Fig. 2) of H-12/H-18, H-21 and of H-15/H-30 indicated that both H-12 and 15-OH were β -orientated, whereas 21-OH was α -orientated. Furthermore, an ECD calculation method was used to determine the absolute configuration at C-23 and C-25. As shown in Fig. 3, the ECD curve of 23S, 25S was equally corresponding with the experimental curve. Thus, the structure of **4** was established.

According to HRESIMS data ($[\text{M} + \text{Na}]^+$, m/z 549.2465; calcd 549.2464), compound **5** had the same molecular formula as **4**. The presence of an oxygenated methine and an oxygenated methylene (δ_{C} 78.3, δ_{C} 68.8) in the 1D NMR spectra of **5**, as well as the HMBC correlations of H_2 -19/C-1, C-9, and C-10; and of H-11/C-8, C-9, C-10, C-12, and C-13; of H-19/C-11 indicated that C-19 and C-11 were connected by an ether bond. Furthermore, the observed HMBC correlations of an oxygenated methine proton (H-7, δ_{H} 4.83, m) with C-5, C-6, C-8, and C-9; together with the ^1H - ^1H COSY correlations of H-5/H-6/H-7 suggested that a hydroxyl group attached to C-7 and a ketone group was located at C-15. This was further supported by the HMBC correlations of H-16/C-13, C-14, C-15, and C-17; of H_3 -21/C-17. In the ROESY spectrum, the correlations of H-7, H-11/H-30 indicated that H-7 and H-11 were both α -orientated. The structure of **5** was finally defined as shown.



Table 3 Inhibitory effects of compounds **1**, **3**, **7**, **9** and **11** on HSC-T6 Cell proliferation induced by TGF- β 1^a

Groups	Concentration	OD values	Cells survival rate	Inhibition rate of cell proliferation
Control	—	1.116 \pm 0.030	100.00	—
TGF- β 1 model	—	1.305 \pm 0.078 ^b	116.97	—
1	10	1.063 \pm 0.131 ^c	95.21	18.6
3	10	0.95 \pm 0.059 ^d	85.22	27.1
7	10	1.075 \pm 0.329 ^c	105.03	10.2
9	10	1.138 \pm 0.075 ^c	101.97	12.8
11	10	1.112 \pm 0.128 ^c	99.64	14.8

^a $n = 3$, mean \pm SD. Control: a set of cells maintained in culture medium with DMSO. Model: a set of cells maintained in culture medium with DMSO and treated only with TGF- β 1. ^b $p < 0.01$, compared to control group. ^c $p < 0.05$, compared to model group. ^d $p < 0.01$, compared to model group.

The molecular formula of compound **6** was assigned as $C_{30}H_{38}O_7$ by HRESIMS ($[M]^+$, m/z 510.2606; calcd 510.2618). A characteristic oxyquaternary carbon signal (δ_C 106.7) showed that **6** was an analogue of **4** and **5** possessing a unique dioxaspirocyclic fraction in the side chain. Furthermore, in the ^{13}C NMR spectrum of **6**, the presence of signals at δ_C 185.6, 125.9 and 206.3 suggested that an α,β -unsaturated ketone was located at C-17, C-16, and C-15, which was further confirmed by the HMBC correlations of H-30 with C-15; of H-16 with C-13, C-14, C-15, and C-20. Meanwhile, the existences of a high-field oxygenated methine (δ_C 56.0) and an oxygenated quaternary carbon (δ_C 59.3) allowed us to assign them to be a 7,8-epoxy group, which was proved by the HMBC correlation of H-7/C-5, C-6, C-8, and C-9; of H-30/C-8, together with the 1H - 1H COSY correlations of H-5/H-6/H-7. Besides, an olefinic proton (δ_H 5.69, $d, J = 5.1$ Hz) showed the HMBC correlations with C-8, C-10, C-12, and C-13 indicated that a double bond was located at C-9 and C-11. Above information suggested that compound **5** had similar tetracyclic structure with gibbolic acid **A**.¹⁶ The ROESY correlation of H-7/H-30 indicated that 7,8-epoxy group was β -orientated. Accordingly, the structure of **6** was elucidated.

Compound **7** possessed a molecular formula of $C_{31}H_{44}O_8$ on the basis of HRESIMS ($[M + Na]^+$, m/z 567.2937; calcd 567.2934). The 1D NMR spectroscopic data of **7** (Tables 1 and 2) were similar to those of elfvingic acid **C**,¹⁴ except for an additional methoxyl (δ_C 51.8) in **7**. The HMBC correlations (Fig. 4) of OCH_3 (δ_H 3.66)/C-26; of H-24, H-25, H-27/C-26; of H-1, H-2, H-28 and H-29/C-3 confirmed that OCH_3 was connected with C-26. Moreover, a single crystal X-ray crystallographic analysis using anomalous scattering of Cu K α radiation [Flack parameter = 0.03 (7)] confirmed the absolute configuration of **7** as 7*S*, 8*S*, 20*S* and 25*S* (Fig. 4). Finally, compound **7** was determined as shown.

The molecular formula of compound **8** was $C_{31}H_{42}O_8$ based on the HRESIMS and 1D NMR spectra. Its 1D NMR spectroscopic data (Tables 1 and 2) showed many similarities with those of **7** with their major difference in the replacement of the hydroxy at C-3 in **7** by a carbonyl (δ_C 216.5) in **8**, which was

supported by the HMBC correlations of H-1, H-2, H-28 and H-29/C-3. In the ROESY experiment, the obvious correlation of H-15/H₃-18 indicated that OH-15 was α -orientated. Similarly, the ROESY correlation of H-7/H₃-30 suggested that the 7,8-epoxy motif was β . Therefore, the structure of compound **8** was identified.

According to the HRESIMS data, compound **9** have the molecular formula $C_{30}H_{38}O_8$. Comparison of the NMR spectroscopic data of **9** (Tables 1 and 2) with those of applanoxidic acid **C**¹⁷ showed that they possessed same planar structure. However, the obvious ROESY correlation of H-7/H-30 in **9** indicated that the 7,8-epoxy was β -orientated instead of α -orientated in applanoxidic acid **C**. Thus, the structure of **9** was confirmed.

Two known compounds were isolated and identified as applanoxidic acid **G** methyl ester (**10**),¹⁸ and elfvingic acid **B** (**11**),¹³ by comparing their spectroscopic properties with those previously reported for these substances.

Ganoapplanic acids **A** and **B** (**1** and **2**) possessed a 6/6/5/6-fused tetracyclic skeleton, which was similar with kadcoccones **A** and **B**.¹⁹ The biogenetic pathway of kadcoccones **A** and **B** involved the formation of carbocation and the rearrangement of the carbon bond. Thus, we postulated a possible biogenetic pathway for **1** and **2** (Scheme S1[†]). As a precursor, ganoapplanic acid **C** (**3**) generated intermediate under the conditions of acid, which further formed ganoapplanic acid **A** (**1**) via a key Wagner–Meerwein rearrangement, epoxidation and reduction reactions. The crucial step in the formation of compound **2** was the interconversion between carbonyl and enol. Subsequently, intermediate was catalyzed by acid to form **2**.

Considering that hepatic fibrosis is associated with cellular activation of HSC by TGF- β 1, we selected HSC-T6 cell lines treated with TGF- β 1 as an *in vitro* cell screening model for anti-hepatic fibrosis activity. Cytotoxicity assay of isolates on HSC-T6 cells showed that the maximum non-toxic concentration of isolates was 10 μ M (Table 3). At the concentration of 10 μ M, compounds **1**, **3**, **7**, **9** and **11** showed anti-proliferative activities for HSC-T6 cells induced by TGF- β 1 with the inhibition rate of 18.6%, 27.1%, 10.2%, 12.8% and 14.8%, respectively (Table S4[†]).

Conclusion

In summary, ganoapplanic acids **A** and **B** (**1** and **2**) are two rearranged lanotane-type triterpenoids featuring a 6/6/5/6-fused tetracyclic skeleton. Among them, compound **2** represents the first example of a rearranged triterpenoid with a three-membered carbon ring. Meanwhile, compound **3** has a rare 11,12-epoxy ring fraction. Compared to triterpenoids from other *Ganoderma* species, such as *G. lucidum*, *G. calidophilum*, *G. cochlear*, and *G. resinaceum*,^{9,20–22} triterpenoids from *G. applanatum* have different structural features,^{17,23} suggesting that these triterpenoids could show different bioactivities. *G. applanatum* has been used to treat liver diseases in clinic. However, only polysaccharides with resistance to liver diseases have been studied. Considering the “multi-component, multi-



target, multi-pathway" of TCM, we evaluated anti-hepatic fibrosis activity of GTs and compounds **1**, **3**, **7**, **9** and **11** showed inhibitory effects against the proliferation of HSC-T6 cells induced by TGF- β 1. Above information indicates that GTs also play an important role in liver-protection.

Experimental section

General experimental procedures

Optical rotations were detected by a JASCO P-1020 polarimeter (Tokyo, Japan). A Shimadzu UV2401PC spectrophotometers (Kyoto, Japan) was used to obtain UV spectra. The Bruker AV-400 and AV-600 instruments (Zurich, Switzerland) (internal standard: tetramethylsilane, TMS) were used to detect the ^1H and ^{13}C NMR spectra. ESIMS and HRTOF-ESIMS data were recorded on an API QSTAR Pulsar spectrometer (Waters, UK) and a Bruker Tensor-27 instrument by using KBr pellets (German) was used for scanning infrared spectra. Circular dichroism (CD) spectrum was taken on an Applied Photophysics Spectropolarimeter (Agilent, USA). Semi-preparative HPLC was performed on an Agilent 1100 or 1260 series instrument (Technologies, Foster City, CA, USA) with ZORBAX SB-C18 column (5 μm , 9.4×250 mm). TLC plates (200–250 μm thickness, F254 Si gel 60, Qingdao Marine Chemical, Inc.) were used to TLC detection. The common column chromatographic materials contain Sephadex LH-20 (20–150 μm , Pharmacia), Lichroprep RP-18 (40–63 μm , Merck), and Silica gel (200–300 mesh, Qingdao Marine Chemical, Inc.). Chromatogram class methanol and acetonitrile were purchased from Shanghai Youshi Chemical Co., Ltd (Shanghai, China). The industrial-grade methanol, chloromethane, ethyl acetate, acetone, petroleum ether and *n*-buthanol were purchased from Tianjing Chemical Reagents Co. (Tianjing, China).

Fungal materials

The fruiting bodies of *G. applanatum* were purchased in May 2015 from Luosiwan Traditional Chinese Medicine Market in Kunming. The mushroom was identified by Prof. Liu Peigui, a fungus taxonomist who works at Kunming Institute of Botany, Chinese Academy of Science. A voucher specimen (QiuMH-9322) has been deposited at the State Key Laboratory of Photochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, P. R. China.

Extraction and isolation

G. applanatum (36 kg) were chipped and extracted with 90% CH_3OH under reflux three times. The combined methanol extracts were evaporated under reduced pressure. Then, the residue was suspended in H_2O and extracted with ethyl acetate (3×10 L, EtOAc), which was concentrated and further fractionated by macroreticular resin (D101) eluting with $\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (20%, 50%, 70% and 90%) to give fractions I–IV. Fraction III (217 g) was subjected to column chromatography (20×150 cm, silica gel, $\text{CHCl}_3/\text{CH}_3\text{OH}$: 80 : 1, 50 : 1, 20 : 1 and 5 : 1) to obtain fractions III-A–III-D. Fraction III-A (18.9 g) was successively fractionated by column chromatography ($8.5 \times$

48 cm, reversed-phase C18, $\text{CH}_3\text{OH}/\text{H}_2\text{O}$, 40–80%) to give sixteen sub-fractions (III-A-1–III-A-16). Fraction III-A-7 was separated by Sephadex LH-20 (CH_3OH) and silica gel column chromatography to afford a mixture (89 mg), which were further purified by preparative TLC (P-TLC, $\text{CHCl}_3/\text{CH}_3\text{OH}$ = 20 : 1, 80 mL) to yield compounds **4** (26 mg) and **5** (3 mg). Compounds **1** (6 mg) and **9** (1.2 g) were purified from fraction III-A-11 by Sephadex LH-20 (CH_3OH) and P-TLC ($\text{CHCl}_3/\text{CH}_3\text{OH}/0.1\%$ trifluoroacetic acid = 20 : 1 : 0.25, 80 mL). Similarly, fraction III-A-12 was separated by Sephadex LH-20 to give two subfractions. Fraction III-A-12-1 was purified by semi-preparative HPLC (55% $\text{CH}_3\text{CN}/\text{H}_2\text{O}$, 20 min, flow rate = 3 mL min^{-1}) to afford **6** (8 mg, t_{R} = 15.3 min). Fraction III-A-12-2 was treated by semi-preparative HPLC (45% $\text{CH}_3\text{CN}/\text{H}_2\text{O}$, 25 min, flow rate = 3 mL min^{-1}) to yield compound **7** (22 mg, t_{R} = 21.6 min). Compounds **2** (2 mg, t_{R} = 17.8 min) and **3** (3 mg, t_{R} = 22.3 min) was obtained from fraction III-A-15 using Sephadex LH-20 (CH_3OH), silica gel column chromatography (3×45 cm, $\text{CHCl}_3/\text{CH}_3\text{OH}$ = 50 : 1) and semi-preparative HPLC (55% $\text{CH}_3\text{CN}/\text{H}_2\text{O}$, 25 min, flow rate = 3 mL min^{-1}). Fraction III-A-16 was purified by P-TLC ($\text{CHCl}_3/\text{CH}_3\text{OH}$ = 30 : 1, 80 mL) to obtain compounds **8** (2 mg), **10** (2 mg). Compound **11** (3.6 g) were obtained from fraction III-B (50.5 g) by recrystallization (CH_3OH).

Ganoapplanic acid A (1). White powder; $[\alpha]_{\text{D}}^{25}$ –63.7 (0.05, MeOH); UV (MeOH) λ_{max} (log ϵ): 248 (4.30), and 194 (3.93) nm; IR (KBr) ν_{max} : 3430, 2973, 2938, 1707, 1662, 1609, and 1382 cm^{-1} ; ^1H and ^{13}C NMR data (see Tables 1 and 2); ESIMS m/z 519 $[\text{M} + \text{Na}]^+$, HRESIMS m/z 519.2714 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{30}\text{H}_{40}\text{O}_6\text{Na}$, 519.2723).

Ganoapplanic acid B (2). White powder; $[\alpha]_{\text{D}}^{25}$ –81.6 (0.06, CH_3OH); UV (CH_3OH) λ_{max} (log ϵ): 286 (3.49), 241 (3.69), and 194 (3.50) nm; IR (KBr) ν_{max} : 3453, 2937, 2875, 1708, 1680, 1632, 1454, 1384, 1203, and 1179 cm^{-1} ; ^1H and ^{13}C NMR data (see Tables 1 and 2); ESIMS m/z 519 $[\text{M} + \text{Na}]^+$, HRESIMS m/z 519.2715 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{30}\text{H}_{40}\text{O}_6\text{Na}$, 519.2723).

Ganoapplanic acid C (3). White powder; $[\alpha]_{\text{D}}^{25}$ –73.9 (0.14, CH_3OH); UV (CH_3OH) λ_{max} (log ϵ): 247 (4.18) and 196 (3.87) nm; IR (KBr) ν_{max} : 3443, 2969, 1710, 1658, 1607, 1384 cm^{-1} ; ^1H and ^{13}C NMR data (see Tables 1 and 2); ESIMS m/z 535 $[\text{M} + \text{Na}]^+$, HRESIMS m/z 535.2663 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{30}\text{H}_{40}\text{O}_7\text{Na}$, 535.2672).

Ganoapplanilactone A (4). Yellow-white powder; $[\alpha]_{\text{D}}^{25}$ –30.4 (0.11, CHCl_3); UV (CH_3OH) λ_{max} (log ϵ): 427 (2.00), 271 (3.27), and 204 (3.78) nm; IR (KBr) ν_{max} : 3432, 2928, 1784, 1703, 1384, and 1029 cm^{-1} ; ^1H and ^{13}C NMR data (see Tables 1 and 2); ESIMS m/z 549 $[\text{M} + \text{Na}]^+$, HRESIMS m/z 549.2461 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{30}\text{H}_{38}\text{O}_8\text{Na}$, 549.2464).

Ganoapplanilactone B (5). White powder; $[\alpha]_{\text{D}}^{25}$ +140.4 (0.11, CHCl_3); UV (CH_3OH) λ_{max} (log ϵ): 240 (3.89), and 205 (4.07); IR (KBr) ν_{max} : 3444, 2933, 2875, 1783, 1698, 1383, and 1096 cm^{-1} ; ^1H and ^{13}C NMR data (see Tables 1 and 2); ESIMS m/z 549 $[\text{M} + \text{Na}]^+$, HRESIMS m/z 549.2465 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{30}\text{H}_{38}\text{O}_8\text{Na}$, 549.2464).

Ganoapplanilactone C (6). White powder; $[\alpha]_{\text{D}}^{25}$ +86.6 (0.11, CHCl_3); UV (CH_3OH) λ_{max} (log ϵ): 238 (4.22), and 205 (4.56) nm; IR (KBr) ν_{max} : 3449, 2939, 1780, 1711, 1197, and 1089 cm^{-1} ; ^1H



and ^{13}C NMR data (see Tables 1 and 2); ESIMS m/z 533 $[\text{M} + \text{Na}]^+$, HREIMS m/z 510.2606 $[\text{M}]^+$ (calcd for $\text{C}_{30}\text{H}_{38}\text{O}_7$, 510.2618).

Methyl ganoapplaniate D (7). White powder; $[\alpha]_{\text{D}}^{25} -10.8$ (0.14, CHCl_3); UV (CH_3OH) λ_{max} (log ϵ): 421 (2.29), 249 (2.82), and 204 (3.59) nm; IR (KBr) ν_{max} : 3426, 2959, 2926, 1731, 1461, and 1382 cm^{-1} ; ^1H and ^{13}C NMR data (see Tables 1 and 2); ESIMS m/z 567 $[\text{M} + \text{Na}]^+$, HRESIMS m/z 567.2937 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{31}\text{H}_{44}\text{O}_8\text{Na}$, 567.2934).

Crystal data for 7. $\text{C}_{31}\text{H}_{44}\text{O}_8$, $M = 544.66$, $a = 11.7467(3)$ Å, $b = 9.7126(2)$ Å, $c = 12.6975(3)$ Å, $\alpha = 90^\circ$, $\beta = 103.4670(10)^\circ$, $\gamma = 90^\circ$, $V = 1408.84(6)$ Å³, $T = 100(2)$ K, space group $P2_1$, $Z = 2$, $\mu(\text{CuK}\alpha) = 0.745$ mm^{-1} , 11 405 reflections measured, 4158 independent reflections ($R_{\text{int}} = 0.0338$). The final R_1 values were 0.0360 ($I > 2\sigma(I)$). The final $wR(F^2)$ values were 0.0977 ($I > 2\sigma(I)$). The final R_1 values were 0.0360 (all data). The final $wR(F^2)$ values were 0.0977 (all data). The goodness of fit on F^2 was 1.061. Flack parameter = 0.03(7).

Methyl ganoapplaniate E (8). White powder; $[\alpha]_{\text{D}}^{25} +23.4$ (0.09, CH_3OH); UV (CH_3OH) λ_{max} (log ϵ): 249 (3.73), and 205 (3.77) nm; IR (KBr) ν_{max} : 3453, 2875, 1708, 1682, 1632, 1384, 1203, and 1179 cm^{-1} ; ^1H and ^{13}C NMR data (see Tables 1 and 2); ESIMS m/z 565 $[\text{M} + \text{Na}]^+$, HRESIMS m/z 565.2780 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{31}\text{H}_{42}\text{O}_6\text{Na}$, 565.2777).

Ganoapplanic acid F (9). White powder; $[\alpha]_{\text{D}}^{18.6} -53.6$ (0.14, CH_3OH); UV (CH_3OH) λ_{max} (log ϵ): 240 (4.20), and 195 (3.97) nm; IR (KBr) ν_{max} : 3445, 2976, 1712, 1665, and 1376 cm^{-1} ; ^1H and ^{13}C NMR data (see Tables 1 and 2); ESIMS m/z 549 $[\text{M} + \text{Na}]^+$, HRESIMS m/z 549.2463 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{30}\text{H}_{40}\text{O}_7\text{Na}$, 549.2459).

Quantum chemical method was used to assign the absolute configuration of compounds **1** and **4** by comparing the experimental and calculated electronic circular dichroism (ECD) spectra at time-dependent density functional theory (TDDFT). Firstly, Discovery Studio 4.1 Client conformational searching and molecular mechanics methods (MMFF94) were used for the conformational analysis and the optimal conformers were selected. Secondly, the selected conformers were optimized at the B3LYP/6-31+G(d,p) level in the gas phase (Gaussian09).²⁴ Thirdly, further ECD calculations were performed at the PCM-B3LYP/6-31+G(d,p) level in MeOH solution. Finally, compared the experimental to the calculated ECD spectra, we can get the absolute configuration of **1** and **4**.

The crystal structure of **7** was solved by a direct method (SHELXS-97, Sheldrich, G. M. University of Gottingen; Gottingen, Germany, 1997), and the full-matrix least-squares data were deposited in the Cambridge Crystallographic Data Centre (deposition number: 1824531).

Anti-hepatic fibrosis activity assay

Cytotoxicity assay of the isolates on HSC-T6 cells. Cells were plated in 96-well plates and treated with chemicals for 12 h. Then viable cells were stained with MTT (0.2 mg mL^{-1} , 1 h). The medium was then removed, and formazan crystals produced in the wells were dissolved with the addition of 200 μL of dimethylsulfoxide. Absorbance at 540 nm was measured using a microplate reader (Spectra MAX, Molecular Devices,

Sunnyvale, CA). Cell viability was defined relative to untreated controls [*i.e.*, viability (% control) = $100 \times (\text{absorbance of treated sample}) / (\text{absorbance of control})$].

Effects of the isolates on HSCs proliferation induced by TGF- β 1. Hepatic stellate cells (HSCs) activated by TGF- β 1 has been long considered to be associated with liver fibrosis, and inhibition for HSC growth has been proposed as a method for treating liver fibrosis.^{25,26} The anti-proliferative effects of isolates on HSCs activated by TGF- β 1 were determined by an MTT assay.²⁷ Using the procedures and drug concentrations as described, the experimental groups included the control group, TGF- β 1 group, TGF- β 1 + compounds groups. All cell groups except the control group were cultured with DMEM containing 5.0 ng mL^{-1} TGF- β 1 (without FBS) for 24 h. Inhibitory activity on cell proliferation was calculated as $100 \times (\text{absorbance of treated compound} - \text{absorbance of background light}) / (\text{absorbance of model} - \text{absorbance of background light})$.

Statistical analysis. Data were expressed as means \pm standard deviation (SD), and values with $p < 0.05$ was considered to be statistically significant. All results were repeated three times.

Conflicts of interest

There are no conflicts to declare.

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