

Lanostane-triterpenoids from the fungus *Phellinus gilvus*

Hui-Kang Liu^a, Tung-Hu Tsai^b, Tun-Tschu Chang^c, Cheng-Jen Chou^a, Lie-Chwen Lin^{a,b,d,*}

^a National Research Institute of Chinese Medicine, 155-1, Li-Nong St., Sec. 2, Taipei 112, Taiwan

^b Institute of Traditional Medicine, National Yang-Ming University, Taipei, Taiwan

^c Division of Forest Protection, Taiwan Forestry Research Institute, Taipei, Taiwan

^d Graduate Institute of Integrated Medicine, China Medical University, Taichung, Taiwan

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ABSTRACT

Triterpenoids gilvins A–D (**1–4**), with oxygenated lanostane skeletons, were isolated from the fruiting body of *Phellinus gilvus*, together with two known compounds, 24-methylenelanost-8-ene-3 β , 22-diol and 5 α -ergosta-7,22-diene-3-one. The structures of **1–4** were deduced from analysis of spectroscopic data. The absolute configuration at C-22 of **1** was determined by the modified Mosher's method and the structure of **1** was confirmed by X-ray analysis. The hypoglycemic activities of the crude extract of *P. gilvus* and the isolated compounds were also evaluated, but were not promising for further investigation.

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1. Introduction

Phellinus gilvus (Schw.) Pat. (Hymenochaetaceae) is a parasitic fungus, commonly found, with fatal results, in trees of the Fagaceae. *P. gilvus* can be found at low to mid-elevation in forests of Taiwan (Chou and Chang, 2005). In addition, it has been used as a Formosan folk medicine for treatment of cancer, diarrhea, hepatic fibrosis as well as being used as an immunomodulatory agent. Previous chemical studies of *P. gilvus* have shown the presence of ergosterols, triterpenoids, and polysaccharides (Ahmaol et al., 1976; Bae et al., 2005; Hosoe et al., 2006). The related species, *P. baumii*, has hypoglycemic activity in streptozotocin (STZ)-induced diabetic rats (Hwang et al., 2005). In this paper, we report the isolation and structural elucidation of four new lanostane triterpenoids together with two known compounds from the fruiting body of *P. gilvus*. The hypoglycemic activity of the crude extract of *P. gilvus* and the isolated compounds was also evaluated.

2. Results and discussion

2.1. Characterization of the compounds

The aqueous ethanol (1:1) extract of the dried fruit of *P. gilvus* was separated by silica gel column chromatography and prepara-

tive HPLC, leading to purification of compounds **1–6**. Compounds **5** and **6** were identified as the known compounds, 24-methylenelanost-8-ene-3 β , 22-diol (Baumert et al., 1997) and 5 α -ergosta-7, 22-diene-3-one (Protiva et al., 1980), respectively. The structures of the new compounds **1–4** are shown in Fig. 1.

Compound **1**, named gilvins A, was obtained as needles and its IR spectrum displayed hydroxyl (3544 cm⁻¹), carbonyl (1708 cm⁻¹) and alkene (1645 cm⁻¹) absorptions. The molecular formula of **1** was established as C₃₁H₅₀O₂ from HREIMS at *m/z* 454.3817 [M]⁺. The EIMS of **1** showed similar fragmentation patterns with those of **5**. The ¹H NMR spectrum of **1** (Table 1) indicated the presence of five tertiary methyl groups [δ 0.78 (s, Me-18), 0.92 (s, Me-30), 1.08 (s, Me-29), and 1.10 (s, Me-28), and 1.14 (s, Me-19)], three secondary methyl groups [δ 0.95 (d, Me-21), 1.06 (d, Me-27), and 1.09 (d, Me-26)], a vinyl methylene group at δ 4.84/4.94, and one oxygenated methine at δ 3.78 (d, *J* = 12.5 Hz, H-22). The ¹H and ¹³C NMR spectra of **1** exhibited signals similar to those of **5**. The structural difference between **1** and **5** was only in the A-ring, with the resonance of C-3 at δ 79.0 (d) in **5** being replaced by a C=O group [δ 217.8 (s)] in **1**. The signals of C-2, C-4, C-29 and C-30 in **1** shifted +6.8, +8.5, -1.9 and +5.9 ppm, respectively, compared with those of **5**, confirming that the ketone group was at the C-3 position (Knight 1974). Significant cross-peaks were observed from Me-29 (δ 1.08) to C-3, C-5 (δ 51.2), and C-28 (δ 26.1), from Me-28 (δ 1.10) to C-3, C-5, and C-29 (δ 21.3), and from Me-19 (δ 1.14) to C-5 and C-9 (δ 133.4) in the HMBC experiment, which also confirmed the above deductions. Thus, structure **1** was established as 22-hydroxy-24-methylenelanost-8-en-3-one and its relative configuration was confirmed by X-ray crystallographic analysis. A 3-D structure of the molecule of

* Corresponding author. Address: National Research Institute of Chinese Medicine, 155-1, Li-Nong St., Sec. 2, Taipei 112, Taiwan. Tel.: +886 2 28201999x7101; fax: +886 2 28264276.

E-mail address: lcclin@nricm.edu.tw (L.-C. Lin).

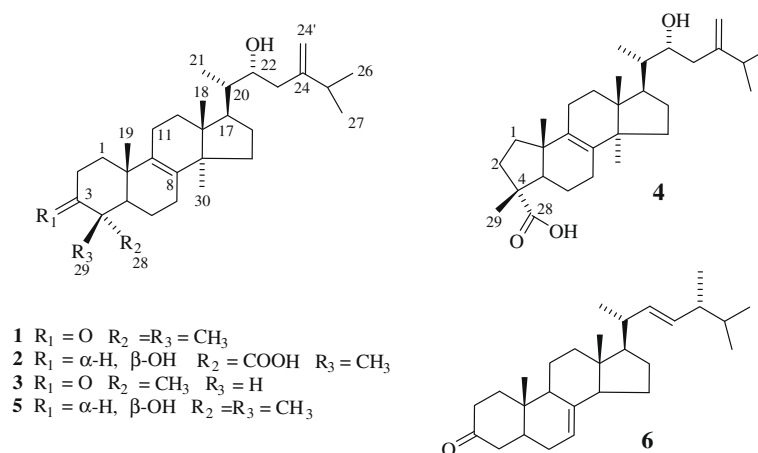
Fig. 1. Chemical constituents from *Phyllinus gilvus*.

Table 1

¹H NMR spectroscopic data for compounds 1–5^a.

Proton	5	1	2	3	4
H-1	1.23/1.76	1.62/2.00	1.37/1.77	1.57/2.07	1.50/1.63
H-2	1.70	2.41/2.60	1.64/1.77	2.36/2.47	2.49
H-3	3.25 dd (4.5, 11.5)	–	4.03 dd (4.0, 11.5)	–	–
H-4	–	–	–	2.32	–
H-5	1.07	1.62	1.86	1.41	2.08 dd (3.5, 14.0)
H-6	1.70	1.62	1.72	1.79	1.73
H-7	2.05	2.10	2.02	2.07	2.15
H-11	2.02	2.07	2.05	2.10	2.01
H-12	1.76	1.77	1.77	1.79	1.80
H-15	1.23/1.70	1.27/1.67	1.23/1.72	1.24/1.65	1.23/1.71
H-17	1.56	1.55	1.56	1.57	1.56
Me-18	0.75 s	0.78 s	0.75 s	0.80 s	0.78 s
Me-19	1.00 s	1.14 s	1.03 s	1.21 s	0.99 s
H-20	1.85 ^b	1.85 ^b	1.86 ^b	1.87 ^b	1.88
Me-21	0.94 d (7.0)	0.95 d (6.5)	0.94 d (7.0)	0.95 d (6.5)	0.95 d (6.5)
H-22	3.78 d (11.5)	3.78 d (12.5)	3.78 d (11.0)	3.80 d (12.5)	3.79 d (11.0)
H-23	1.95 dd (14.0, 11.0)/2.24 d (14.0)	1.96 dd (14.0, 11.0)/2.24 d (14.0)	1.96 dd (14.0, 11.0)/2.24 d (14.0)	1.96 dd (14.0, 11.0)/2.26 d (14.0)	1.95 dd (14.0, 11.0)/2.25 d (14.0)
H-25	2.24	2.24	2.24	2.26	2.25
Me-26 ^b	1.09 d (7.0)	1.09 d (7.0)	1.09 d (7.0)	1.09 d (7.0)	1.09 d (7.0)
Me-27 ^b	1.06 d (7.0)	1.06 d (7.0)	1.06 d (7.0)	1.06 d (7.0)	1.06 d (7.0)
>C=CH ₂ -24	4.84 s/4.94 s	4.84 s/4.94 s	4.84 s/4.94 s	4.84 s/4.95 s	4.84 s/4.95 s
Me-28	1.02 s	1.10 s	–	1.04 d (6.5)	–
Me-29	0.83 s	1.08 s	1.20 s	–	1.25 s
Me-30	0.91 s	0.92 s	0.92 s	0.91 s	0.91 s

^a Measured in CDCl₃; coupling constant (*J* in Hz) assigned in parentheses.^b Assignments in some column are interchangeable.

1 is shown in Fig. 2. The absolute stereochemistry of C-22 was determined using the modified Mosher's method (Ohtani et al., 1991; Ovenden and Capon, 1999). Compound **1** was esterified to afford (R)- and (S)-MTPA ester products. The $\Delta\delta$ values ($\delta_{(S)\text{-MTPA ester}} - \delta_{(R)\text{-MTPA ester}}$) of the H₂-24', Me-26 and Me-27 were positive and the $\Delta\delta$ values of the Me-18, Me-21 and Me-30 were negative. These results indicated that the configuration at C-22 in **1** was *R*.

Compound **2**, gilvsin B, was obtained as a white amorphous powder and its IR spectrum displayed hydroxyl (3435 cm^{−1}), carboxyl (1704 cm^{−1}), and alkene (1640 cm^{−1}) absorptions. The molecular formula was established as C₃₁H₅₀O₄ from analysis, the HREIMS at *m/z* 486.3708 [M]⁺. Compounds **1** and **2** had similar MS fragmentation patterns with those of **5**, suggesting that they shared the same structural skeleton. The ¹H NMR spectrum (Table 1) indicated the presence of four tertiary methyl groups [δ 0.75 (s, Me-18), 0.92 (s, Me-30), 1.03 (s, Me-19), and 1.20 (s, Me-29)], three secondary methyl groups [δ 0.94 (d, Me-21), 1.06 (d, Me-27), and

1.09 (d, Me-26)], a vinyl methylene group at δ 4.84/4.94, and two oxygenated methine moieties at δ 3.78 (d, *J* = 11.0 Hz, H-22), and 4.03 (dd, *J* = 11.5, 4.0 Hz, H-3). The ¹³C NMR spectrum of **2** (Table 2) provided evidence for a lanostane skeleton. Of the seven degrees of unsaturation suggested by C₃₁H₅₀O₄, three were accounted for by a carboxyl [δ 182.0 (s)] group and two double bonds. One is an exocyclic methylene [δ 109.8 (t), 153.5 (s)] and the other is the lanosterol Δ^8 -double bond [δ 134.0 (s), 134.6 (s)] (Lobo et al., 1988). Comparison of the ¹³C NMR spectroscopic data between **2** and **5** (Table 2) suggested the former was structurally different from **5** only in the A-ring. The methyl signal (δ 15.4) of C-28 in **5** was replaced by a carboxyl group (δ 182.0) in **2**. Significant cross-peaks were observed from Me-29 to C-3, C-5, and C-28, and Me-19 to C-5 in the HMBC experiment (Fig. 3); these observations confirmed the above deduction. The similarity of the shifts and coupling constant of H-22 and C-22 to those of **1** suggested that the configuration at C-22 was also *R*. The NOESY spectrum

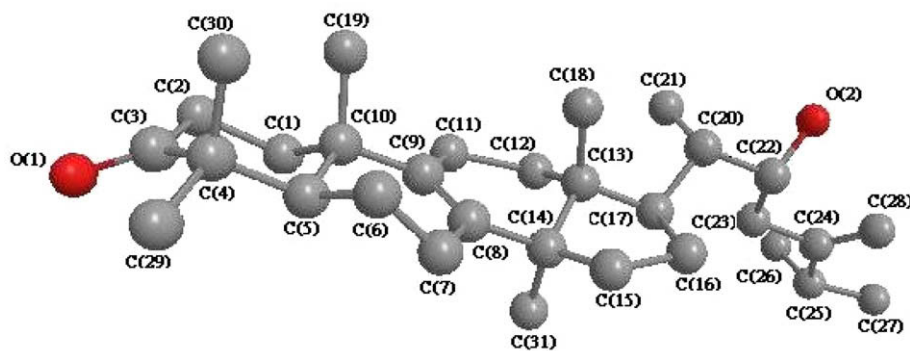


Fig. 2. 3-D structural drawing with atomic labeling of compound **1**. Hydrogen atoms are omitted for clarity.

(Fig. 3) showed a correlation between for H-3 α with H-5 α indicating that the C-3 hydroxyl group was β . Thus gilvsin B (**2**) is 3 β , 22R-dihydroxy-24-methylenelanost-8-en-28-oic acid.

The molecular formula of gilvsin C (**3**) was established as C₃₀H₄₈O₂ from HREIMS at m/z 440.3648 [M]⁺. The ¹H NMR spectrum indicated the presence of three tertiary methyl groups [δ 0.80 (s, Me-18), 0.91 (s, Me-30), and 1.21 (s, Me-19)], four secondary methyl groups [δ 0.95 (d, Me-21), 1.04 (d, Me-28), 1.06 (d, Me-27), and 1.09 (d, Me-26)], a vinyl methylene group at δ 4.84/4.95, and an oxygenated methine at δ 3.80 (d, J = 12.5 Hz, H-22). Comparison of the ¹³C NMR spectroscopic data between **3** and **1** (Table 2) suggested that **3** was structurally different from **1** in the A-ring and had one fewer methyl group than **1**. A secondary methyl proton signal at δ 1.04 (3H, d), which correlated with the carbon resonances at δ 11.4 in the HMQC spectrum, was assigned as Me-28. Significant cross-peaks were observed from Me-28 to C-3 (δ 213.5) and C-5 (δ 49.7), and Me-19 (δ 1.21) to C-5 in the HMBC

experiment, indicating the secondary methyl in the cyclohexanone ring A. The NOESY data exhibited cross-peak correlations for Me-19 with H-4 β and Me-28 with H-5 α , indicating that the methyl group at C-4 was α . Thus structure **3** was established as 22R-hydroxy-24-methylene-29-norlanost-8-en-3-one.

The ¹H and ¹³C NMR spectra of gilvsin D (**4**) indicated the presence of four tertiary methyl, three secondary methyl, a vinyl methylene, an oxygenate methine, and a carboxylic acid group similar to those of **2** (Tables 1 and 2) but lacking the C-3 hydroxyl substituent at δ_H 4.03/ δ_C 75.3 in **2**. It also suggested the presence of a degraded lanosterol skeleton which is consistent with the mass spectroscopic result. The molecular formula of **4** was established as C₃₀H₄₈O₃, CH₂O less than **2** by HREIMS. The EIMS of **4** showed a base peak at m/z 357 [M – CH₃–C₆H₁₂]⁺, accompanied by a fragment ion at m/z 299 [M – CH₃–C₉H₁₈O]⁺ and m/z 311 [357 – COOH–H]⁺. A tertiary methyl proton signal at δ 1.25 (3H, s), which is correlated with carbon resonance at δ 21.3 in the HMQC spectrum, was assigned as Me-29. As observed in the HMBC spectrum, the correlations of Me-19 (δ 0.99) with C-1 (δ 36.0) and C-5 (δ 52.9), Me-29 (δ 1.25) with C-4 (δ 48.1), C-5 (δ 52.9) and C-28 (δ 184.2), H₂-2 (δ 2.49) with C-28 confirmed the presence of a degraded ring A. The chemical shift of Me-29 showed that the C-4 carboxyl group was α (Gonzalez et al., 1986). Thus, the structure of gilvsin D (**4**) is 22R-hydroxy-24-methylene-3-norlanost-8-en-oic acid.

2.2. Hypoglycemic activity testing

Abnormal PEPCK (phosphoenolpyruvate carboxykinase) expression is considered as one of the pathological characteristics in diabetic subjects (Hofmann et al., 1992; Noguchi et al., 1993; Sun

Table 2
¹³C NMR spectroscopic data for compounds **1–5** (in CDCl₃).

Carbon	5	1	2	3	4
1	35.6	36.0	35.1	37.1	36.0
2	27.8	34.6	27.2	38.0	36.9
3	79.0	217.8	75.3	213.5	–
4	38.9	47.4	53.5	45.0	48.1
5	50.4	51.2	46.1	49.7	52.9
6	18.2	19.4	20.8	22.0	18.6
7	26.5	26.4	26.0	25.5	25.9
8	134.2	135.1	134.6	135.4	134.1
9	134.6	133.4	134.0	132.6	135.0
10	37.0	36.9	36.4	36.6	45.6
11	21.0	21.1	21.0	21.9	22.5
12	31.0	30.9	31.0	30.9	30.5
13	44.9	44.9	44.9	44.9	45.4
14	49.4	49.5	49.4	49.5	48.9
15	31.0	31.0	30.9	30.9	30.6
16	27.2	27.2	27.1	27.2	27.3
17	47.2	47.2	47.2	47.2	47.0
18	15.7	15.8	15.7	15.8	15.5
19	19.1	18.7	19.4	17.5	19.3
20	41.1	41.0	41.0	41.0	41.0
21	12.5	12.5	12.5	12.6	12.6
22	70.2	70.2	70.2	70.2	70.2
23	36.0	36.0	36.0	36.0	36.0
24	153.5	154.5	153.5	153.5	153.5
24	109.8	109.8	109.8	109.8	109.8
25	33.1	33.1	33.1	33.1	33.1
26 ^a	21.6	21.6	21.6	21.6	21.6
27 ^a	22.3	22.3	22.3	22.3	22.3
28	28.0	26.1	182.0	11.4	184.2
29	15.4	21.3	10.5	–	21.3
30	24.3	24.4	24.3	24.5	24.5

^a Assignments in some column are interchangeable.

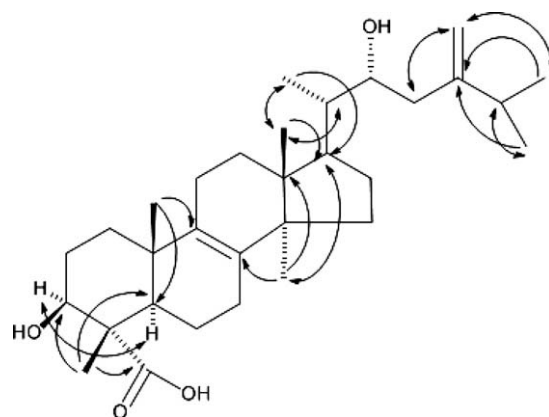


Fig. 3. Key correlations in HMBC (H→C) and NOESY (←→) spectra of **2**.

et al., 2002). In contrast, knocking down PEPCK expression could ameliorate both hyperglycemia and insulin resistance (Robinson et al., 2000; Gomez-Valades et al., 2006). Therefore, restoration of elevated PEPCK transcription was considered as a good therapeutic target for diabetes therapy (O'Brien et al., 1995; Sutherland et al., 1996; O'Brien et al., 2001). As shown in Fig. 4, the dexamethasone and 8-bromo-cAMP induced PEPCK mRNA and protein expression was significantly reduced in presence of insulin (0.06 $\mu\text{g/ml}$) after 4 h and 6 h treatment, respectively. In the presence of the ethanol–H₂O (1:1) extract of *P. gilvus* (PG-Et), a significant reduction of PEPCK mRNA and protein expression was also noted when H4IIE cells were treated with PG-Et at a concentration of 20 $\mu\text{g/ml}$. At a concentration of 80 $\mu\text{g/ml}$, the inhibitory potency of PG-Et was comparable to insulin. To explain such activity found in the crude extract, isolated compounds were employed to evaluate for hypoglycemic activity. Unfortunately, no promising activity of isolated com-

pounds could be demonstrated at the concentrations from 50 μM up to 150 μM (Data not shown).

2.3. Concluding remarks

These results show that *P. gilvus* lanostanes are mainly 24-methylene compounds containing an 8-ene system, with structural variations occurring in ring A. Unfortunately, those compounds were not responsible for the hypoglycemic activity observed from *P. gilvus* crude extract.

3. Experimental

3.1. General experimental procedures

Mps were determined with a Yanagimoto micro-melting point apparatus and are uncorrected. IR spectra were obtained as KBr pellets on a Perkin–Elmer 781 IR spectrometer, whereas UV spectra were obtained on a Hitachi U-3200 spectrophotometer in MeOH. Optical rotations were measured on a JASCO DIP-370 polarimeter. ¹H, ¹³C and 2D NMR spectra were acquired using a Varian Inova-500 spectrometer with deuterated solvent as internal standard. EIMS and HREIMS were recorded on a FINNIGAN LCQ and FINNIGAN MAT-95XL spectrometer, respectively. The X-ray data were acquired on a Nonius, Kappa CCD Single Crystal XRD.

3.2. Plant material

The fruiting body of *P. gilvus* was collected at Jui-Yuan-Hsi, Nantou, Taiwan, in May 2006, and identified by Dr. Tun-Tschu Chang, Division of Forest Protection, Taiwan Forestry Research Institute. A voucher specimen (No. TFRI-1028) is deposited in the herbarium of the Taiwan Forestry Research Institute.

3.3. Extraction and isolation

The dry fruiting body of *P. gilvus* (275 g) was cut into small pieces and extracted with EtOH–H₂O (1:1, v/v; 1 L $\times$ 4) at 60 °C for 24 h. The combined solutions were evaporated *in vacuo* at ca. 50 °C to give a crude extract (6 g), which was partitioned successively between H₂O (0.5 L) and EtOAc (0.5 L), followed by *n*-BuOH (0.5 L). Due to their similar TLC profiles, the EtOAc and *n*-BuOH extracts were combined, (5.5 g total), then being subjected to silica gel MPLC (3.6 $\times$ 48 cm) with a gradient of EtOAc in *n*-hexane from 0% to 100% in 120 min at a flow rate of 3 mL/min, with 20 fractions being collected. Compound **6** (14.6 mg) was crystallized from Fr. 3 in MeOH solution. Frs. 6 (0.65 g), 7 (0.32 g), 9 (46.7 mg), 11 (56.2 mg) and 14 (0.31 g) were individually purified by HPLC [column: Cosmosil C18, 5 μm , 20 $\times$ 250 mm, solvent: CH₃CN–H₂O (85:15, v/v for Frs. 6, 7 and 9; 75:25, v/v for Frs. 11 and 14), flow rate: 20 mL/min, detector: 210 nm], to give **1** (135.2 mg) and **3** (20.4 mg) from Fr. 6, **5** (7.8 mg) from Fr. 7, **4** (7.1 mg) from Fr. 9, and **2** (18.4 mg) from Fr. 14.

3.4. Gilvinsin A (**1**)

Colorless needles (MeOH), mp 122–124 °C; [α]_D²⁴ 52.2 (c 0.23, CHCl₃); IR (KBr) ν_{max} 3544 (OH), 3073 (C=CH), 1708 (C=O), 1645 (C=C), 1456, 1386, 1375 cm^{−1}; for ¹H and ¹³C NMR spectroscopic data (CDCl₃), see Tables 1 and 2, respectively. EIMS (30 eV) *m/z* (rel. Int.) 454 [M]⁺ (24), 355 [M – CH₃–C₆H₁₂]⁺ (100), 338 [355 – H₂O + H]⁺ (48), 297 [M – CH₃–C₆H₁₈O]⁺ (18); HREIMS *m/z* 454.3817 [M]⁺ (calcd 454.3811 for C₃₁H₅₀O₂).

X-ray structure analysis of **1**: A suitable colorless crystal (0.20 $\times$ 0.15 $\times$ 0.10 mm³), grown by slow evaporation of MeOH

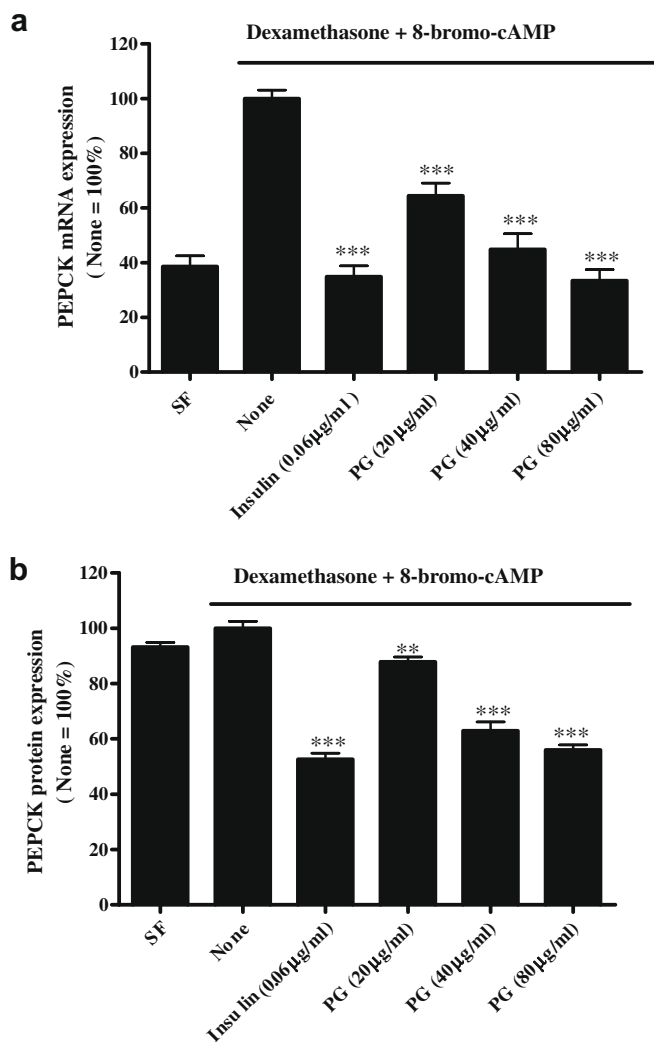


Fig. 4. *Phellinus gilvus* (PG) dose-dependent suppression of PEPCK mRNA and protein expression. (A) Semi-quantified effect of dexamethasone (500 nM) and 8-bromo-cAMP (100 μM) induced PEPCK mRNA expression in presence or absence of ethanol–H₂O (1:1) extract of *Phellinus gilvus* (PG-Et). Insulin (0.06 $\mu\text{g/ml}$) served as the experimental control. Quantified data was presented as mean $\pm$ SEM (*n* = 4). ****P* < 0.001 when compared with control (none). (B) Semi-quantified effect of dexamethasone (500 nM) and 8-bromo-cAMP (100 μM) induced PEPCK protein expression in presence or absence of PG-Et or insulin (0.06 $\mu\text{g/ml}$). Quantified data was presented as mean $\pm$ SEM (*n* = 4). ***P* < 0.01 and ****P* < 0.001 when compared with control (none).

solution, was mounted on a Nonius CCD diffractometer equipped with Mo radiation ($\lambda = 0.71073 \text{ \AA}$). Crystal data: $\text{C}_{31}\text{H}_{50}\text{O}_2$, $M_r = 454.71 \text{ g/mol}$, triclinic, $P1$, $a = 7.3237(2) \text{ \AA}$, $b = 12.1169(3) \text{ \AA}$, $c = 16.1701(4) \text{ \AA}$, $V = 1381.95(6) \text{ \AA}^3$, $Z = 2$, $D_{\text{calc}} = 1.093 \text{ mg/m}^3$, $F(000) = 504$. A total of 12303 reflections were collected (12303 unique, $R_{\text{int}} = 0.0000$) in the range $2.53^\circ < \theta < 28.91^\circ$. The structure was solved using direct methods and refined by Full-matrix least-squares on F^2 values. The non-hydrogen atoms were refined anisotropically. All hydrogen atoms were fixed at calculated positions. The final indices were $R_1 = 0.0478$, $wR_2 = 0.1211$ with goodness-of-fit = 0.868. Atomic coordinates for this structure have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication No. CCDC 688411. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

(R)-MTPA derivative (**1a**): To a solution of **1** (9 mg) in dry CH_2Cl_2 (5 mL) were added 1,3-dicyclohexyl-carbodiimide (30 mg), 4-dimethylaminopyridine (23 mg), and (R)-MTPA acid (20 mg). After being stirred at room temperature for 20 h, the reaction mixture was evaporated to dryness. The residue was purified by silica gel column (EtOAc: *n*-hexane, 1:9, v/v), to yield the (R)-MTPA ester **1a**: ^1H NMR (CDCl_3) δ 0.72 (3H, s, Me-18), 0.87 (3H, d, $J = 7.0 \text{ Hz}$, Me-21), 0.88 (3H, s, Me-30), 0.92/0.95 (each 3H, d, $J = 7.5 \text{ Hz}$, Me-26/-27), 1.05/1.07/1.10 (each 3H, s), 4.58/4.65 (H_2 -24'), 5.35 (1H, d, $J = 9.0 \text{ Hz}$, H-22).

(S)-MTPA derivative (**1b**): As above, except for use of (S)-MTPA acid (20 mg). (S)-MTPA ester **1b**: ^1H NMR (CDCl_3) δ 0.71 (3H, s, Me-18), 0.86 (3H, d, $J = 7.0 \text{ Hz}$, Me-21), 0.87 (3H, s, Me-30), 1.00/1.01 (each 3H, d, $J = 7.0 \text{ Hz}$, Me-26/-27), 1.05/1.07/1.10 (each 3H, s), 4.76/4.83 (H_2 -24'), 5.36 (1H, d, $J = 9.5 \text{ Hz}$, H-22).

3.5. Gilvsin B (**2**)

White powder (MeOH), mp 238–240 °C; $[\alpha]_{\text{D}}^{25}$ 25.0 (c 0.24, CHCl_3); IR (KBr) ν_{max} 3435 (OH), 1704 (C=O), 1640 (C=C), 1470, 1370, 1260 cm^{-1} ; for ^1H and ^{13}C NMR spectroscopic data (CDCl_3), see Tables 1 and 2, respectively. EIMS (30 eV) m/z (rel. Int.) 486 $[\text{M}]^+(20)$, 387 $[\text{M} - \text{CH}_3 - \text{C}_6\text{H}_{12}]^+ (100)$, 369 $[\text{387} - \text{H}_2\text{O}]^+ (38)$, 341 $[\text{369} - \text{CO}]^+ (38)$, 329 $[\text{M} - \text{CH}_3 - \text{C}_9\text{H}_{18}\text{O}]^+ (47)$; HREIMS m/z 486.3708 $[\text{M}]^+$ (calcd 486.3709 for $\text{C}_{31}\text{H}_{50}\text{O}_4$).

3.6. Gilvsin C (**3**)

Amorphous powder; $[\alpha]_{\text{D}}^{24}$ 29.2 (c 0.24, CHCl_3); IR (KBr) ν_{max} 3434 (OH), 1698 (C=O), 1635 (C=C), 1456, 1374, 1127 cm^{-1} ; for ^1H and ^{13}C NMR spectroscopic data (CDCl_3), see Tables 1 and 2, respectively. EIMS (30 eV) m/z (rel. Int.) 440 $[\text{M}]^+(27)$, 356 $[\text{M} - \text{C}_6\text{H}_{12}]^+ (17)$, 341 $[\text{M} - \text{CH}_3 - \text{C}_6\text{H}_{12}]^+ (100)$, 323 $[\text{341} - \text{H}_2\text{O}]^+ (52)$, 283 $[\text{M} - \text{CH}_3 - \text{C}_9\text{H}_{18}\text{O}]^+ (18)$; HREIMS m/z 440.3648 $[\text{M}]^+$ (calcd 440.3655 for $\text{C}_{30}\text{H}_{48}\text{O}_2$).

3.7. Gilvsin D (**4**)

Amorphous powder; $[\alpha]_{\text{D}}^{24}$ 6.5 (c 0.31, CHCl_3); for ^1H and ^{13}C NMR spectroscopic data (CDCl_3), see Tables 1 and 2, respectively. EIMS (30 eV) m/z (rel. Int.) 456 $[\text{M}]^+ (21)$, 357 $[\text{M} - \text{CH}_3 - \text{C}_6\text{H}_{12}]^+ (100)$, 311 $[\text{357} - \text{H}_2\text{O} - \text{CO}]^+ (48)$, 299 $[\text{M} - \text{CH}_3 - \text{C}_9\text{H}_{18}\text{O}]^+ (40)$, 253 $[\text{299} - \text{HCOOH}]^+ (22)$; HREIMS m/z 456.3600 $[\text{M}]^+$ (calcd 456.3604 for $\text{C}_{30}\text{H}_{48}\text{O}_3$).

3.8. H4IIE cell culture

The rat liver cell line H4IIE was cultured with Dulbecco's Modified Eagle's Medium (DMEM) containing 1000 mg/L glucose, 5%

(v/v) fetal calf serum (GIBCO, Carlsbad, CA) and maintained at 37 °C in an atmosphere of 5% CO_2 and 95% air.

3.9. Measurement of gene expression

Based on the procedure previously described (Chen et al., 2008) total RNA was extracted using TRI-reagent according to the manufacturer's instructions. 50 ng of reverse transcribed cDNA was employed for further PCR. PCR products were separated by gel electrophoresis, visualized and photographed with a digital camera, and quantified with Genetools 3.06 (Syngene).

3.10. Preparation of cell extract for SDS PAGE and immunoblotting

Based on the procedure previously described (Chen et al., 2008), cells were washed with ice-cold PBS and scraped into ice-cold lysis buffer. Equal amounts of protein (40 μg) were separated using SDS 10% polyacrylamide gels. Following transfer to nitrocellulose membrane, blots were blocked with 5% (w/v) non-fat milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 for 1 h and incubated with primary antibodies prior to incubation with the secondary antibody. Finally, results were visualized after development of films with the aid of ECL kit (Amersham Biosciences).

3.11. Statistics

The significance of various treatments was determined by the Student's *t*-test. The results were expressed as mean $\pm$ S.E.M. Differences were considered significant if $P < 0.05$.

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