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FULL PAPER

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Phytochemicals from the Leaves of *Cyclocarya paliurus* and their 11β-HSD1 Enzyme Inhibitory Effects

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Two new dammarane-type triterpenoid saponins, 3β-(α-L-arabinopyranosyloxy)-24,25-dihydroxydammar-20-en-12α-yl 6-deoxy-β-D-glucopyranoside (**1**) and (24*R*)-3β-[(4-*O*-acetyl-α-L-arabinopyranosyl)oxy]-25-hydroxy-20,24-epoxydammaran-12β-yl 6-deoxy-β-D-glucopyranoside (**2**), and fourteen known triterpenoids were isolated from the 70% MeOH extract of the leaves of *Cyclocarya paliurus*. Their structures were established based on analyses of spectroscopic data. All compounds were tested for their inhibitory activities against the 11β-HSD1 enzyme. Hederagenin (**13**) exhibited moderate inhibitory effect for mouse 11β-HSD1 with an IC_{50} value of 0.16 $\pm$ 0.04 μM.

Keywords: *Cyclocarya paliurus*, dammarane-type triterpenoid saponins, 11β-HSD1 enzyme, inhibitory activity.

Introduction

11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) is the enzyme primarily responsible for the regulation of intracellular cortisol levels. Inhibition of 11β-HSD1 is a new strategy for the treatment of metabolic disorder diseases, such as type 2 diabetes.[1,2] In modern medicine, a number of synthetic 11β-HSD1 inhibitors have been reported,[3] however, natural products are rarely.[4,5] Herein, we report the isolation and structure determination of two new compounds from *Cyclocarya paliurus* and their inhibitory activities against 11β-HSD1.

Cyclocarya paliurus (Batalin) Iljinsk., a Chinese endemic plant, belongs to the family Juglandaceae.[6,7] Because of the sweet taste of its leaves, this species have long been used as the herbal tea known as 'sweet tea tree', In addition, *C. paliurus* can serve as a dietary supplement for trace elements and a good food resource for maritime people.[8] Furthermore, the leaves of *C. paliurus* is an essential ingredient of Tujia ethnomedicine in P. R. China, in light of treating diabetes mellitus.[9,10] Previous research shows that *C. paliurus* ethanol extract displayed antihyperglycemic and improved insulin resistance bioactivities.[11] Besides, structurally diverse secondary metabolites, such as *seco*-dammarane triterpenoids, lignans, flavonoids, and phenolic compounds, have been isolated from *C. paliurus* so far.[12] These results inspired our great interest in exploring the bioactive phytochemicals of this plant. As a result, two dammarane-type triterpenoid saponins, named 3β-(α-L-arabinopyranosyloxy)-24,25-dihydroxydammar-20-en-12α-yl 6-deoxy-β-D-glucopyranoside (**1**) and (24*R*)-3β-[(4-*O*-acetyl-α-L-arabinopyranosyl)oxy]-25-hydroxy-20,24-epoxydammaran-12β-yl 6-deoxy-β-D-glucopyranoside (**2**), along with 14 known triterpenoids (*Figure 1*) were obtained. The known compounds were identified by detailed comparison of their NMR and MS date with those reported in previous literatures as (20*S*,24*R*)-epoxydammarane-3β,12β,25-trihydroxy-12-*O*-β-D-quinvopyranosyl-3-*O*-α-L-arabinpyranoside (**3**),[13] cyclocarioside I (**4**),[14]

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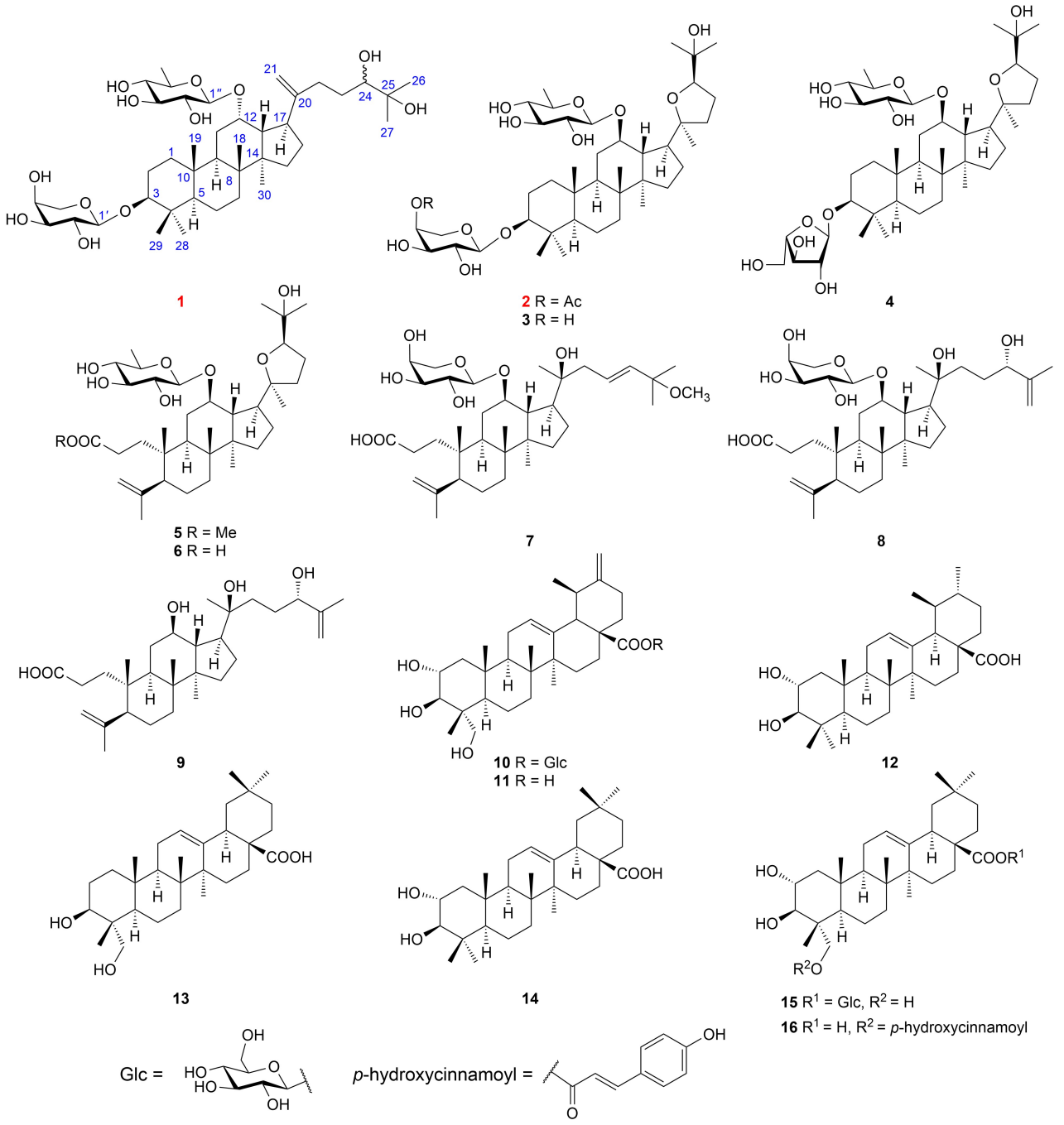

Figure 1. Chemical structures of **1**–**16** isolated from *C. paliurus.*

(20*S*,24*R*)-20,24-epoxy-25-hydroxy-12β-(β-D-quinovopyranosyloxy)-3,4-*seco*-dammar-4(28)-en-3-oic acid methyl ester (**5**),[15] cyclocarioside III (**6**),[16] cyclocarioside F (**7**),[17] cyclocarioside J (**8**),[18] cyclocariol D (**9**),[19] 2α,3β,23-trihydroxylurs-12,20(30)-dien-28-oic acid-β-D-glucopyranoside (**10**),[20] actinidia acid (**11**),[21] 2α-hydroxyursolic acid (**12**),[22] hederagenin (**13**),[23] maslinic acid (**14**),[24] chebuloside II (**15**),[25] and 23-*trans*-*p*-counmaroyloxy-2α,3β-dihydroxyplen-12-en-28-acid (**16**),[26] respectively. In conclusion, This article reports the isolation, structural elucidation, and 11β-HSD1 enzyme activities of these triterpenoids.

Results and Discussion

Compound **1**, white amorphous powder, had the molecular formula of $C_{41}H_{70}O_{12}$ as established by HR-EI-MS data (*m/z* 754.4867 $[M]^+$), indicating seven unsaturated degrees. The ^{1}H-NMR spectrum (*Table 1*) of **1** exhibited eight methyl singlets at δ_H 1.58 (3H, s, CH_3-6″), 1.52 (3H, s, CH_3-27), 1.48 (3H, s, CH_3-26), 1.39 (3H, s, CH_3-19), 1.25 (3H, s, CH_3-28), 1.04 (3H, s, CH_3-18), 0.98 (3H, s, CH_3-29) and 0.60 (3H, s, CH_3-30); one pair of terminal olefinic protons at δ_H 4.91 (1H, br. s, H-21) and 4.90 (1H, br. s, H-21); and partially overlapped multiples of methylene and methine protons were observed between δ_H 0.94 and 2.70. Combined with two anomeric protons at δ_H 4.88 (d, J = 11.6, H-1″) and 4.72 (d, J = 6.4, H-1′), with other oxygen-bearing methine and one oxymethylene protons of the sugar units, these spectroscopic data suggested that **1** was a glycoside with two glycosyl moieties. Acid hydrolysis of **1**, one set of carbon signals for an L-arabinopyranose and another set of carbon signals for a D-quinovopyranose were identified by comparison on HPLC with authentic sample. The ^{13}C-NMR and DEPT spectra of **1** displayed 41 carbon signals, 11 of the 41 carbons were assigned to the glycosyl groups, and other 30 carbons to aglycone which have seven methyl groups, nine methylene groups (one olefinic), seven methine groups (three oxygenated), and six quaternary carbons (one olefinic). The ^{1}H,^{1}H-COSY correlations (*Figure 2*) revealed six structural fragments by correlations of H_2-1/H_2-2/H-3, H-5/H_2-6/H_2-7, H-9/H_2-11/H-12/H-13/H-17, H_2-16/H_2-15/H-17, H_2-22/H_2-23/H-24, H-1′/H-2′/H-3′/H-4′/H-5′ and H-1″/H-2″/H-3″/H-4″/H-5″/H_3-6″. Besides, ten key HMBCs signals, H_3-19 (δ_H 1.39) with C-1/C-5/C-9/C-10, H_3-18 (δ_H 1.04) with C-7/C-8/C-9/C-14, H_3-30 (δ_H 0.60) with C-8/C-14/C-15, H_2-21 (δ_H 4.91 and 4.90) with C-17/C-20/C-22, H_3-26 (δ_H 1.48) with C-24/C-25, H_3-27 (δ_H 1.52) with C-24/C-25, H_3-28 (δ_H 1.25) with C-4/C-5,and H_3-29 (δ_H 0.98) with C-3/C-4, a anomeric proton H-1′ (δ_H 4.72) with C-3, and another anomeric proton at H-1″ (δ_H 4.87) with C-12, were observed. Overall, the above spectral data indicated that compound **1** was a dammarane-type triterpenoid saponin similar to cyclocarioside Q,[27] and their major differences are located at the side chains. The terminal double bond at C-20 and C-21 was elucidated by HMBCs of H_2-21 (δ_H 4.91 and 4.90) with C-20/C-17/C-22 (*Figure 2*). The position of two hydroxy groups were supported by the key HBMC correlations of H_3-26 (δ_H 1.48) with C-24/C-25. Thus, the planar structure of **1** was elucidated as shown in *Figure 2*.

The relative orientation of H-3 and H-12 were established by the ROESY experiment (*Figure 3*), in which correlations of H-3/H-5α, H-12/H_3-18/H_3-19/H-13β revealed that H-3 and H-12 shared α- and β-orientation, respectively. Based on the aforementioned data, the structure of **1** was deduced as 3β-(α-L-

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of **1** and **2** in (D_5)pyridine (δ in ppm, J in Hz).

No.	**1**		**2**		No.	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C		δ_H	δ_C	δ_H	δ_C
1	3.09–3.12 (m)	35.6 (t)	3.13–3.17 (m)	35.6 (t)	22	2.64–2.70 (m)	32.1 (t)	1.73 – 1.78 (m)	34.2 (t)
	2.01–2.08 (m)		2.09–2.15 (m)			2.24–2.31 (m)		1.55 – 1.60 (m)	
2	1.88–1.92 (m)	30.0 (t)	1.73–1.77 (m)	26.8 (t)	23	2.04 (overlap)	31.3 (t)	2.05 (overlap)	26.4 (t)
	1.48–1.54 (m)		1.54–1.58 (m)			1.79–1.85 (m)		1.94 (overlap)	
3	3.58–3.62(m)	81.4 (d)	3.57–3.60 (m)	81.6 (d)	24	3.75–3.78 (m)	78.5 (d)	3.94–3.97 (m)	84.3 (d)
4		38.1 (s)		38.0 (s)	25		72.7 (s)		71.3 (s)
5	1.57 (overlap)	51.1 (d)	1.61 (overlap)	51.0 (d)	26	1.48 (s)	26.0 (q)	1.45 (s)	26.0 (q)
6	1.51–1.53 (m)	18.3 (d)	1.50–1.53 (m)	18.4 (t)	27	1.52 (s)	26.1 (q)	1.41 (s)	27.7 (q)
	1.42–1.45 (m)		1.46–1.47 (m)		28	1.25 (s)	30.0 (q)	1.00 (s)	23.2 (q)
7	1.44–1.51 (m)	36.6 (t)	1.50 (overlap)	36.5 (d)	29	0.98 (s)	23.2 (q)	1.27 (s)	30.0 (q)
	1.12 (d, J = 11.6)		1.19–1.20 (m)		30	0.60 (s)	16.1 (q)	0.60 (s)	16.8 (q)
8		41.6 (s)		41.6 (s)	Ara(*p*)				
9	1.79–1.86 (m)	54.0 (d)	1.87 (overlap)	53.9 (d)	1′	4.72 (d, J = 6.4)	101.9 (d)	4.72 (d, J = 7.6)	102.4 (d)
10		40.0 (s)		40.0 (s)	2′	4.35–4.40 (m)	72.5 (d)	4.24–4.26 (m)	75.1 (d)
11	2.50–2.53 (m)	32.1 (t)	2.87–2.90 (m)	34.6 (t)	3′	4.20 (dd, J = 3.2, 12)	74.7 (d)	4.01 (overlap)	75.1 (d)
	1.40 (overlap)		1.48–1.50 (m)		4′	4.33–4.35 (m)	69.2 (d)	5.36–5.42 (m)	73.4 (d)
12	4.38–4.43 (m)	76.8 (d)	4.36–4.43 (m)	77.5 (d)	5′	4.29 (dd, J = 3.2, 8.0)	66.4 (t)	4.30–4.33 (m)	63.4 (t)
13	1.93 (overlap)	44.1 (d)	1.80–1.84 (m)	41.3 (d)		3.76 (overlap)		3.52 (t, J = 10.6)	
14		49.6 (s)		50.1 (s)	*C*OOMe				170.8 (s)
15	1.43–1.48 (m)	31.3 (t)	1.34–1.37 (m)	31.6 (t)	COO*Me*			1.97 (s)	21.0 (q)
	0.98 (overlap)		0.95–0.98 (m)		Qui				
16	1.82–1.87 (m)	21.9 (t)	2.08–2.12 (m)	22.1 (t)	1″	4.88 (d, J = 11.6)	101.8 (d)	5.02 (d, J = 7.6)	101.9 (d)
	1.80–1.87 (m)		1.90–1.93 (m)		2″	3.92 (t, J = 8.4)	75.6 (d)	3.94–3.98 (m)	75.6 (d)
17	2.23–2.28 (m)	48.3 (d)	1.86–1.91 (m)	49.2 (d)	3″	4.06 (t, J = 8.4)	78.3 (d)	4.17 (t, J = 9.0)	78.5 (d)
18	1.04 (s)	17.2 (q)	1.09 (s)	17.1 (q)	4″	3.64 (overlap)	76.9 (d)	3.68 (t, J = 9.0)	76.9 (d)
19	1.39 (s)	16.8 (q)	1.41 (s)	16.8 (q)	5″	3.64 (overlap)	72.7 (d)	3.80–3.86 (m)	72.9 (d)
20		153.2 (s)		86.2 (s)	6″	1.57 (d, J = 4.7)	18.6 (q)	1.61 (d, J = 6.0)	18.7 (q)
21	4.91 (br. s)	108.3 (d)	1.17 (s)	24.5 (q)					
	4.90 (br. s)								

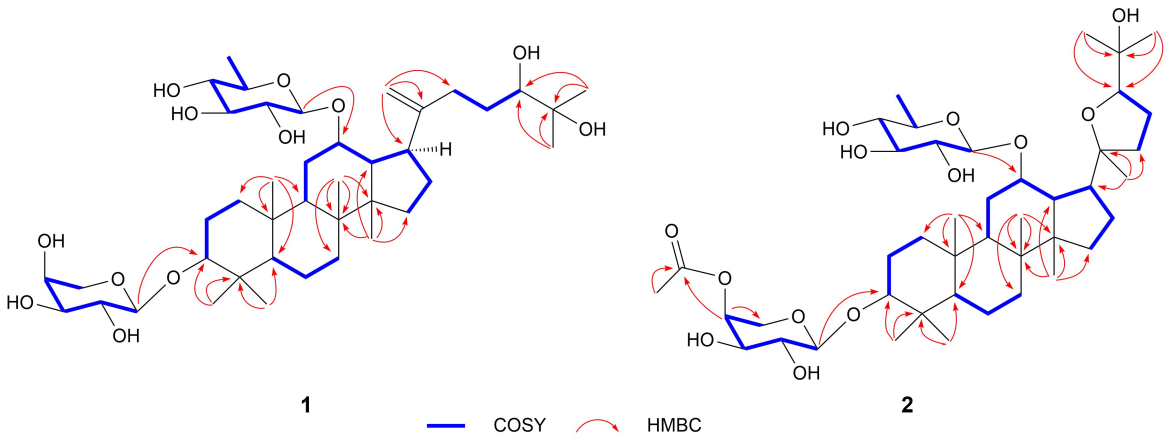

Figure 2. The ^{1}H,^{1}H-COSY and key HMBC data of **1** and **2**.

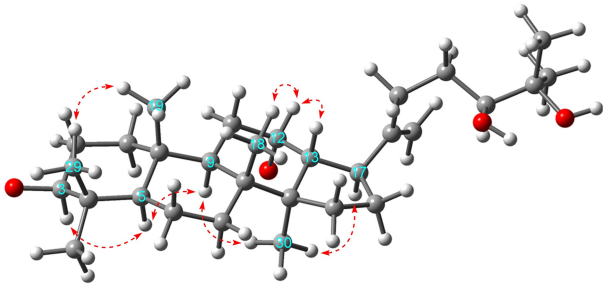

Figure 3. Key ROESY correlations for the aglycone moiety of **1**.

arabinopyranosyloxy)-24,25-dihydroxydammar-20-en-12α-yl 6-deoxy-β-D-glucopyranoside.

Compound **2** was obtained as colorless flake crystals. Its molecular formula, $C_{43}H_{72}O_{13}$, was confirmed by the HR-EI-MS peak at *m/z* 796.4978 $[M]^+$ (calc. for $C_{43}H_{72}O_{13}$, 796.4973). Detailed inspection of the ^{1}H-NMR and ^{13}C-NMR data (*Table 1*) of **2** disclosed that it was also a dammarane-type triterpenoid derivative with two sugar moieties. Comparison the NMR data of **2** with the data of (24*R*)-3β-(α-L-arabinopyranosyloxy)-25-hydroxy-20,24-epoxydammaran-12β-yl 6-deoxy-β-D-glucopyranoside[12] indicated that they shared the same aglycone, except for the occurrence of additional signals for an acetyl group. The acetyl group was assigned to be placed at C-4′ of the arabinopyranosyl unit by the observed correlations of H-4′ (δ_H 5.39) with C=O/C-3′/C-5′ in the HMBC spectrum (*Figure 2*). Thus, the structure of **2** was elucidated to be (24*R*)-3β-[(4-*O*-acetyl-α-L-arabinopyranosyl)oxy]-25-hydroxy-20,24-epoxydammaran-12β-yl 6-deoxy-β-D-glucopyranoside.

Compounds **1**-**16** were evaluated for their inhibitory activity on murine and human 11β-HSD1. However, the results showed that only **13** had moderate bioactivity for mouse 11β-HSD1 with an IC_{50} value of 0.16 ± 0.04 μM, compared with positive control glycyrrhizinic acid (6.60 ± 1.49 nM).

Conclusions

The phytochemical study of *C. paliurus* let to the isolation of two new dammarane-type triterpenoid saponins **1** and **2**, along with fourteen known triterpenoids including seven dammarane-type triterpenoids **3**-**9**, three ursane-type triterpenoids **10**-**12**, and four oleanane-type triterpenoid lactones **13**-**16**. The isolates were tested for their inhibitory activities against the 11β-HSD1 enzyme. Compound **13** exhibited moderate inhibitory effect for mouse 11β-HSD1 with an IC_{50} value of 0.16 ± 0.04 μM.

Experimental Section

General

Optical rotations were measured with a JASCO P-1020 digital polarimeter. IR Spectra were recorded on a Bruker-Tensor-27 infrared spectrophotometer with KBr pellets. NMR Spectra were obtained on DRX-500 and AM-400 instruments with TMS as internal standard. ESI-MS and HR-ESI-MS spectra were measured by Bruker HTC/Esquire and API-Qstar-Pulsar spectrometers, respectively. Semi-preparative HPLC was carried out by an Agilent 1100 apparatus with a Zorbax SB-C_{18} column (9.4 mm × 25 cm, 5 μm, Agilent) under the detect guidance of 203 nm. Column chromatography (CC) was performed on silica gel (SiO_2, 200–300 mesh,

Qingdao Marine Chemical Inc., China) or SiO_2 H (10–40 μm, Qingdao Marine Chemical Inc., China), MCI-gel CHP20P (75–150 μm, Mitsubishi Chemical Co., Japan), RP-18 (50 μm, Merck, Germany), and Sephadex LH-20 (GE Healthcare, USA). Fractions were monitored by TLC and spots were detected with a UV254 lamp followed by heating SiO_2 plates sprayed with 10% H_2SO_4 in EtOH.

Plant Material

The leaves of *C. paliurus* collected in Xinning County, Hunan Province, P. R. China, in August 2013. The materials were identified by Dr. Yun-Heng Ji from the Kunming Institute of Botany. A voucher specimen (No. HY201307) has been deposited at the Sate Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China.

Extraction and Isolation

The air-dried and powdered leaves of *C. paliurus* (5.0 kg) were extracted three times with 70% ethanol under reflux. The filtrate was concentrated under vacuum to give a residue, which was suspended in H_2O and extracted with ethyl acetate (AcOEt) for three times. The AcOEt fraction was submitted to an MCI-gel CC eluted with MeOH/H_2O (1:1 to 1:0, v/v) to yield six fractions A-H. Fr. C (190 g) was subjected to a silica gel (200–300 mesh) column eluted with CH_3Cl/MeOH (30:1 to 1:7, v/v) to afford six fractions (Frs. C1–C6). Frs. C2 (3.0 g) and C3 (5.0 g) were separately applied to Rp-C_{18} gel (MeOH/H_2O, 40:60 to 90:10, v/v), Sephadex LH-20 (MeOH), silica gel column (200–300 mesh, petroleum ether/acetone, 1:1 to 1:2, v/v; $CHCl_3$/MeOH, 60:1 to 0:1, v/v), and semi-preparative HPLC (MeCN/H_2O, 30:70 to 65:35, v/v, flow rate: 3.0 mL/min), to give **11** (10 mg, t_R = 12.0 min), **12** (6 mg, t_R = 17.1 min), **13** (10 mg, t_R = 19.0 min) and **14** (20 mg, t_R = 17.9 min) from Fr. C2 as well as **9** (50 mg, t_R = 18.9 min) and **16** (4 mg, t_R = 20.1 min) from Fr. C3. Fr. C4 (15 g) was purified successively by an Rp-C_{18} gel CC (MeOH/H_2O, 40:60 to 90:10, v/v) and a Sephadex LH-20 (MeOH) column to give **2** (1.5 g), **5** (200 mg), and **6** (1.5 g). Likewise, Fr. C5 (26 g) was submitted to diverse CC (Rp-C_{18} gel, MeOH/H_2O, 45:55 to 90:10, v/v; silica gel, 200–300 mesh, $CHCl_3$/MeOH, 25:1 to 0:1, v/v; Sephadex LH-20, MeOH) to give **3** (1 g), **4** (100 mg), **7** (100 mg), and **8** (15 mg). Fr. C6 was submitted to Sephadex LH-20 (MeOH) to provide three fractions: Frs. C6.1–C6.3. Fr. C6.1 was separated by a silica gel column (200–300 mesh, AcOEt/MeOH, 7:1 to 1:1, v/v) and purified by semi-preparative HPLC (MeCN/H_2O, 40:60 to 80:20, v/v, flow rate: 3.0 mL/min) to give **1** (4 mg, t_R = 6.26 min), **10** (6 mg, t_R = 12.1 min), and **15** (30 mg, t_R = 10.2 min).

3*β*-(*α*-L-Arabinopyranosyloxy)-24,25-dihydroxy-dammar-20-en-12*α*-yl 6-Deoxy-*β*-D-glucopyranoside (**1**). White amorphous powder. $[\alpha]_D^{22} = -30.2$ (c = 0.1, CH_3OH). IR (KBr): ν_{max} 3440, 2935, 1633, 1066 cm^{-1}. ^{1}H-NMR (500 MHz, (D_5)pyridine) and ^{13}C-NMR (125 MHz, (D_5)pyridine): see *Table 1*. HR-EI-MS: *m/z* 754.4882 ([M]$^+$, calc. for $C_{41}H_{70}O_{12}$, *m/z* 754.4867).

(24*R*)-3*β*-[(4-*O*-Acetyl-*α*-L-arabinopyranosyl)oxy]-25-hydroxy-20,24-epoxydammaran-12*β*-yl 6-Deoxy-*β*-D-glucopyranoside (**2**). White and flake crystals. $[\alpha]_D^{22} = -20.0$ (c = 0.12, CH_3OH). IR (KBr): ν_{max} 3429, 2937, 1638, 1066 cm^{-1}. ^{1}H-NMR (500 MHz, (D_5) pyridine) and ^{13}C-NMR (125 MHz, (D_5)pyridine): see *Table 1*. HR-EI-MS: *m/z* 796.4978 ([M]$^+$, calc. for $C_{43}H_{72}O_{13}$, *m/z* 796.4973).

Acid Hydrolysis of Compounds **1–2** *and Determination of the Absolute Configuration of the Sugars by HPLC*

Compounds **1** (1.0 mg) and **2** (2.0 mg) in 4 M CF_3COOH (1,4-dioxane/H_2O 1:1, 2.0 mL) were heated at 99 °C for 3 h, respectively. The reaction mixture was diluted with H_2O (1 mL) and then, extracted with $CHCl_3$ (3×2 mL). Next, each aqueous layer was evaporated to dryness using rotary evaporation. Each dried residue was dissolved in pyridine (1.0 mL) mixed with L-cysteine methyl ester hydrochloride (1.0 mg) (Aldrich, Japan) and heated at 60 °C for 1 h. Then, *O*-tolyl isothiocyanate (5.0 μL) (Tokyo Chemical Industry Co., Ltd., Japan) was added to each mixture, this being heated at 60 °C for 1 h. Each reaction mixture was directly analyzed by reversed-phase HPLC following the above procedure. The reaction mixture was directly analyzed by analytical HPLC on a Poroshell 120 SB-C_{18} column (100×4.6 mm, 2.7 μm, Agilent) using an elution of CH_3CN/H_2O (20:75→40:60, v/v) at a flow rate of 0.6 mL/min. Under UV detection at 254 nm, retention time for the standard monosaccharides: L-arabinose (13.079 min), D-quinovose (15.748 min) was used for comparison with retention times from reaction mixtures for the saponin. The absolute configurations of the sugars for **1** and **2** were identified as L-arabinose (13.077 min) and D-quinovose (15.752 min).

11β-HSD1 Enzyme Activity Assay

The inhibitory activities of the isolated compounds on human or mouse 11β-HSD1 was evaluated using the scintillation proximity assay (SPA). The full-length cDNAs of human or murine11β-HSD1 was isolated from the cDNA libraries provided by NIH Mammalian Gene Collection. Then, the cDNAs were cloned into pcDNA3 expression vectors by PCR. HEK-293 cells were transfected with the pcDNA3-derived expression plasmid and selected by cultivation in the presence of 700 μg/mL of G418. Non-resistant cells were removed by replacing the cell culture medium every other day for 12–14 days. The single surviving colony was picked up and expanded. The microsomal fraction overexpressing 11β-HSD1 was prepared from the HEK-293 cells, which were stably transfected with either human or mouse 11β-HSD1. The fraction was then used as the enzyme source for SPA. Different concentrations of compound were added to 96-well microtiter plates, followed by the addition NADPH and [^{3}H] cortisone for 11β-HSD1 assay. The product, [^{3}H] cortisol, was specifically captured by a monoclonal antibody coupled to protein A-coated SPA beads. All tests were performed for three independent replicates with glycyrrhizinic acid as a positive control. The % inhibition was calculated relative to a non-inhibited control. IC_{50} (X + SD, $n = 3$) values were calculated by using Prism Version 4 (GraphPad Software, San Diego, CA).[28]

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Author Contribution Statement

H. Yan, X. Li, W. Ni and Q. Zhao were responsible for the isolation, structure elucidation and preparing the manuscript. Y. Leng carried out the bioassays. H.-Y. Liu designed and supervised this research.

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