Prenylated Flavonoids from the Stems and Roots of *Tripterygium wilfordii*

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ABSTRACT: Phytochemical investigation on the stems and roots of *Tripterygium wilfordii* led to the isolation and characterization of three new prenylated flavanones, tripteryols A-C (**1-3**), along with ($\pm$)-5,4'-dihydroxy-2'-methoxy-6',6''-dimethypyraro-(2'',3'':7,8)-6-methylflavanone (**4**), and ((2*S*)-5,7,4'-trihydroxy-2'-methoxy-8,5'-di(3-methyl-2-butenyl)-6-methylflavanone (**5**). Structures of the compounds **1-5** were elucidated using spectroscopic techniques, such as UV, IR, NMR (1D and 2D), and HRESI-MS. Tripteryols B (**2**) was found active in the antimicrobial assay against *Cryptococcus neoformans*, *Pseudomonas aeruginosa*, vancomycin-resistant *Enterococcus faecalis* (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) with IC₅₀ values in the range of 2.95-8.59 μ g/mL. Compounds **4** and **5** showed significant antimicrobial activities against *C. neoformans*, MRSA and *Staphylococcus aureus* with IC₅₀ values in the range of 1.06-2.60 μ g/mL. Additionally, significant antimalarial activities of tripteryols A-B (**1-2**) against chloroquine-sensitive D6 and resistant W2 clones of *Plasmodium falciparum* were observed and none of the compounds **1-5** were cytotoxic to Vero cells.

Key words: *Tripterygium wilfordii*; Celastraceae; Flavanone; Antimicrobial; Antimalarial; NMR.

1. Introduction

The stems and roots of *T. wilfordii* are named Lei-gong-teng in Chinese, and have been used as Chinese herbal medicine for hundreds of years. It is mainly distributed in regions of Eastern and Southern China, Korea, and Japan [1-2]. A variety of preparations of *T. wilfordii*, such as “Tripterygium glycosides tablet”, and “Tripterygium-loaded solid lipid nanoparticles”, have been widely used to treat auto-immune and inflammatory diseases [3-5]. Previous phytochemical investigations revealed sesquiterpenoid alkaloids, diterpenoids and triterpenes as main chemical constituents of *T. wilfordii* [6-7]. However, limited study focused on the isolation of flavonoids from *T. wilfordii*, except for one of our previous phytochemical investigations on the plant, which reported two prenylated and C-methylated flavanones with moderate antiproliferative activity [8].

In our ongoing phytochemical investigation on *T. wilfordii*, three new prenylated flavanones, tripteryols A-C (**1-3**), along with two known flavanones ($\pm$)-5,4'-dihydroxy-2'-methoxy-6',6''-dimethylpyraro-(2'',3'':7,8)-6-methylflavanone (**4**), and ((2*S*)-5,7,4'-trihydroxy-2'-methoxy-8,5'-di(3-methyl-2-butenyl)-6-methylflavanone (**5**) were isolated and characterized from ethanol extract of the stems and roots. Compounds **1-5** were tested for their antimicrobial, antimalarial, and cytotoxicity activities. Tripteryol B (**2**) showed good antimicrobial activity, while **4** and **5** were significantly active in the antimicrobial assay. Tripteryols A-B (**1-2**) also showed significant antimalarial activities, while **1-5** were found inactive in the cytotoxicity assay.

2. Experimental

2.1. General experimental procedures

Optical rotations were determined on a Rudolph Research Analytical Autopol IV automatic polarimeter. UV spectra were measured in methanol on a Varian Cary 50 Bio UV-visible

spectrophotometer. IR spectra were recorded on an Agilent Technologies Cary 630 FTIR spectrometer. NMR spectra were recorded on a Bruker DRX-500 spectrometers with TMS as the internal standard. HRESI-MS data was obtained on an Agilent Technologies 6200 series mass spectrometer or Waters Xevo G2-XS Q TOF mass spectrometer. Preparative HPLC, Agilent Series 1200 system consisting of a quaternary delivery system, an auto-sampler with a diode array detector and a semi-preparative column Eclipse XDB-C₁₈ column (250mm×9.4mm, 5μm) was used for compounds purification.

2.2. Plant material

Debarked stems and roots of *Tripterygium wilfordii* were collected from Tian Heng drugstore, Beijing, China. The plant material was identified by Prof. De-an Guo. A voucher specimen No. 20080220 was deposited at *TCM and Ethnomedicine Innovation & Development Laboratory, Hunan University of Chinese Medicine*.

2.3. Extraction and isolation

Air-dried debarked stems and roots of *T. wilfordii* (9.5 kg) were extracted by refluxing with 95% ethanol for 2h. The pooled extract was concentrated under vacuum to yield a crude extract (380 g). The crude extract (380 g) was suspended in water (1 L) and partitioned successively with petroleum ether (PE) and ethyl acetate (EtOAc) to afford 20 g and 135 g fractions, respectively. The ethyl acetate soluble part was subjected to column chromatography (CC) over silica gel (100 × 10 cm) with gradient CHCl₃/MeOH (100:0-50:50) to obtain 9 fractions (Fr. 1-Fr. 9). Fr. 2 (50 g) was applied to CC over silica gel using PE/Acetone gradients (5:0-5:2) to obtain 9 subfractions (Fr. 2A-Fr. 2I). Fr. 2C was further purified over Sephadex LH-20 (methanol as mobile phase), followed by semipreparative HPLC to obtain **2** (10.6 mg) and **1** (4.3 mg). Fr. 2F was subjected to Sephadex LH-20 column using methanol as solvent, followed by

semipreparative HPLC using 85% MeOH-H₂O solvent system to yield **3** (3.5 mg). Compounds **4** (6.8 mg) and **5** (7.2 mg) were obtained from Fr. 2E by Sephadex LH-20 column, using methanol as solvent.

2.3.1 Tripteryol A (1): obtained as white amorphous powder; $[\alpha]_D^{25}$ -9.4 (*c* 1.1, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 294 (3.99) nm; IR (KBr) $\nu_{\max}$ 3401, 2926, 1627, 1450, 1371, 1295, 1191, 1127 and 1020 cm⁻¹; ¹H NMR (Pyridine-*d*₅, 500 MHz) and ¹³C NMR (Pyridine-*d*₅, 125 MHz), see **Table 1**; Negative HRESI-MS *m/z* 449.1967 [M-H]⁻ (calcd for C₂₇H₂₉O₆, 449.1964).

2.3.2 Tripteryol B (2): obtained as yellowish amorphous powder; $[\alpha]_D^{25}$ 0 (*c* 1.75, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 291 (3.82) nm; IR (KBr) $\nu_{\max}$ 3364, 2920, 2361, 1630, 1508, 1442, 1379, 1298, 1168, 1079 and 1017 cm⁻¹; ¹H NMR (CD₃OD, 500 MHz) and ¹³C NMR (CD₃OD, 125 MHz), see **Table 1**; Negative HRESI-MS *m/z* 437.1984 [M-H]⁻ (calcd for C₂₆H₂₉O₆, 437.1964).

2.3.3 Tripteryol C (3): obtained as yellow amorphous powder; $[\alpha]_D^{25}$ -115.6 (*c* 1.09, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 292 (4.04) nm; IR (KBr) $\nu_{\max}$ 3382, 2925, 2360, 1633, 1473, 1435, 1382, 1299, 1161, 1096 and 1011 cm⁻¹; ¹H NMR (Pyridine-*d*₅, 500 MHz) and ¹³C NMR (Pyridine-*d*₅, 125 MHz), see **Table 1**; Negative HRESI-MS *m/z* 393.1316 [M-H]⁻ (calcd for C₂₃H₂₁O₆, 393.1338).

2.4 Antimalarial bioassay

The *in vitro* antimalarial activities of tripteryol A-C (**1-3**) were determined against chloroquine-sensitive (D6) and resistant (W2) clones of *Plasmodium falciparum*, which based on the determination of the parasitic lactate dehydrogenase (pLDH) activity using Malstat reagent, utilizing an assay described previously [9]. IC₅₀ values were computed from the dose response curves. Artemisinin (Aldrich-Sigma, St. Louis, MO) was used as positive control. DMSO was used as a vehicle control.

2.5 Antimicrobial bioassay

Microorganisms, *Cryptococcus neoformans* ATCC 90113, *Pseudomonas aeruginosa* ATCC 27853, Low-level vancomycin-resistant *Enterococcus faecalis* ATCC 51299, methicillin-resistant *Staphylococcus aureus* ATCC 43300 (MRSA), and *Staphylococcus aureus* ATCC 29213 were obtained from American Type Culture Collection (Manassa, VA). Susceptibility testing were done using modified versions of the NCCLS methods [10-12]. Briefly, Samples (dissolved in DMSO) were serially diluted in saline, and transferred in duplicates to 96-well flat bottom microplates. Microbial inocula were prepared by correcting the OD₆₃₀ of microbe suspensions in incubation broth to afford recommended inocula [9]. Amphotericin B, ciprofloxacin, and vancomycin (ICN Biomedicals, Ohio) were used as positive control drugs in each assay.

2.6 Cytotoxicity

Compounds **1-5** were also tested for their *in vitro* cytotoxicities against mammalian kidney fibroblasts (VERO) cells. The assay was performed in 96-well tissue culture-treated microplates as described earlier [13]. In brief, cells were seeded to the wells of the plate at a density of 25,000 cells/well and incubated for 24 h at 37 °C. Following 48 h of incubation with samples, the number of viable cells were determined using Neutral Red procedure.

3. Results and discussion

The ethyl acetate soluble part from ethanol extract of *T. wilfordii* was successively chromatographed over silica gel, Sephadex LH-20, and semi-preparative reverse-phase HPLC to obtain three new prenylated flavanones, tripteryol A-C (**1-3**).

Tripteryol A (**1**) was obtained as white powder. Its molecular formula was determined as C₂₇H₃₀O₆ by pseudomolecular ion peak [M-H]⁻ in HRESI-MS at *m/z* 449.1967 (calcd. for

449.1964), indicating 13 degrees of unsaturation. IR spectrum showed absorptions for hydroxyl and carbonyl groups at 3401 and 1627 cm^{-1} , respectively. ^1H NMR spectrum (**Table 1**) of **1** displayed one hydroxyl group [δ_{H} 13.10 (1H, s, 5-OH)], two singlet aromatic protons [δ_{H} 6.64 (1H, s, H-3') and δ_{H} 7.50 (1H, s, H-6')], a methoxy group [δ_{H} 3.71 (3H, s, 2'-OCH₃)], an isoprenyl group [δ_{H} 3.65 (2H, m, H-1''), δ_{H} 5.60 (1H, m, H-2''), δ_{H} 1.76 (3H, s, H-4'') and δ_{H} 1.68 (3H, s, H-5'')], a 2,2-dimethylpyran ring unit [δ_{H} 1.46 (6H, d), the two doublets at δ_{H} 5.58 (d, J = 9.7 Hz, H-2''') and 6.49 (d, J = 9.7 Hz, H-1''')] and a C-methyl group [δ_{H} 2.45 (3H, s)]. ^{13}C NMR spectrum (**Table 1**) of **1** suggested a flavanone skeleton [8]. The heteronuclear multiple quantum correlations (HMQC) and heteronuclear multiple bond correlations (HMBC) were used to determine connections of substituents (**Fig. 2**). The HMBC of H-1'''/C-4' and C-3''', H-2'''/C-5', H-6'/C-2, C-2', C-4' and C-1''', H-3'/C-1' and C-5' revealed that dimethylpyran group should be fused to C-4' and C-5' of ring B. A C-methyl group [δ_{H} 2.45 (3H, s), δ_{C} 9.1] at C-6 and two hydroxy group attached to C-5 and C-7 respectively were determined by HMBC cross-peaks of a methyl (δ_{H} 2.45) with C-7 and C-5, and HMBC of δ_{H} 13.10 to C-5, C-6 and C-4a. A methoxy group at C-2' was identified by HMBC of H₃-MeO/C-2'. The absolute configuration was determined as “S” for the chiral center at C-2 based on the negative optical rotation value [14]. Therefore, **1** was assigned as (2*S*)-5,7-dihydroxy-2'-methoxy-8-(3-methyl-2-butenyl)-4',5'-(3,3-dimethylpyrano)-6-methylflavanone.

Tripteryol B (**2**) was obtained as yellowish powder. HRESI-MS at m/z 437.1984 [$\text{M-H}]^-$ (calcd. for 437.1964), demonstrated the molecular formula of **2** to be C₂₆H₃₀O₆. The IR spectrum showed presence of hydroxyl (3363 cm^{-1}) and carbonyl groups (1630 cm^{-1}). The ^1H and ^{13}C NMR spectra (**Table 1**) indicated that **2** was also a flavanone. The resonances for two isoprenyl groups were observed at δ_{H} 3.21 (2H, m, H-1''), δ_{H} 3.26 (2H, d, H-1'''), δ_{H} 5.19 (1H, m, H-2''), δ_{H}

5.31 (1H, m, H-2''), and δ_H 1.64, 1.63, 1.74, 1.71 (4Me, s). The locations of the substituents were further determined by HMBCs, in which correlations of H-1''/C-7 and C-8a, H-6/C-4a and C-8, H-6'/C-2 and C-1'', H-3'/C-1' and C-5', H-1'''/C-4' and C-6' revealed that two isoprenyl groups should be attached at C-8 and C-5' respectively. The three single aromatic protons were assigned to H-6, H-6' and H-3'. The assignments of OH-5, MeO-2' and OH-4' were confirmed by the HMBC (**Fig. 2**). The optical rotation was found zero, which implied it should be a racemate [8, 15]. Based on the above evidence, **2** was established as ($\pm$)-5,7,4'-trihydroxy-2'-methoxy-8,5'-di(3-methyl-2-butenyl)-flavanone.

Tripteryol C (**3**) was obtained as yellow powder. Its molecular formula was determined as C₂₃H₂₂O₆ by pseudomolecular ion peak [M-H]⁻ in HRESI-MS at m/z 393.1316 (calcd. for 393.1338). Similar to **1**, its characteristic IR (ν_{max} 3381, 1633 and 1473 cm⁻¹), ¹H and ¹³C NMR data suggested a flavanone basic skeleton [8]. Furthermore, the ¹H NMR spectrum showed a hydroxyl group [δ_H 12.86 (1H, s, OH-5)], three aromatic protons [δ_H 8.07 (1H, s, H-6'), 7.85 (1H, s, H-3'), 6.55 (1H, s, H-6)] and a isoprenyl group [δ_H 3.70 (2H, m, H-1''), 5.71 (1H, t, H-2''), 1.80 (3H, s, H-4''), 1.71 (3H, s, H-5'')]. The isoprenyl group was located at C-8 according to the HMBC of H-1''/C-7, C-8 and C-8a. A pair of olefinic protons at δ_H 6.91 (1H, d, J = 2.1 Hz, H-1''') and δ_H 7.85 (1H, d, J = 2.1 Hz, H-2''') were located at δ_C 107.6 (C-1''') and 145.7 (C-2'''), respectively, suggesting a benzofuran moiety in the structure of **3**. The locations of the furan ring, OH-7, OH-5, and MeO-2' were confirmed by HMBCs (**Fig. 2**). The (2*S*) absolute configuration was assigned according to its negative optical activity. Therefore, **3** was identified as (2*S*)-5,7-dihydroxy-2'-methoxy-8-(3-methyl-2-butenyl)-4',5'-furanone-flavanone.

The two known flavanones were identified as ($\pm$)-5,4'-dihydroxy-2'-methoxy-6',6''-dimethypyraro-(2'',3''':7,8)-6-methylflavanone (**4**) and ((2*S*)-5,7,4'-trihydroxy-2'-methoxy-8,5'-

di(3-methyl-2-butenyl)-6-methylflavanone (**5**), based on the spectroscopic analyses, and comparison with literature data [8].

To best of our knowledge there are many reports of C-methylated flavonoids [16-18], while B ring furanized flavanones are rare in nature. Tripteryol C (**3**) (the furanized flavanone) was found as *O*-methylated derivative of cudraflavanone E. Chen et al., proposed a biogenetic pathway for cudraflavanone E, suggesting cudraflavanone D as precursor [19].

Compounds **1-5** were tested for their antimalarial, antimicrobial and cytotoxic activities (Table 2). The results showed that compounds **2** and **5** possessed promising antimicrobial activities against *C. neoformans* with IC₅₀ values of 2.95 µg/mL and 1.06 µg/mL, respectively. Compound **2** was also active against *P. aeruginosa*, VRE, and MRSA displaying IC₅₀ values of 8.591, 4.316 and 4.47 µg/mL, respectively. Compounds **4** and **5** exhibited activities against MRSA (IC₅₀ values of 2.07 and 2.60 µg/mL, respectively) and *S. aureus* (2.01 and 2.16 µg/mL, respectively). In addition, moderate antimalarial activities for **1** and **2** (IC₅₀ = 3.15 and 4.63 µg/mL for the D6 clone, and 3.35 and 4.18 µg/mL for the W2 clone, respectively) were observed. Compounds **1-5** were also screened for their cytotoxic activities against VERO cells, but were found inactive.

Conflict of interest

Authors declare no conflicts of interest.

Acknowledgements

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Table 1.¹H NMR (500 MHz) and ¹³C NMR (125 MHz) spectroscopic data for **1-3**.

	1 ^a		2 ^b		3 ^a	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}
2	5.85 (dd 2.9, 12.9)	74.7	5.61 (dd 3.2, 12.1)	75.6	5.99 (dd 3.8, 12.0)	75.3
3a	3.13 (dd 17.0, 12.9)	43.2	2.94 (dd 17.1, 12.1)	43.3	3.12 (m)	43.3
3b	2.98 (dd 17.0, 2.9)		2.77 (dd 17.1, 3.2)			
4		197.8		198.7		197.3
4a		103.4		103.4		103.3
5		160.7		163.2		163.5
6		105.0	5.95 (s)	96.3	6.55 (s)	97.0
7		164.4		166.1		166.9
8		109.0		109.1		109.1
8a		159.2		162.0		161.4
1'		120.8		119.3		125.1
2'		158.1		156.7		155.5
3'	6.64 (s)	100.7	6.49 (s)	99.6	7.3 (s)	95.6
4'		155.3		157.0		156.5
5'		115.0		121.1		121.3
6'	7.50 (s)	125.7	7.20 (s)	128.6	8.07 (s)	119.7
1''	3.65 (overlap)	23.3	3.21 (m)	22.5	3.70 (m)	23.0
2''	5.60 (t)	124.6	5.19 (t)	124.1	5.71 (t)	124.6
3''		131.3		131.5		131.2
4''	1.76(s)	18.4	1.63(s)	18.0	1.80(s)	18.4
5''	1.68(s)	26.4	1.64(s)	26.1	1.71(s)	26.4
1'''	6.49 (d 9.7)	122.6	3.26 (d 7.1 Hz)	28.6	6.91 (d 2.1)	107.6
2'''	5.58 (d 9.7)	128.9	5.31 (t)	124.3	7.85 (d 2.1)	145.7
3'''		77.3		132.8		
4'''	1.46 (s)	28.6	1.74 (s)	26.0		
5'''	1.46 (s)	28.6	1.71 (s)	17.9		
CH ₃ O	3.71 (s)	56.1	3.82 (s)	56.0	3.76 (s)	56.4
CH ₃ -	2.45 (s)	9.1				
OH-5	13.10 (s)				12.86 (s)	

^a NMR spectral data of **1** and **3** in Pyridine-*d*₅.^b NMR spectral data of **2** in CD₃OD.

Table 2.**Anti-infective Activities and Cytotoxicity of Compound 1-5.**

Compounds	Antimalarial ^a		Antimicrobial ^b					Cytotoxicity ^c
	<i>P. falciparum</i>	<i>P. falciparum</i>	<i>C. neoformans</i>	<i>P. aeruginosa</i>	VRE	MRS	<i>S. aureus</i>	Vero (IC ₅₀)
	D6 (IC ₅₀)	W2 (IC ₅₀)	(IC ₅₀)	(IC ₅₀)	(IC ₅₀)	(IC ₅₀)	(IC ₅₀)	
1	3.15	3.35	na	na	na	na	nt	nc
2	4.63	4.18	2.95	8.59	4.32	4.47	nt	nc
3	na	na	na	na	na	na	nt	nc
4	nt	nt	na	nt	nt	2.07	2.60	nc
5	nt	nt	1.06	nt	nt	2.01	2.16	nc
Artemisinin	0.0085	0.0090	nt	nt	nt	nt	nt	nt
Amphotericin B	nt	nt	0.75	nt	nt	nt	nt	nt
Ciprofloxacin	nt	nt	nt	0.06	nt	0.15	0.10	nt
Vancomycin	nt	nt	nt	nt	3.3	nt	nt	nt

^a highest test concentration at 4.76 µg/mL.

^b highest test concentration at 20 µg/mL.

^c highest test concentrations at 4.76 µg/mL.

na, not active. nt, not tested. nc, not cytotoxic.

IC₅₀ is the concentration (µg/mL) that affords 50% inhibition of growth.

Both anti-infective activities and cytotoxicity are expressed as mean values of three experimental data.

Fig. 1. Structures of **1-5**.

Fig. 2. Key HMBC and ^1H - ^1H COSY correlations in **1-3**.