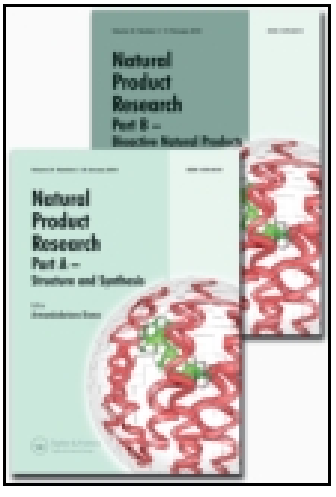

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Two new compounds from the roots of Ilex pubescens

Ting Wu [a], Qing-Wen Zhang [b], Xiao-Qi Zhang [a c], Ge Liu [a], Lei Wang [a], Miao-Miao Jiang [d], Yi-Fan Feng [d] & Wen-Cai Ye [a c]

[a] Institute of Traditional Chinese Medicine and Natural Products, College of Pharmacy, Jinan University, Guangzhou 510632, P.R. China

[b] Institute of Chinese Medical Sciences, University of Macau, Taipa, Macau

[c] Guangdong Province Key Laboratory of Pharmacodynamic Constituents of TCM and New Drugs Research, Jinan University, Guangzhou 510632, P.R. China

[d] Central Laboratory, Guangdong Pharmaceutical University, Guangzhou 510006, P.R. China

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Two new compounds from the roots of *Ilex pubescens*

Ting Wu[a], Qing-Wen Zhang[b], Xiao-Qi Zhang[ac], Ge Liu[a], Lei Wang[a], Miao-Miao Jiang[d], Yi-Fan Feng[d] and Wen-Cai Ye[ac]*

[a]*Institute of Traditional Chinese Medicine and Natural Products, College of Pharmacy, Jinan University, Guangzhou 510632, P.R. China;* [b]*Institute of Chinese Medical Sciences, University of Macau, Taipa, Macau;* [c]*Guangdong Province Key Laboratory of Pharmacodynamic Constituents of TCM and New Drugs Research, Jinan University, Guangzhou 510632, P.R. China;* [d]*Central Laboratory, Guangdong Pharmaceutical University, Guangzhou 510006, P.R. China*

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A new triterpenoid glycoside, heterobetulinic acid 3-*O*-*β*-D-glucopyranosyl $(1 \rightarrow 2)$-*β*-D-xylopyranoside (**1**), together with a new phenylacrylic acid derivative, 3′*S*,4′-dihydroxyl-2′-methylene-but-1′-enyl caffeate (**2**), were isolated from the roots of *Ilex pubescens*. Their structures were elucidated by HR–ESI–MS, 1-D and 2-D NMR analyses and chemical methods. In addition, the absolute configuration of **2** was established by the application of a modified Mosher's method.

Keywords: *Ilex pubescens*; triterpenoid glycoside; phenylacrylic acid derivative; absolute configuration

1. Introduction

The plant *Ilex pubescens* Hook. *et* Arn. is widely distributed in the south of China. The dried roots of the plant have been used as a traditional Chinese medicine for the treatment of cardiovascular and cerebrovascular diseases and hypercholesterolemia (Jiang et al., 2005; Wang, Zhou, Jiang, Wong, & Liu, 2008). Previous phytochemical and pharmacological studies of this plant had led to the isolation of anticoagulant and anti-inflammatory triterpenoid saponins (Han, Song, & Rhee, 1993; Wang, Zhou, Jiang, & Liu, 2008), anti-platelet aggregating phenylacrylic acid compounds (Jiang et al., 2005), as well as lignans and iridoid glycosides (Yang, Ding, & Zhang, 2007; Zhou, Wang, X.M. Li, & X.Li, 2008). As a part of our investigation on bioactive constituents from the plants of genus *Ilex* (Sun, Wang, Zhang, Zhao, & Ye, 2009; Sun, Zhang, et al., 2009; Wu, Zhang, Wang, Ye, & Zhao, 2009), two new compounds including a triterpenoid glycoside (**1**) and a phenylacrylic acid derivative (**2**) (Figure 1) were obtained from the roots of *I. pubescens*. Herein we report the isolation and structural elucidation of the new compounds.

2. Results and discussion

Compound **1** was obtained as colourless needles. The HR–ESI–MS of **1** exhibited a quasi-molecular ion at m/z 749.4465 $[M-H]^-$ (Calcd for $C_{41}H_{65}O_{12}$, 749.4482), consistent with a molecular formula $C_{41}H_{66}O_{12}$. The IR spectrum showed absorptions at 3389 (OH), 1698 (C=O) and 1601 (C=C) cm^{-1}. Acid hydrolysis of **1** yielded D-glucose and D-xylose, which

*Corresponding author. Email: chywc@yahoo.com.cn

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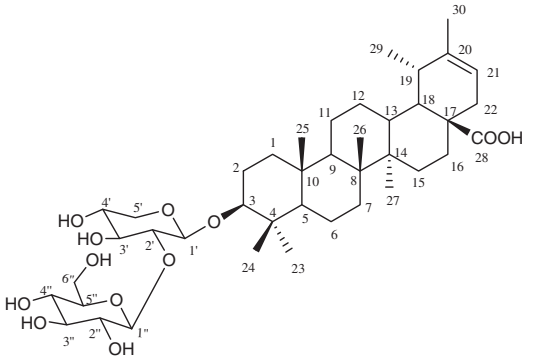

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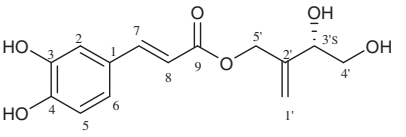

2

Figure 1. Chemical structures of compounds **1** and **2**.

were identified by gas chromatography of their aldononitrile peracetate derivatives using authentic samples as references (Section 3). The ^{1}H-NMR spectrum of **1** displayed the signals for seven methyl groups at δ_H 0.84 (3H, s, H-25), 1.05 (3H, s, H-23), 1.06 (3H, s, H-27), 1.10 (3H, s, H-26), 1.12 (3H, d, $J = 6.6$ Hz, H-29), 1.29 (3H, s, H-24) and 1.73 (3H, s, H-30), an oxygenated methine proton at δ_H 3.30 (1H, dd, $J = 11.6$, 4.1 Hz, H-3), two anomeric protons at δ_H 4.87 (1H, d, $J = 6.8$ Hz, H-1 of xylose) and 5.38 (1H, d, $J = 7.6$ Hz, H-1 of glucose), as well as an olefinic proton at δ_H 5.48 (1H, m, H-21). The ^{13}C-NMR and DEPT spectra showed 41 carbon signals including 7 methyls and 2 olefinic carbon signals. All the above data indicated that **1** was an urs-20-en type triterpenoid glycoside. Comparison of the NMR data of **1** with those of heterobetulinic acid (3β-hydroxy-urs-20-en-28-oic acid) revealed that the aglycone of **1** was heterobetulinic acid (Lobo-Echeverri et al., 2005). The sequence and glycosylation position of the sugar chain could be deduced by an HMBC experiment. Thus, in the HMBC spectrum, correlations between H-1 (δ_H 5.38) of glucose and C-2 (δ_C 83.4) of xylose as well as between H-1 (δ_H 4.87) of xylose and C-3 (δ_C 88.8) of aglycone were observed. Hence, the structure of **1** was elucidated as heterobetulinic acid 3-*O*-β-D-glucopyranosyl(1 $\rightarrow$ 2)-β-D-xylopyranoside.

Compound **2** was obtained as amorphous powder. The molecular formula of **2** was determined as $C_{14}H_{16}O_6$ on the basis of a quasi-molecular ion at m/z 279.0796 $[M-H]^-$ (Calcd for $C_{14}H_{15}O_6$, 279.0784) in the HR–ESI–MS. The IR spectrum showed absorptions at 3405 (OH), 1692 (C=O), 1630 (C=C), 1605 and 1447 (Ar) cm^{-1}. The ^{1}H-NMR and ^{1}H–^{1}H COSY spectra indicated the presence of a set of aromatic protons [δ_H 7.06 (1H, d, $J = 1.7$ Hz, H-2), 7.00 (1H, dd, $J = 8.1$, 1.7 Hz, H-6) and 6.76 (1H, d, $J = 8.1$ Hz, H-5)], a couple of *trans*-olefinic protons [δ_H 7.50 (1H, d, $J = 15.9$ Hz, H-7) and 6.29 (1H, d, $J = 15.9$ Hz, H-8)], an oxygenated methine [δ_H 4.05 (1H, m, H-3′)], two oxygenated methylenes [δ_H 4.67 (2H, br s, H-5′) and 3.42 (2H, m, H-4′)], and an exo-methylene [δ_H 5.18 (1H, br s, H-1′a) and 5.10 (1H, br s, H-1′b)]. The ^{13}C- and DEPT-NMR spectra displayed 14 carbon signals. The above data indicated that **2** was a caffeate derivative with a 3,4-dihydroxyl-2-methylene-1-but-enyl moiety (Jiang et al., 2005). The HMBC correlation between H-5′ (δ_H 4.67) and C-9 (δ_C 166.1) demonstrated that the isoprene unit was connected to C-9 through an oxygen atom. To determine the absolute configuration of C-3′, a modified Mosher's method was applied (Su et al., 2002). The differences of proton chemical shift values ($\Delta\delta = \delta_S - \delta_R$, ppm) between (*S*)-MTPA ester (**2a**) and (*R*)-MTPA ester (**2b**) (Supplementary Figure S1 – online only) suggested the presence of *S*

configuration at C-3′ in **2**. Therefore, **2** was identified as 3′*S*,4′-dihydroxyl-2′-methylene-but-1′-enyl caffeate.

3. Experimental

3.1. *General experimental procedures*

Melting points were determined on an X-5 melting point apparatus. Optical rotations were measured on a JASCO P-1020 digital polarimeter. IR spectra were recorded on a JASCO FT/IR-480 Plus spectrometer in KBr pellets. The NMR data were obtained on a BRUKER AV-400 spectrometer using TMS as an internal standard. HR–ESI–MS data were determined on an Agilent 6210 LC/MSD TOF mass spectrometer. Column chromatographies were run on silica gel (200–300 mesh, Qingdao Marine Chemical Group Co. Ltd, P.R. China), Sephadex LH-20 (Pharmacia, Sweden) and ODS (YMC, Japan). TLC analyses were carried out using precoated silica gel GF_{254} plates (Qingdao Marine Chemical Group Co. Ltd, P.R. China). (*S*)- and (*R*)-α-methoxy-α-(trifluoromethyl) phenylacetic chloride were purchased from Alfa-Aesar Chemicals Ltd. (Tianjin, China). All solvents used in column chromatographies and HPLC chromatography were of analytical grade (Shanghai Chemical Plant, Shanghai, P.R. China) and chromatographic grade (Fisher Scientific, New Jersey, USA), respectively.

3.2. *Plant material*

The roots of *I. pubescens* were collected from Conghua city, Guangdong province of China, in July of 2007. The plant was identified by Prof. Guang-Xiong Zhou of College of Pharmacy, Jinan University. A voucher specimen (No. 2008071101) was deposited in the Institute of Traditional Chinese Medicine and Natural Products, Jinan University, Guangzhou, P.R. China.

3.3. *Extraction and isolation*

The air-dried and powdered roots (5.0 kg) were extracted with 70% EtOH at room temperature. After the removal of EtOH under reduced pressure, the remaining aqueous solution was chromatographed over D101 macroporous resin using H_2O, 20%, 50% and 95% EtOH as eluents. The 50% EtOH fraction (120.0 g) was subjected to silica gel eluted with $CHCl_3$:MeOH (100:0 to 50:50) to give 20 subfractions (1–20). Subfraction 14 (300 mg) was further purified over an ODS column (50% MeOH) and a Sephadex LH-20 column (MeOH) to yield **1** (3.0 mg). The 95% EtOH fraction (266.0 g) was chromatographed on silica gel with a gradient elution by $CHCl_3$:MeOH (100:0 to 50:50) to afford **2** (5.0 mg).

3.3.1. *Heterobetulinic acid 3-O-β-D-glucopyranosyl(1 → 2)-β-D-xylopyranoside* (**1**)

Colourless needles, m.p. 290–292°C; IR (KBr) ν_{max}: 3389, 2944, 1698, 1601, 1455, 1117, 1079 and 1042 cm^{-1}; HR–ESI–MS: *m/z* 749.4465 $[M-H]^-$ (Calcd for $C_{41}H_{65}O_{12}$, 749.4482); ^1H (400 MHz, C_5D_5N) δ: 5.48 (1H, d, *J* = 6.8 Hz, H-21), 5.38 (1 H, d, *J* = 7.6 Hz, H-1″), 4.87 (1 H, d, *J* = 6.8 Hz, H-1′), 4.50 (1H, m, H-6″b), 4.48 (1H, m, H-6″a), 4.39 (1H, m, H-4″), 4.32 (1H, m, H-5′a), 4.27 (1H, m, H-3″), 4.25 (1H, m, H-3′), 4.24 (1H, m, H-2′), 4.18 (1H, m, H-4′), 4.14 (1H, m, H-2″), 3.94 (1H, m, H-5″), 3.70 (1H, m, H-5′b), 3.30 (1H, dd, *J* = 11.6, 4.1 Hz, H-3), 2.84 (1H, m, H-13), 2.64 (1H, dd, *J* = 15.2, 7.2 Hz, H-22b), 2.46 (1H, m, H-19), 2.33 (1H, d, *J* = 9.8 Hz, H-12b), 2.20 (1H, m, H-2b), 2.02 (1H, m, H-22a), 1.92 (1H, m, H-2a), 1.81 (1H, m, H-15b), 1.78 (1H, m, H-16b), 1.73 (3H, s, H-30), 1.68 (1H, m, H-1a), 1.56 (1H, m, H-11b), 1.52 (1H, m, H-12a), 1.47 (1H, m, H-6b), 1.45 (1H, m,

H-7b), 1.36 (1H, dd, $J = 11.2$, 6.4 Hz, H-9), 1.35 (1H, m, H-18), 1.33 (1H, m, H-7a), 1.30 (1H, m, H-6a), 1.29 (3H, s, H-24), 1.26 (1H, m, H-11a), 1.25 (1H, m, H-16a), 1.17 (1H, m, H-15a), 1.12 (3H, d, $J = 6.6$ Hz, H-29), 1.10 (3H, s, H-26), 1.06 (3H, s, H-27), 1.05 (3H, s, H-23), 0.94 (1H, m, H-1b), 0.84 (3H, s, H-25) and 0.75 (1H, d, $J = 10.2$ Hz, H-5); ^{13}C-NMR (100 MHz, C_5D_5N) δ: 178.1 (C-28), 143.1 (C-20), 117.9 (C-21), 106.1 (C-1″), 105.7 (C-1′), 88.8 (C-3), 83.4 (C-2′), 78.3 (C-5″), 78.0 (C-3′, 3″), 77.1 (C-2″), 71.7 (C-4″), 71.0 (C-4′), 66.7 (C-5′), 62.7 (C-6″), 56.0 (C-5), 50.9 (C-9), 49.4 (C-18), 49.1 (C-17), 42.3 (C-14), 41.3 (C-8), 39.8 (C-4), 39.4 (C-13), 39.2 (C-1), 38.5 (C-22), 37.8 (C-19), 37.1 (C-10), 34.7 (C-7), 33.7 (C-12), 29.6 (C-16), 28.0 (C-24), 27.9 (C-15), 27.0 (C-2), 23.7 (C-29), 22.2 (C-30), 21.9 (C-11), 18.5 (C-6), 16.6 (C-25), 16.5 (C-23), 16.4 (C-26) and 15.0 (C-27).

3.3.2. *3′S,4′-Dihydroxyl-2′-methylene-but-1′-enyl caffeate (**2**)*

Amorphous powder, m.p. 170–171°C, [α]25 D = +2.3° ($c = 0.13$, MeOH); IR (KBr) ν_{max}: 3405, 1692, 1630, 1605, 1447, 1208, 1162 cm^{-1}; HR–ESI–MS: m/z 279.0796 [M − H]$^-$ (Calcd for $C_{14}H_{15}O_6$, 279.0784); ^{1}H (400 MHz, DMSO-d_6) δ: 7.50 (1H, d, $J = 15.9$ Hz, H-7), 7.06 (1H, d, $J = 1.7$ Hz, H-2), 7.00 (1H, dd, $J = 8.1$, 1.7 Hz, H-6), 6.76 (1H, d, $J = 8.1$ Hz, H-5), 6.29 (1H, d, $J = 15.9$ Hz, H-8), 5.18 (1H, br s, H-1′a), 5.10 (1H, br s, H-1′b), 4.67 (2H, br s, H-5′), 4.05 (1H, m, H-3′), and 3.42 (2H, m, H-4′); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 166.1 (C-9), 148.6 (C-4), 145.6 (C-3), 145.5 (C-2′), 145.3 (C-7), 125.3 (C-1), 121.4 (C-6), 111.9 (C-1′), 115.7 (C-5), 114.7 (C-2), 113.6 (C-8), 72.7 (C-3′), 65.2 (C-4′) and 63.5 (C-5′).

3.4. *Acid hydrolysis and identification of sugars for* 1

Compound **1** (2.0 mg) was heated in an ampoule with 5.0 mL of 2N HCl (MeOH–H_2O, 1:1) at 80°C for 10 h. The aglycone was extracted with $CHCl_3$ for three times and the water layer was evaporated under reduced pressure. Pyridine (1 mL) and 3 mg of $NH_2OH{\cdot}HCl$ were added to the residue, and the mixture was heated at 100°C for 1 h. Followed by the addition of Ac_2O (2.0 mL), the mixture was incubated in a water bath at 100°C for 1 h and partitioned using $CHCl_3$ as solvent. The $CHCl_3$ layer was concentrated for GC analysis (front inlet 250°C, column temperature 80°C → 230°C, 5°C min^{-1}) using standard aldononitrile peracetates as reference samples. The monosaccharides of **1** were identified as D-xylose [t_R (min): 20.956 (from **1**); 20.977 (reference D-xylose aldononitrile peracetate derivative)] and D-glucose [t_R (min): 35.626 (from **1**); 35.662 (reference D-glucose aldononitrile peracetate derivative), 37.501(reference L-glucose aldononitrile peracetate derivative)].

3.5. *Preparation of MTPA esters for* 2

Compound **2** (1.5 mg) was treated with (*R*)-α-methoxy-α-(trifluoromethyl) phenylacetic chloride (10 µL) in 0.5 mL of dried and deuterated pyridine. After being stirred at room temperature for 10 h, the mixture was then evaporated to dryness. The residue was poured into water (5 mL) and extracted with EtOAc (5 mL). The EtOAc extract was further purified by silica gel column chromatography eluted with *n*-hexane:EtOAc (70:30) to yield (*S*)-MTPA ester (**2a**, 1.6 mg). (*R*)-MTPA ester (**2b**, 1.7 mg) was obtained using the same method by treatment of **2** (1.5 mg) with (*S*)-α-methoxy-α- (trifluoromethyl) phenylacetic chloride.

3.5.1. *(S)-MTPA ester (**2a**)*

^{1}H-NMR (400 MHz, Pyridine-d_5) δ: 6.32 (1H, m, H-3′), 5.58 (1H, m, H-1′a), 5.55 (1H, m, H-1′b), 5.09 (1H, m, H-4′a), 5.03 (2H, br s, H-5′) and 4.82 (1H, m, H-4′b).

3.5.2. *(R)-MTPA ester (**2b**)*

^{1}H-NMR (400 MHz, Pyridine-d_5) δ: 6.17 (1H, m, H-3′), 5.47 (1H, m, H-1′a), 5.33 (1H, m, H-1′b), 5.13 (1H, m, H-4′a), 4.94 (2H, br s, H-5′) and 4.82 (1H, m, H-4′b).

Supplementary material

Figure S1 relating to this article is available online.

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