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Phyllaciduloids E and F, two new cleistanthane diterpenoids from the leaves of *Phyllanthus acidus*

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ABSTRACT

Phyllaciduloids E (**1**) and F (**2**), two new cleistanthane diterpenoids, were isolated from the leaves of *Phyllanthus acidus* (L.) Skeels (Phyllanthaceae). Their planar structures were established by spectroscopic analysis and comparison with literature values. The relative configurations of phyllaciduloids E and F were confirmed by DFT-NMR chemical shift calculations and subsequent CP3 probability methods. Phyllaciduloids E and F were evaluated for their cytotoxicity. However, no significant activities were detected at concentrations up to 40 μM.

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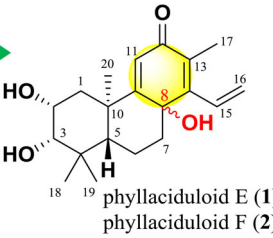

phyllaciduloid E (**1**) 8*R*
phyllaciduloid F (**2**) 8*S*

1. Introduction

The genus *Phyllanthus*, belonging to the family Phyllanthaceae, consists of more than 700 species (Tan et al. 2020), which is widespread in tropical and subtropical areas. Among them, *Phyllanthus acidus* (L.) Skeels, a tropical and subtropical species commonly distributed in Malaysia, Thailand, Indonesia, Philippines, Vietnam, Laos, and India, is also cultivated as a potential medicinal plant in the south of Yunnan province, China (Tan et al. 2020). It is widely served as a valuable medicinal source to treat

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many diseases, such as inflammatory, bronchitis, asthma, rheumatism, hepatopathy and diabetes in Asia, the Caribbean region, and Central and South America (Tan et al. 2020). Previous phytochemical studies on the leaves and roots of *P. acidus* mainly resulted in the isolation and identification of several biological cleistanthane diterpenoids (Duong et al. 2017; Zheng et al. 2018; Duong et al. 2020), norbisabolane sesquiterpenoids (Vongvanich et al. 2000; Lv et al. 2014; Xin et al. 2020), and rare sulfonic acid-containing flavonoids or normal flavonoids (Duong et al. 2018). Our further phytochemical investigation of *P. acidus* (Lv et al. 2014; Zheng et al. 2018; Xin et al. 2020) afforded two new cleistanthane diterpenoids, phyllaciduloids E (**1**) and F (**2**) from the leaves. Their relative configurations were confirmed by DFT-NMR chemical shift calculations and subsequent CP3 probability methods. Herein, we report their structure elucidation and cytotoxic activity against five human cancer cell lines.

2. Results and discussion

The ethanol extract of the leaves was repeatedly purified by column chromatography on silica gel, and preparative or semi-preparative HPLC to yield two new compounds, phyllaciduloids E (**1**) and F (**2**). Their structures were shown in Figure 1.

Compounds **1** and **2** exhibited the same molecular formula $C_{20}H_{28}O_4$, as deduced from the HRESIMS [*m/z* 331.1916 $[M - H]^-$ (**1**) and *m/z* 355.1883 $[M + Na]^+$ (**2**)], indicating 7 indices of hydrogen deficiency. The ^{1}H NMR spectrum of **1** exhibited signals due to one terminal vinyl moiety (δ_H 6.58, 1H, ddd, $J = 18.0$, 11.9, 0.7 Hz; 5.52, 1H, dd, $J = 18.0$, 2.0 Hz, and 5.64, 1H, dd, $J = 11.9$, 2.0 Hz), two oxygenated methines (δ_H 4.19, 1H, q, $J = 3.5$ Hz; 3.13, 1H, d, $J = 3.5$ Hz) and four methyls (δ_H 1.94, 0.99, 1.11, and 1.73, each 3H, s). The ^{13}C NMR and DEPT spectra revealed 20 carbon resonances, consisting of four methyls, three aliphatic and one olefinic methylenes, five methines (including two oxygenated and two olefinic methines), and seven quaternary carbons (including a carbonyl, three olefinic and one oxygen-bearing carbons). The aforementioned NMR features of **1** were closely related to those of ovoideal E (Su et al. 2014; Duong et al. 2020), a known cleistanthane diterpenoid also isolated from the leaves of *P. acidus*. The only difference was the occurrence of an additional oxymethine (δ_C 72.1, δ_H 4.19) in **1** instead of an aliphatic methylene at C-2 in ovoideal E, indicating that compound **1** was a C-2 hydroxy analogue of ovoideal E. This was supported by the ^{1}H−^{1}H COSY correlations of H-1/H-2/H-3 as well as the key HMBC correlations from H-2 to C-4 and

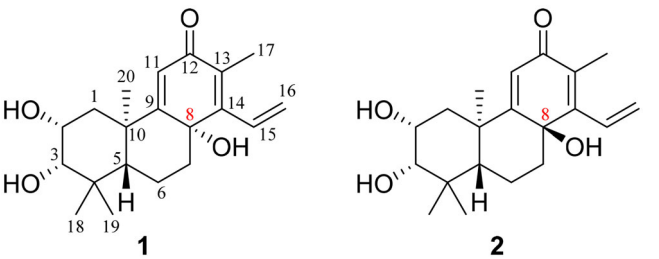

Figure 1. The structures of phyllaciduloids E (**1**) and F (**2**).

C-10, from H-3 to C-5, from H-11 to C-8 and C-13, and from H-15 to C-8, C-13 and C-14 (Figure S1).

The MS and NMR spectral features of compound **2** were quite similar to those of compound **1**. However, the ^{13}C NMR chemical shift of C-8 for **2** was observed at upper field ($\Delta\delta_C$ −10.0 ppm), whereas resonances for C-7, C-9, and C-14 were shifted lower field ($\Delta\delta_C$ +1.9, +3.5, and +3.3 ppm, respectively), when compared with those of compound **1**. These differences indicated that the two compounds are a pair of C-8 epimers. The planar structures of **1** and **2** were thus constructed as shown in Figure 1.

The relative stereochemistry of **1** and **2** was established by the ^{13}C chemical shifts and ROESY experiment. Firstly, the similar ^{13}C chemical shifts at C-2 and C-3 exhibited same relative configuration at C-2 and C-3 in **1** and **2** to those of phyllanflexoid A (Zhao et al. 2013). Furthermore, the ROESY correlations of H-3 with H-1β, H-5 and Me-18, and of H-2 with H-3 and H-1β revealed their β-orientation, while the correlations of H-1α with Me-19 and Me-20 revealed that all of these protons were α-orientation (Figure S2). Unfortunately, no reliable NOESY correlations could be observed to determine the relative stereochemistry of the oxygen-binding quaternary carbon at C-8 in both compounds.

In order to define the relative configuration at C-8 of **1** and **2**, density functional theory (DFT) NMR chemical shift calculations and subsequent CP3 probability method were performed on two different candidates (8β-OH and 8α-OH) (Duong et al. 2020) as shown in Figure S25, demonstrating the structural equivalence of diastereoisomer **1** with 99.0% probability, proposing the 8*R* configuration of **1** and the 8*S* configuration of **2**. Their absolute configurations were further established as 2*R*,3*S*,5*S*,10*R* by similar Cotton effects in their CD spectra (Figures S11 and S21), which displayed positive Cotton effects at approximately 220 nm and 250 nm in compounds **1** and **2** (Zhao et al. 2013; Lv et al. 2015). Thus, the structures of **1** and **2** including their absolute configuration were established as shown in Figure 1 and given trivial names of phyllaciduloids E (**1**) and F (**2**), respectively.

Previously, cleistanthane-type diterpenoids from this genus exhibited potential or selective cytotoxicities *in vitro* against several human tumor cell lines (Zhao et al. 2013; Duong et al. 2017; Zheng et al. 2018). Compounds **1** and **2** were evaluated for cytotoxic activity on five human tumor cell lines (HL-60, SMMC-7721, A-549, MCF-7, and SW-480) using the MTS method (Zheng et al. 2018). However, no significant activities were detected at concentrations up to 40 μM.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured with a JASCO DIP-370 digital polarimeter. Circular dichroism spectra were measured on a Chirascan instrument. UV data were obtained on a Shimadzu UV-2401A spectrophotometer (Shimadzu, Kyoto, Japan). A BioRad FtS-135 spectrophotometer (Bio-Rad, Richmond, CA, USA) was used for scanning IR spectrophotometry with KBr pellets. The NMR spectroscopic data were recorded on an Avance III 600 NMR spectrometer (Bruker, Karlsruhe, Germany) with TMS as internal standard, and chemical shifts (δ) are expressed in ppm with reference to the TMS

signal. ESIMS and HRESIMS analyses were measured on Agilent 1290 UPLC/6540 Q-TOF mass spectrometer. Preparative or semi-preparative HPLC was performed on an Agilent 1100 HPLC (Agilent Technologies, Foster City, CA, USA) with Zorbax SB-C18 (21.2 mm × 25 cm) or Zorbax SB-C18 (9.4 mm × 25 cm) columns. Column chromatography was performed using silica gel (200–300 mesh, Qingdao Marine Chemical, Inc., Qingdao, China), MCI gel (75-150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan). Column fractions were monitored by TLC visualized by spraying with 8% H_2SO_4 in ethanol.

3.2. Plant material

The leaves of *P. acidus* were collected from Yuanjiang county of Yunnan Province, People's Republic of China, on May 2018. The identification of plant material was verified by Dr. En-De Liu. A voucher specimen (Kib-18-05-022) has been deposited in State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, P. R. China.

3.3. Extraction and isolation

The air-dried and powdered leaves of *P. acidus* (30 kg) were extracted with 95% aqueous ethanol solution (100 L × 3 times) under reflux (approximately 60 °C). The combined solution was concentrated in vacuo (at 45 °C) to yield a residue (4.5 kg), which was partitioned further between water and EtOAc. The EtOAc part (2.0 kg) was subjected to a silica gel column with a gradient elution of petroleum ether-EtOAc (20:1, 10:1, 8:1, 2:1, 1:1 and 0:1) to yield six main fractions A-F. Further separation of fraction C (18 g) on silica gel, eluted with petroleum ether-acetone (8:2-1:2), afforded subfractions C_1−C_6. Fraction C_2 (8:2, 1.0 g) was chromatographed repeatedly by preparative HPLC (25% MeCN-H_2O, flow rate 12 mL/min) and followed purified by semi-preparative HPLC (42% MeOH-H_2O, flow rate 3 mL/min) to yield compounds **1** (5.8 mg) and **2** (4.2 mg), respectively.

3.5. Cytotoxicity assays

Five human tumor cell lines (HL-60, SMMC-7721, A-549, MCF-7, and SW-480) were used in cytotoxic assay, which were obtained from ATCC (Manassas, VA, USA). Cells were cultured in RMPI-1640 or DMEM medium (Biological Industries, Kibbutz Beit-Haemek, Israel) supplemented with 10% fetal bovine serum (Biological Industries) at 37 °C in 5% CO_2. The assay was performed by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)- 2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) (Promega, Madison, WI, USA) method. Briefly, cells were seeded into each well of a 96-well cell culture plate. After 12 h of incubation at 37 °C, the test compound (40 μM) was added. After incubated for 48 h, cells were subjected to the MTS assay (Zheng et al. 2018). Compounds with a growth inhibition rate of 50% were further evaluated under the concentrations of 40, 8, 1.6, 0.32, and 0.064 μM in triplicate, with cisplatin and paclitaxel (Sigma, St. Louis, MO, USA) as positive controls. The IC_{50} value of each compound was calculated with Reed and Muench's method (Reed and Muench 1938).

3.6. Spectroscopic data

Phyllaciduloid E (**1**): $C_{20}H_{28}O_4$; yellow powder; $[\alpha]_D^{19.0}$ −7.17 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 192 (4.22), 196 (3.12) nm; CD (c 0.18, MeOH) $\Delta\varepsilon 197 - 5.990$, $\Delta\varepsilon 219 + 1.106$, $\Delta\varepsilon 247 + 3.679$; IR (KBr) ν_{max} 3440, 2964, 2945, 2875, 1645, 1622, 1368, 1069, 1039, 590 cm^{-1}; negative ESIMS *m/z* 331 $[M - H]^-$; HRESIMS (negative ion mode) *m/z* 331.1916 $[M - H]^-$ (calcd 331.1915 for $C_{20}H_{27}O_4$); ^1H NMR (600 MHz, CD_3OD) δ_H 6.58 (1H, ddd, *J* = 18.0, 11.9, 0.7 Hz, H-15), 6.16 (1H, s, H-11), 5.64 (1H, dd, *J* = 11.9, 2.0 Hz, H-16a), 5.52 (1H, dd, *J* = 18.0, 2.0 Hz, H-16b), 4.19 (1H, q, *J* = 3.5 Hz, H-2), 3.13 (1H, d, *J* = 3.5 Hz, H-3), 2.35 (1H, dt, *J* = 14.5, 3.1 Hz, H-7a), 2.17 (1H, dd, *J* = 13.7, 3.5 Hz, H-1α), 1.94 (3H, s, H-17), 1.90 (1H, m, H-6a), 1.74 (1H, dd, *J* = 13.7, 3.5 Hz, H-1*β*), 1.73 (3H, s, H-20), 1.65 (1H, m, H-6b), 1.35 (1H, td, *J* = 14.5, 4.6 Hz, H-7b), 1.11 (3H, s, H-19), 1.06 (1H, dd, *J* = 12.6, 2.5 Hz, H-5), 0.99 (3H, s, H-18); ^{13}C NMR (125 MHz, CD_3OD) δ_C 189.9 (C-12), 169.2 (C-9), 156.2 (C-14), 133.8 (C-15), 133.2 (C-13), 124.6 (C-11), 123.3 (C-16), 81.6 (C-8), 78.8 (C-3), 72.1 (C-2), 55.7 (C-5), 42.9 (C-1), 42.4 (C-10), 40.4 (C-4), 39.1 (C-7), 30.4 (C-18), 20.8 (C-20), 18.8 (C-6),17.8 (C-19), 12.6 (C-17).

Phyllaciduloid F (**2**): $C_{20}H_{28}O_4$; yellow powder; $[\alpha]_D^{19.1}$ −46.39 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 192 (4.01), 196 (4.11), 219 (3.93) nm; CD (c 0.16, MeOH) $\Delta\varepsilon 195 - 24.932$, $\Delta\varepsilon 212 + 2.720$, $\Delta\varepsilon 247 + 11.492$, $\Delta\varepsilon 277 - 7.245$; IR (KBr) ν_{max} 3425, 2955, 2926, 2879, 1650, 1620, 1360, 1048, 998, 532 cm^{-1}; positive ESIMS *m/z* 355 $[M + Na]^+$; HRESIMS (positive ion mode) *m/z* 355.1883 $[M + Na]^+$ (calcd 355.1880 for $C_{20}H_{28}O_4Na$); ^1H NMR (600 MHz, CD_3OD) δ_H 6.62 (1H, ddd, *J* = 18.0, 11.9, 0.7 Hz, H-15), 6.03 (1H, s, H-11), 5.65 (1H, dd, *J* = 11.9, 2.1 Hz, H-16a), 5.50 (1H, dd, *J* = 18.0, 2.1 Hz, H-16b), 4.18 (1H, q, *J* = 3.6 Hz, H-2), 3.14 (1H, d, *J* = 3.6 Hz, H-3), 2.34 (1H, dt, *J* = 13.8, 3.1 Hz, H-7a), 2.16 (1H, dd, *J* = 13.7, 3.6 Hz, H-1α), 2.05 (1H, dd, *J* = 13.0, 3.1 Hz, H-6a), 1.93 (3H, s, H-17), 1.79 (3H, s, H-20), 1.73 (1H, m, H-1*β*), 1.69 (1H, m, H-6b), 1.22 (1H, td, *J* = 13.8, 4.3 Hz, H-7b), 1.12 (3H, s, H-19), 1.06 (1H, dd, *J* = 13.0, 2.4 Hz, H-5), 1.01 (3H, s, H-18); ^{13}C NMR (125 MHz, CD_3OD) δ_C 189.7 (C-12), 172.7 (C-9), 159.5 (C-14), 134.1 (C-15), 129.7 (C-13), 123.4 (C-16), 121.4 (C-11), 78.8 (C-3), 72.0 (C-2), 71.6 (C-8), 55.8 (C-5), 42.8 (C-1), 42.5 (C-10), 41.0 (C-7), 40.3 (C-4), 30.3 (C-18), 22.4 (C-20), 18.5 (C-6),17.7 (C-19), 12.5 (C-17).

3.7. Computational details

All DFT calculations were carried out using Gaussian 16 package. The stable conformations were optimized at B3LYP/6-311++G(2d,2p) level of theory, as confirmed by the absence of imaginary frequencies at the same level. Theoretical ^{13}C NMR chemical shifts were deduced from the isotropic magnetic shielding tensors by using Gauge-Independent Atomic Orbital (GIAO) methodology at B3LYP/6-311tG(d,p) (Duong et al. 2020). The CP3 probability was performed to assign the exact conformer using online implementation available from http://www-jmg.ch.cam.ac.uk/tools/nmr/CP3/ (Grimblat et al. 2015).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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References

Duong TH, Beniddir MA, Nguyen VK, Aree T, Gallard JF, Mac DH, Nguyen HH, Bui XH, Boustie J, Nguyen KPP, et al. 2018. Sulfonic acid-containing flavonoids from the roots of *Phyllanthus acidus*. J Nat Prod. 81(9):2026–2031.

Duong TH, Bui XH, Le Pogam P, Nguyen HH, Tran TT, Nguyen TAT, Chavasiri W, Boustie J, Nguyen KPP. 2017. Two novel diterpenes from the roots of *Phyllanthus acidus* (L.) Skeel. Tetrahedron. 73(38):5634–5638.

Duong TH, Trung Nguyen T, Dang PC, Nguyen VD, Nguyen HC, Dao TBN, Mai DT, Niamnont N, Tran TNM, Sichaem J. 2020. A new diterpenoid from the leaves of *Phyllanthus acidus*. Nat Prod Res. https://doi.org/10.1080/14786419.2020.1789980.

Grimblat N, Zanardi MM, Sarotti AM. 2015. Beyond DP4: An improved probability for the stereochemical assignment of isomeric compounds using quantum chemical calculations of NMR shifts. J Org Chem. 80(24):12526–12534.

Lv JJ, Yu S, Wang YF, Wang D, Zhu HT, Cheng RR, Yang CR, Xu M, Zhang YJ. 2014. Anti-hepatitis B virus norbisabolane sesquiterpenoids from *Phyllanthus acidus* and the establishment of their absolute configurations using theoretical calculations. J Org Chem. 79(12):5432–5447.

Lv JJ, Yu S, Xin Y, Zhu HT, Wang D, Cheng RR, Yang CR, Xu M, Zhang YJ. 2015. Stereochemistry of cleistanthane diterpenoid glucosides from *Phyllanthus emblica*. RSC Adv. 5(37):29098–29107.

Reed LJ, Muench H. 1938. A simple method for estimating 50% endpoints. Am J Epidemiol. 27(3):493–497.

Su D, Yang XY, Feng X, Shang MY, Cai SQ. 2014. The diterpenes ovoideal A-G from Tirpitzia ovoidea. Molecules. 19(11):18966–18979.

Tan SP, Tan NY, Lim QY, Nafiah MA. 2020. *Phyllanthus acidus* (L.) Skeels: A review of its traditional uses, phytochemistry, and pharmacological properties. J Ethnopharmacol. 253:112610.

Vongvanich N, Kittakoop P, Kramyu J, Tanticharoen M, Thebtaranonth Y. 2000. Phyllanthusols A and B, cytotoxic norbisabolane glycosides from *Phyllanthus acidus* Skeels. J Org Chem. 65(17):5420–5423.

Xin Y, Xu M, Wang YF, Zheng XH, Zhu HT, Wang D, Yang CR, Zhang YJ. 2020. Phyllanthacidoid U: a new *N*-glycosyl norbisabolane sesquiterpene from *Phyllanthus acidus* (L.) skeels. Nat Prod Res. https://doi.org/10.1080/14786419.2020.1712387.

Zhao JQ, Lv JJ, Wang YM, Xu M, Zhu HT, Wang D, Yang CR, Wang YF, Zhang YJ. 2013. Phyllanflexoid C: first example of phenylacetylene-bearing 18-nor-diterpenoid glycoside from the roots of *Phyllanthus flexuosus*. Tetrahedron Lett. 54(35):4670–4674.

Zheng XH, Yang J, Lv JJ, Zhu HT, Wang D, Xu M, Yang CR, Zhang YJ. 2018. Phyllaciduloids A-D: Four new cleistanthane diterpenoids from Phyllanthus acidus (L.) Skeels. Fitoterapia. 125:89–93.