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Phyllanthacidoid U: a new *N*-glycosyl norbisabolane sesquiterpene from *Phyllanthus acidus* (L.) skeels

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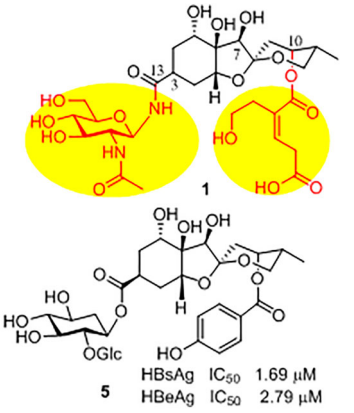
ABSTRACT

A novel highly oxygenated norbisabolane sesquiterpene, namely phyllanthacidoid U (1), along with nine known sesquiterpenes (2–10) was isolated from the roots and stems of *Phyllanthus acidus* (L.) Skeels (Phyllanthaceae), collected from Xishuangbanna, Yunnan province, China. Their structures were elucidated by means of extensive spectroscopic analysis and by comparison of their data with reported values in literatures. Instead of the C-13 ester *O*-glycosyl found mostly in the titled plant growing in Thailand, compound **1** possessed a rare *N*-*β*-glucosamine-2-*N*-acetate moiety linked directly to the carbonyl at C-13 through an amido bond. Moreover, the acyl group at C-10 in **1** was (*Z*)-2-(2-hydroxyethyl)-pent-2-enedioyl group, instead of benzoic or *p*-hydroxybenzoic moieties found commonly in the reported norbisabolane sesquiterpenes. The known sesquiterpene **5** displayed stronger anti hepatitis B virus (HBV) activity with IC_{50} values of 1.69 ± 0.22 and 2.79 ± 0.69 μM towards HBV surface antigen (HBsAg) and HBV excreted antigen (HBeAg) secretion, respectively.

Phyllanthus acidus



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1. Introduction

The genus *Phyllanthus* (Phyllanthaceae) with most 900 species (Unander et al. 1995; Zhao et al. 2014) are widely distributed throughout the tropical and subtropical countries of the world. Many *Phyllanthus* species have been used medicinally for the treatment of various diseases such as kidney, intestinal infections and so on (Piers and Gavai 1990; Yaoita et al. 1999; Rocha et al. 2007; Li et al. 2012; Zhao et al. 2014; Jang et al. 2016). *Phyllanthus acidus* (L.) Skeels is widely growing in the tropical countries of the world, e.g., Thailand, Vietnam, and Malaysia. It is also introduced and cultivated in the south part of Yunnan province, China. As a traditional medicine in Thailand, the leaves have been used as anti-hypertensive agent, while the roots are used for the treatment of fever and to cure alcoholism (Mackeen et al. 1997; Calixto et al. 1998; Meléndez and Capriles 2006; Satish et al. 2007; Jain and Singhai 2011; Chakraborty et al. 2012; Hossen et al. 2015; Ling et al. 2015; Duong et al. 2017; Tram et al. 2017). However, long-term use will cause serious side effects (Vongvanich et al. 2000).

Previous phytochemical studies on the roots of the titled plant from Thailand led to identification of a series of novel bisabolane type sesquiterpenoids with mostly *scyllo* quercitol-2-*O*-β-glucosamine-*N*-acetate as sugar moiety linked to the aglycone C-13 through an ester bond (Vongvanich et al. 2000; Lv et al. 2014). In order to compare the chemical composition of the titled plant from different distribution and find new bioactive ingredients, the roots and stems of *P. acidus* collected from Xishuangbanna of Yunnan province, China was studied chemically. This led to the identification of 10 highly oxygenated norbisabolane sesquiterpenes (**1**–**10**). Compound **1**, namely phyllanthacidoid U, is a rare *N*-glycosyl norbisabolane sesquiterpene with *N*-β-glucosamine-2-*N*-acetate as saccharide moiety connecting with the carbonyl at C-13 through an amido bond. Moreover, the acyl at C-10 in **1** was a rare (*Z*)-2-(2-hydroxyethyl)-pent-2-enedioyl group. All the isolates **1–10** were evaluated for their anti-hepatitis B virus (HBV) activities against HBsAg and HBeAg secretion by texting their cytotoxicity against HepG2.2.15 and HepGRL1. Herein, we report the structure determination of these compounds and their anti-HBV activities.

2. Results and discussion

The MeOH extract of the air-dried roots and stems of *P. acidus* collected from Xishuangbanna district of Yunnan province, China, was partitioned with *n*-BuOH and H_2O. After removal of most of the polyphenols by polyamide, the organic layer was applied to repeated column chromatography (CC) of Diaion HP 20SS, Sephadex LH-20, RP-18, CG161M, and Toyopearl HW-40F, followed with semi-preparative HPLC to yield 10 norbisabolane sesquiterpenes **1–10**. Nine known compounds **2–9** were identified as phyllanthacidoids A-D (**2**-**5**), H (**6**), L (**7**), M (**8**), S (**9**), and T (**10**) (Lv et al. 2014), respectively, by comparison of their spectroscopic data with those reported in literature.

Phyllanacidoid U (**1**) was obtained as white amorphous powder, and had a molecular formula $C_{29}H_{44}N_2O_{16}$, as deduced from the negative HRESIMS (*m/z* 675.2613 $[M–H]^-$) and ^{13}C NMR (DEPT) spectroscopic data, corresponding to nine degrees of unsaturation. The IR spectrum showed the occurrence of hydroxyl (3425 cm^{-1}) and carbonyl (1647 cm^{-1}) groups. The ^1H and ^{13}C NMR spectra of **1**

displayed obviously the existence of one acetyl group (δ_H 1.95, 3H, s; δ_C 174.5, 23.0). In addition, 27 carbon signals composing of six quaternary carbons including three carbonyl (δ_C 168.7, 174.4, 179.2), one ketal (δ_C 102.6), one olefinic (δ_C 133.1) and one oxygen-bearing (δ_C 76.6) ones, 12 methines including one olefinic (δ_C 138.1), and eight oxygen- (δ_C 72.5, 82.5, 76.1, 71.6, 80.1, 76.5, 72.0, 80.0) and one nitrogen- (δ_C 56.3) bearing ones, eight methylenes with three oxygen-bearing ones (δ_C 62.7, 62.1, 63.0), and one methyl (δ_C 13.1) were observed in the ^{13}C NMR and DEPT spectra. The ^{1}H NMR spectrum of **1** also showed the existence of only one olefinic proton at δ_H 7.15 (1H, t, $J = 7.7$ Hz, H-3′), one doublet methyl at δ_H 0.84 (1H, d, $J = 6.8$ Hz, H-14), and a set of oxymethine and methylene protons at δ_H 3.33-3.89. The aforementioned data were quite similar to those of phyllanthacidoids A-D, H, L, and M (**2**-**8**), reported previously from the titled plant growing in Thailand (Lv et al. 2014), suggesting that **1** was a norbisabolane sesquiterpene, whose aglycone was the same with those of **2–8**.

However, the glycosyl bond with C-13 carbonyl in **1** as well as the acyl group at C-10 was quite different with those of **2–8**, in which an ester *O*-glycosyl to C-13 and a benzoyl or *p*-hydroxybenzoyl group linked to C-10 were existed. Instead of the *scyllo* quercitol-2-*O*-β-glucosamine-*N*-acetate in **2** and **3**, the saccharide moiety in **1** was only an β-glucosamine-*N*-acetate unit [δ_C 80.1 (C-1″), 56.3 (C-2″), 76.5 (C-3″), 72.0 (C-4″), 80.0 (C-5″), 62.7 (C-6″), 174.4 (C-1‴), 23.0 (C-2‴); δ_H 4.94 (1H, d, $J = 8.5$ Hz, H-1″), 1.95 (3H, s, H-2‴)]. Comparing with δ_C 103.1 for an *O*-glucosamine-*N*-acetate unit (Lv et al. 2014), the obviously upper field shifted anomeric carbon (δ_C 80.1) indicated the sugar moiety in **1** was linked to the C-13 carbonyl through an amide bond, by combining with the chemical shift (δ_C 179.3) of C-13 as well. The HMBC correlations of glucosyl H-1″ (δ_H 4.94) and aglycone H-2 (δ_H 1.55, 1.78) with δ_C 179.3 (C-13) also confirmed the linkage of the sugar moiety to aglycone (Supplementary material, Figure S12).

The acyl group at C-10 in **1** was determined by 1D and 2D NMR experiments. Instead of the benzene ring signals in **2–8**, a set of signals composing of two carboxyls (δ_C 174.6, 168.7), one trisubstituted double bond [δ_C 133.1 (C), 138.1 (CH), δ_H 7.15 (t, $J = 7.7$ Hz)] and three methylenes with one oxygen-bearing one [δ_C 62.1 (CH_2)/δ_H 3.67 (t, $J = 6.3$ Hz), δ_C 35.2 (CH_2)/δ_H 3.33 (d, $J = 7.7$ Hz), δ_C 31.3 (CH_2)/δ_H 2.60 (t, $J = 6.3$ Hz)] were observed in the ^{1}H and ^{13}C NMR spectra of **1**. The ^{1}H-^{1}H COSY spectrum showed the existence of two fragments, -$C_{(4')}H_2$-$C_{(3')}$H=, and -$C_{(6')}H_2$-$C_{(7')}H_2$O-. In the HMBC spectrum, correlations from H-3′ (δ_H 7.15) to C-1′ (δ_C 168.7), C-2′ (δ_C 133.1) and C-5′ (δ_C 174.6); H-4′ (δ_H 3.33) to C-2′ and C-5′; H-6′ (δ_H 2.60) to C-1′ and C-2′; H-10 (δ_H 5.13) to C-1′, and in the ROESY spectrum, correlations from H-3′ (δ_H 7.15) to H_2-6′ (δ_H 2.60), verified that the acyl group was linked at C-10 and its structure was determined to be (*Z*)-2-(2-hydroxyethyl)-pent-2-enedioyl.

The relative configuration of **1** was further assigned by the comparison of known compounds and coupling constants analysis. The $J_{H\text{-}1,H\text{-}2}$ (11.4 Hz), and $J_{H\text{-}5,H\text{-}4}$ (3.2 Hz) suggested the axial orientations of H-1, and equatorial orientation of H-5. In the ROESY experiment, correlations of H-1 (δ_H3.88)/H-2eq (δ_H 1.78)/H-5 (δ_H 4.04); H-3 (δ_H 2.60)/H-2ax (δ_H 1.55)/H-7 (δ_H 3.75) indicated that H-1, H-5 and H-2eq were on the same side, H-3, H-2ax, H-7 were on the opposite side. Taking into account of both the axial orientation of H-1 and equatorial orientation of H-5, it can be proposed that ring A had a boat formation. Configurations of C-10 and C-11 were the same as those

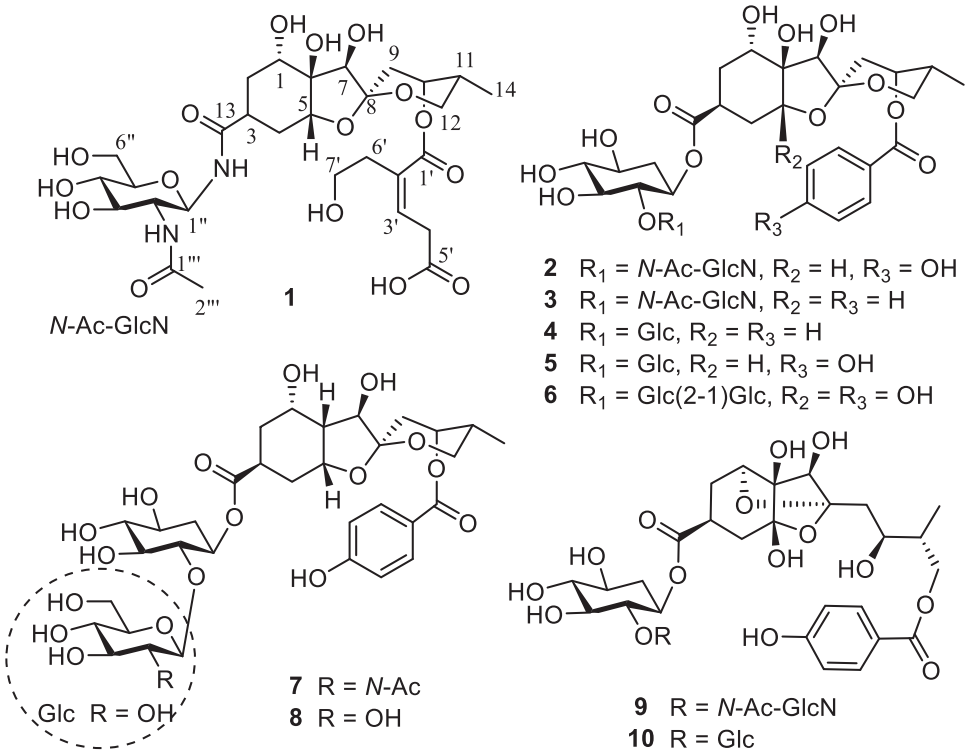

Figure 1. The chemical structures of **1**–**10** from *P. acidus*.

of phyllanthacidoids A-J (Lv et al. 2014). Moreover, the experimental ECD spectra displayed negative Cotton effects at 220 nm, and positive Cotton effects at 260 nm, which agreed well with those of phyllanthacidoids A-M (Lv et al. 2014). The absolute configuration of **1** was therefore the same as that of phyllanthacidoids A-M, as 1*S*,3*S*,5*R*,6*R*,7*R*,8*S*,10*S*,11*R*. Thus, the structure of compound **1** was determined as shown in Figure 1 and named as phyllanthacidoid U.

The isolates **1**-**10** were evaluated for their anti-HBV activities against HBsAg and HBeAg secretion by texting their cytotoxicity against HepG2.2.15 and HepGRL1. As the results, compounds **2**, **3**, **5**, **7**, **10** displayed potential anti-HBV activities. Among which, compound **5** showed stronger anti-HBV activity with IC_{50} values of 1.69 ± 0.22 and $2.79 \pm 0.69\,\mu M$ towards HBsAg and HBeAg secretion, respectively (Supplementary material, Table S1). It is noted that this type of highly oxygenated norbisabolane sesquiterpenoids were specifically effective against HBV.

3. Experimental part

3.1. General

Optical rotations were measured with a P-1020 polarimeter (JASCO, Tokyo, Japan). IR spectra were measured on a Bio-Rad FTS-135 series spectrometer. UV spectra were recorded on a Shimadzu UV2401A ultra-violet-visible spectrophotometer. ESIMS data were recorded on Waters Xevo TQ-S (Waters, Bremen, Germany). HRESIMS were

recorded on an API Qstar Pulsa spectrometer (Applied Biosystems, Framingham, MA, USA). 1D- and 2D-NMR spectra were recorded on Bruker DRX-500 and AV-600 instruments (Bruker Biospin GmbH, Karlsruhe, Germany) operating at 500 and 600 MHz for 1H NMR, and 125 and 150 MHz for ^{13}C NMR. Coupling constants are expressed in Hz, and chemical shifs are given on a ppm scale with tetramethylsilane as internal standard. TLC was performed on precoated TLC plates (0.2–0.25 mm thickness, GF254 Silica gel, Qingdao Hailang Chemical Co., Ltd., Qingdao, China) with compounds visualised by spraying the dried plates with 10% H_2SO_4 in EtOH followed by heating until the plate was dry. Diaion HP20SS (Mitsubishi Chemical Co., Ltd., Tokyo, Japan), Sephadex LH-20 (25–100 μm, Pharmacia, Kyoto, Japan), Toyopearl HW40F (30–60 μm, TOSOH, Kyoto, Japan), CG161M (Amberchrom, Pennsylvania, USA), Lichroprep RP-18 gel (40–63 μm, Merck, Darmstadt, Germany), MCI gel CHP20P (75–150 μm, Mitsubishi Chemical, Tokyo, Japan). An Agilent series 1260 (Agilent Technologies) were used for semi-preparative HPLC with an Agilent Thermo BDS HYPERSIL-C18 column (5 μm, 10 × 250 mm) (Thermo Electron Corporation, New York, USA).

3.2. Material

The roots and stems of *Phyllanthus acidus* (L.) Skeels were collected in Xishuangbanna district, Yunnan province, China, in June, 2013, and identified by Mr. B. Wen from Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences (CAS). A voucher specimen (KIB-Z-1306003) was deposited in the State Key Lab of Phytochemistry and Plant Resource in West China, Kunming Institute of Botany, CAS.

3.3. Extraction and isolation

The air-dried roots and stems of *Phyllanthus acidus* (7.0 kg) was ground into powder and extracted three times with MeOH under reflux at 70 °C. After concentrated under reduced pressure, the combined extract afforded a residue (265 g), which was further suspended into H_2O and partitioned with *n*-butanol. The *n*-butanol extract (143 g) was subjected to a polyamide column chromatography (CC) to remove most of polyphenols, and the yielded fraction (71 g) was chromatographed over Diaion HP20SS, eluted with aqueous MeOH (0%, 30%, 50%, 80%, 100%) to give four fractions (Fr. A - Fr. D).

Fr. C (6.18 g) was applied to a Sephadex LH-20 column, eluting with MeOH/H_2O (0%, 20%, 40%, 70%, 100% v/v) to afford six sub-fractions (subfr. C1–C6). Subfr. C1 (3.9 g) was further purified by CC over RP-18 (MeOH/H_2O, 0:1–1:0), Amberchrom CG161M (MeOH/H_2O, 3:7–10:0), and Toyopearl HW40F (MeOH/H_2O, 2:8–10:0), followed with semi-preparative HPLC (CH_3CN/H_2O, 15%) to yield **1** (10.0 mg, 8 min) and **8** (4.1 mg, 15 min).

Fr. D (42.3 g) was chromatographed over MCI-gel CHP20P, eluting with MeOH/H_2O (0%, 30%, 50%, 80%, 100%) to give eight subfractions (subfr. D1–D8). Subfr. D1 (1.8 g), D2 (2.1 g), D5 (1.2 g), and D7 (3.1 g) were separately applied to CC over RP-18 (MeOH/H_2O, 15%, 30%, 40%, 70%, 100%, v/v) and Amberchrom CG161M (MeOH/H_2O, 0%, 10%, 20%, 50%, 70%, 100%), followed with semi-preparative HPLC (CH_3CN/H_2O, 10%–20%) to yield **10** (3.5 mg, 7.6 min) and **3** (9.9 mg, 8.9 min) from D1, **4** (3.2 mg,

11.5 min), **5** (5.4 mg, 9 min), and **6** (6.7 mg, 15 min) from D2, **9** (2.9 mg, 7.5 min) from D5, and **2** (3.6 mg, 14 min) and **7** (2.6 mg, 16.4 min) from D7, respectively.

3.3.1. Phyllanacidoid U (1)

White amorphous powder, $[\alpha]^{21}{}_{D} + 10.4$ (*c* 0.90, MeOH); IR (KBr) ν_{max} cm^{-1}: 3425 1647 UV (MeOH) λ_{max} (log ε): 202 (0.54) nm; ^{1}H NMR (600 MHz, methanol-d_4): δ_H 3.88 (1H, dd, $J = 11.4$, 6.6 Hz, H-1), 1.55 (1H, m, H-2a), 1.78 (1H, H-2b), 2.60 (1H, m, H-3), 1.91 (1H, dd, $J = 11.4$, 2.9 Hz, H-4a), 1.96 (1H, m, H-4b), 4.40 (1H, t, $J = 4.2$ Hz, H-5), 3.75 (1H, s, H-7), 2.11 (1H, m, H-9a), 1.95 (1H, m, H-9b), 5.13 (1H, brd, $J = 2.2$ Hz, H-10), 2.04 (1H, m, H-11), 3.53 (1H, dd, $J = 10.9$, 4.4 Hz, H-12a), 3.89 (1H, m, H-12b), 0.84 (1H, d, $J = 6.8$ Hz, H-14), 7.15 (1H, t, $J = 7.7$ Hz, H-3′), 3.33 (2H, d, $J = 7.7$ Hz, H-4′), 2.60 (2H, t, $J = 6.3$ Hz, H-6′), 3.67 (2H, t, $J = 6.3$ Hz, H-7′), 4.94 (1H, d, $J = 8.5$ Hz, H-1″), 3.75 (1H, dd, $J = 8.5$, 9.6 Hz, H-2″), 3.46 (1H, dd, $J = 8.1$, 9.6 Hz, H-3″), 3.33 (1H, m, H-4″), 3.32 (1H, H-5″), 3.84 (2H, m, H-6″), 1.95 (3H, s, H-2‴); ^{13}C NMR (150 MHz, methanol-d_4): δ_C 72.5 (C-1), 30.0 (C-2), 36.0 (C-3), 27.4 (C-4), 82.5 (C-5), 76.6 (C-6), 76.1 (C-7), 102.6 (C-8), 35.9 (C-9), 71.6 (C-10), 34.1 (C-11), 63.0 (C-12), 179.3 (C-13), 13.1 (C-14), 168.7 (C-1′), 133.1 (C-2′), 138.1 (C-3′), 35.2 (C-4′), 174.6 (C-5′), 31.3 (C-6′), 62.1 (C-7′), 80.1 (C-1″), 56.3 (C-2″), 76.5 (C-3″), 72.0 (C-4″), 80.0 (C-5″), 62.7 (C-6″), 174.4 (C-1‴), 23.0 (C-2‴); ESIMS: *m/z* 699 $[M + Na]^+$; HRESIMS: *m/z* 675.2613 $[M - H]^-$ (calcd for $C_{29}H_{43}N_2O_{16}$, 675.2613).

3.4. Anti-HBV assay

Anti-HBV activity is measured by HepG2.2.15 and HepGRL1 cells, which were added in 96-well plates and incubated for 24 h (5% CO_2, 37 °C) to form monolayers. The tested compounds at dose below TC_{50} (50 μL per well) each with equal volume were added, and the incubation followed for 6 days. The supernatant was collected and stored at −20 °C. ELISA method was applied to detect the contents of HBsAg and HBeAg. IC_{50} values (the concentration of test compounds required to reduce 50% of HBsAg and HBeAg secretion) were calculated according to the regression analysis of the dose − response curves.

4. Conclusion

In this study, ten norbisabolane sesquiterpenes **1–10** were isolated from the roots and stems of *Phyllanthus acidus,* collected from Xishuangbanna, Yunnan province, China. The new compound, phyllanthacidoid U (**1**), was a novel *N*-glucoside with an *N*-*β*-glucosamine-2-*N*-acetate moiety linked to the C-13 amido bond. Moreover, a rare acyl group, (*Z*)-2-(2-hydroxyethyl)-pent-2-enedioyl, was located on C-10 through an ester bond, instead of benzoic or *p*-hydroxybenzoic moieties found commonly in the reported norbisabolane sesquiterpenes. The known compounds **2**, **3**, **5**, **7**, **10** displayed potential anti HBV activities, especially compound **5** with IC_{50} values of 1.69 ± 0.22 and 2.79 ± 0.69 μM towards HBsAg and HBeAg secretion, respectively. Although the anti-HBV mechanism is not clear and need to be further studied, this kind of norbisabolane sesquiterpene may present a new scaffold of anti-HBV agents.

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Disclosure statement

The authors declare that there are no conflicts of interest.

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