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New anti-HBV norbisabolane sesquiterpenes from *Phyllantus acidus*

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ABSTRACT

The norbisabolane-type sesquiterpenoids bearing a spiroketal functionality have been found in *Phyllanthus* spp. and showed anti-HBV activities. As part of an ongoing effort to search for promising anti-HBV sesquiterpenes from *Phyllanthus* plants, we report four new norbisabolane-type sesquiterpenoids, phyacidusin A (**1**), phyacidusin B (**2**), phllanthacidoid A1 (**3**) and phllanthacidoid N1 (**4**), from stem of *P. acidus* collected in Xishuangbanna, Yunnan province, China. The absolute configuration of new compounds was established by coupling constants and ROESY correlations, as well as comparation of NMR data with those of known compounds. The absolute configuration of new compounds **1** and **2** was further confirmed by X-ray diffraction. Compound **2** showed effect to HBsAg with an IC_{50} value of 11.2 ± 0.01 μM, while compound **3** inhibited HBeAg secretion with an IC_{50} value of 57.1 ± 0.02 μM. The results enriched the diversity of anti-HBV norbisabolane sesquiterpenes.

1. Introduction

Despite the application of effective vaccines, hepatitis B virus (HBV) infection is still a major public health problem worldwide, which continues to cause nearly 1 million deaths each year [1]. HBV infection can lead to liver failure, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [2]. There are approximately 63 million newly-added patients with chronic HBV infections and 17 million HBV-related deaths between 2015 and 2030 [1]. IFN-α is a clinically useful anti-HBV drug but has substantial side effects. Nucleoside/nucleotide analogues (lamivudine, adefovir, dipivoxil, entecavir, telbivudine, tenofovir and so on) acting as competitive inhibitors of viral DNA polymerase have been approved to treat chronic HBV infections [3]. However, these drugs do not possess inhibitory effects on HBeAg or HBsAg expression associated with the occurrence of HCC [4]. Moreover, rapid development of drug-resistant virus becomes a big problem [2]. Hence, there is an urgent need for new drugs with novel target and mechanism.

Sesquiterpenoids, consisting of more than 200 skeleton types, are a major chemical class in natural products. Bisabolane-type sesquiterpenoids, belonging to monocyclic sesquiterpenes, occur mainly in the Euphorbiaceae, Asteraceae, Burseraceae, Zingiberaceae, and Apiaceae families, as well as some sponges and fungi. Among them, the norbisabolane-type sesquiterpenoids with spiroketals have been isolated mainly from *Phyllanthus spp.* (Euphorbiaceae) [5–7] and less frequently in *Glochidion spp.* [8]. The spiroketal contains two oxacyclic rings, which are formed by oxidization and cyclization of 12-methyl group. Moreover, the feature of the norsesquiterpenes in *Phyllanthus* spp. is oxidization of 15-methyl group and esterification of carboxyl group on C-15 with quercitol or a sugar unit. The norsesquiterpenes showed anti-HBV and cytotoxic activities [6]. The highly oxygenated sesquiterpenoids with various bioactivities have stimulated considerable interest in chemical and biological research communities [5–7,9–12].

Our previous studies suggested that norbisabolane sesquiterpenes isolated from *Phyllanthus acidus* collected in Thailand showed anti-HBV activities [6]. As part of an ongoing effort to search for anti-HBV sesquiterpenes from *Phyllanthus* plants [5–7,13,14], herein we report four new norbisabolane sesquiterpenes, phyacidusin A (**1**), phyacidusin B (**2**), phllanthacidoid A1 (**3**) and phllanthacidoid N1 (**4**) (Fig. 1), from stem of *P. acidus* collected in Xishuangbanna, Yunnan province, China, as well as 12 known norbisabolane sesquiterpenes, phyllanthacidoids A-D, F, G, I, J, L, Q and R (Supporting information). Using X-ray diffraction, the absolute configuration of new compounds **1** and **2** was

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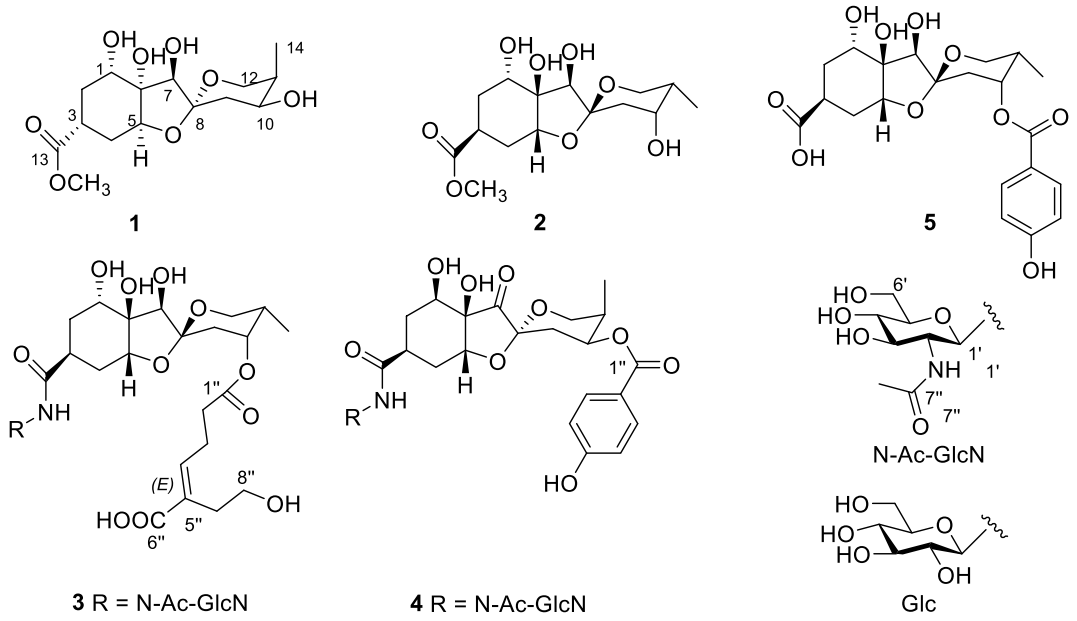

Fig. 1. Structures of **1**–**5**.

confirmed. The absolute configuration of new compounds **3** and **4** was established by coupling constants and ROESY correlations, as well as comparation of NMR data with those of known compounds. Compounds **1**–**3** were tested for anti-HBV activities. Compound **2** showed effect to HBsAg with an IC_{50} value of 11.2 ± 0.01 μM, while no effects were exhibited for hepatitis Be antigen (HBeAg) at 100 μM. However, compound **3** could inhibit HBeAg secretion with the IC_{50} value of 57.1 ± 0.02 μM, while no activity was identified on hepatitis B surface antigen (HBsAg) at 100 μM.

2. Results and discussion

HRESI(+)MS analysis of **1** revealed a pseudomolecular ion (*m/z* 355.1365 $[M + Na]^+$) indicative of a molecular formula ($C_{15}H_{24}O_8$, Δmmu 0.20). The ^{1}H NMR (MeOH-d_4) data for **1** (Table 1) revealed one doublet for methyl (δ_H 1.02) and an *O*-methyl (δ_H 3.67). The ^{13}C NMR (MeOH-d_4) data for **1** (Table 2) suggested the presence of 15 carbons including one methyl (δ_C 9.8), an *O*-methyl (δ_C 52.3), four methylenes including an oxymethylene (δ_C 66.3), six methines including four oxymethines (δ_C 67.2, 70.0, 76.6, 88.7), two oxy quaternary carbon (δ_C 78.2 and 108.0) and one carbonyl carbon (δ_C 176.8). The aforementioned NMR data suggested that compound **1** was a norbisabolane type sesquiterpenoid bearing [5,6]-spiroketal. The aforementioned NMR data of **1** were similar to those of phyllanthacidoid acid methyl ester, but was different due to the lack of benzoyl unit and chiral carbons [6]. Furthermore, HMBC correlations of methyl (δ_H 1.02, C-14) to methine (δ_C 36.2, C-11) and oxymehylene (δ_C 66.3, C-12) confirmed the [5,6]-spiroketal unit and the lack of benzoyl unit, as well as HMBC correlations of oxymethylene (δ_H 3.42, 3.93, C-12) to C-8 (δ_C 108.0), C-10 (δ_C 70.0), C-11 (δ_C 36.2) and C-14 (δ_C 9.8) (Fig. 2). Thus, the planar structure of **1** was assigned as shown in Fig. 1. The relative configuration was established on the basis of coupling constant analysis and ROESY correlations (Fig. 2). The *J* values of H-3 (tt, 12.8, 3.5 Hz) and H-1 (dd, 11.6, 4.1 Hz) proposed the axial orientations of H-1 and H-3. The H-5 showed broad singlet peak at ^{1}H NMR data, supporting equatorial orientation of H-5. The aforementioned data indicated that ring A had a chair conformation. The small coupling constants of $J_{H\text{-}11,\ H\text{-}12}$ showed an equatorial orientation of H-11, while the large coupling constants of H-10 (dt, *J* = 12.5, 4.4 Hz) suggested axial orientation of H-10. Additionally, the ROESY correlation of H-3 and H-1 allowed the construction of the relative configuration of **1**, as well as the ROESY correlation of H-5 and H-7 (Fig. 2). A crystal X-ray diffraction experiment with Cu K*α* radiation finally further confirmed the planar structure of **1** (deposition No. CCDC-1900452) (Fig. 3) and also allowed the unambiguous assignment of the absolute configuration of **1** as 1*S*, 3*R*, 5*S*, 6*R*, 7*R*, 8*R*, 10*S*, 11*R* [the Flack parameter is 0.08(8)] (Fig. 4), and the compound was named phyacidusin A.

Compound **2** was determined to have a molecular formula of $C_{15}H_{24}O_8$ (Δmmu 1.3) by HRESI(+)MS from a pseudomolecular ion (*m/z* 355.1376 $[M + Na]^+$). The ^{1}H NMR (MeOH-d_4) data for **2** (Table 1) showed a doublet methyl (δ_H 0.87) and an *O*-methyl (δ_H 3.67). The ^{13}C NMR (MeOH-d_4) data for **2** (Table 2) suggested 15 carbons including one methyl (δ_C 13.2), an *O*-methyl (δ_C 52.4), four methylenes including an oxymeyhylene (δ_C 62.4), six methines (four oxymethines δ_C 72.1, 82.5, 75.9, 68.8), two oxy quaternary carbon (δ_C 76.3 and 103.2) and one carbonyl carbons (δ_C 178.1). The aforementioned NMR data for **2** (see Table 1) were very similar to those of phyllanthacidoid acid methyl ester [6], with the notable exception of the NMR resonance for C-10 and the lack of a benzoyl unit. It is noted that C-10 (δ_C 71.7) in phyllanthacidoid acid methyl ester was upshifted to δ_C 68.8 in **1**. Furthermore, HMBC correlations of methyl (δ_C 13.2, C-14) to oxymethine (δ_C 68.8, C-10), oxymehylenes (δ_C 62.4, C-12) and methylene (δ_C 35.3, C-11) confirmed the lack of benzoyl at C-10 in compound **2** (Fig. 4). Thus, the planar structure of **2** was assigned as shown in Fig. 1. The relative configuration was established on the basis of coupling constant analysis and ROESY correlations (Fig. 4). The $J_{H\text{-}1,\ H\text{-}2}$ (dd, 10.4, 4.9 Hz) and $J_{H\text{-}5,\ H\text{-}4}$ (t, 3.7 Hz) determined the axial orientations of the H-1 and equatorial orientation of H-5, respectively. The aforementioned data proposed that ring A had a boat conformation. Moreover, the H-10 shows small coupling constants as 6.2 and 3.7 Hz, it was thus determined to be equatorially orientated. Furthermore, the ROESY correlation of H-1 with H-5 and H-7 with H-3 suggested the relative configuration of **2** except for C-8. In order to establish the absolute configuration of **2**, we compared NMR data of **2** with those of **1** and phyllanthacidoid acid methyl ester [6]. It is noted that the chemical shift of C-8 was δ_C 108.0 in **1** featuring 8*R* constructed by X-Ray (Fig. 3), while the chemical shift of C-8 was δ_C 102.6 in phyllanthacidoid acid methyl ester bearing 8*S* established by calculated electronic circular dichroism (ECD). In order to confirm absolute configuration of C-8 of phyllanthacidoid acid methyl ester, we got the crystal of aglycon (**5**) of phllanthacidoid A, a major compound of title plant. A crystal X-ray diffraction experiment with Cu K*α* radiation finally confirmed the

Table 1
^{1}H NMR spectroscopic data for compounds 1–4 at 600 MHz (δ in ppm).

No	1[a]	2[a]	3a[a]	3b[b]	4[a]	5[a]
1	4.11 dd (11.6, 4.1)	3.84 dd (10.4, 4.9)	3.88 dd (11.6, 3.1)	4.19 dd (11.4, 3.1)	3.80 m	3.84 dd (11.1, 5.2)
2a	1.64[c]	1.72 ddd (14.1, 10.4, 9.0)	1.72 ddd (13.9, 11.6, 9.3)	2.09 (overlapped)	1.77 ddd (14.5, 12.3, 3.3)	1.88 m
2b	1.88[d]	2.10[e]	1.56 ddd (13.9, 5.0)	2.67 dt (13.9, 4.7)	2.05 dt (14.5, 3.0)	1.97[i]
3	2.65 (tt, 12.8, 3.5)	2.91 (m)	2.65 (tt, 13.3, 5.0)	3.29 tt (10.9, 4.7)	3.12 dd (12.3, 3.0)	2.48 (m)
4a	2.00 ddd (14.8, 3.5, 1.9)	1.95 ddd (11.1, 3.4)	1.93[f]	2.51 m	1.77 ddd (14.5, 12.3, 3.3)	1.60 m
4b	1.71 ddd (14.8, 12.8, 2.1)	2.10[e]	1.93[f]	2.51 m	2.05 dt (14.5, 3.0)	1.97[i]
5	4.04 brs	4.05 t (3.7)	4.29 dd (4.4, 3.3)	4.67[g]	4.29 dd (3.3, 3.0)	4.11 brs
7	3.97 s	3.78 s	3.74 s	3.88 s	–	3.81 s
9a	1.64[c]	1.91 dd (7.9, 3.2)	1.99 dd (14.9, 3.4)	2.31 dd (14.7, 3.6)	1.99 dd (13.5, 5.1)	1.97[i]
9b	1.88[d]	2.10[e]	2.11 dd (14.9, 2.6)	2.18 dd (14.7, 2.6)	2.23 dd (13.5, 10.1)	2.18 dd (14.3, 3.3)
10	3.95 dt (12.5, 4.4)	3.88 dd (6.2, 3.7)	5.12 d (2.1)	5.32 s	5.45 dt (2.2, 1.2)	5.23 brd (3.1)
11	1.88[d]	1.85 m	2.04 brd (14.4, 10.8)	1.93 ddt (14.4, 10.8, 5.7)	2.21 dt (2.2, 1.2)	1.97[i]
12a	3.42 dd (11.5, 2.3)	3.49 dd (11.1, 4.3)	3.52 dd (11.1, 4.7)	4.05 t (11.4)	3.64 dd (14.2, 1.2)	3.59 m
12b	3.93 dd (11.5, 2.7)	3.79 dd (11.1, 2.3)	3.85 brd (11.1)	4.27 dd (11.4, 2.2)	4.11 dd (14.2, 2.2)	4.01 t (11.4)
14	1.02 d (7.1)	0.87 d (7.0)	0.83 d (6.9)	0.71 d (7.0)	1.15 d (7.1)	0.87 d (6.9)
1′			4.93 d (9.5)	5.81 t (9.3)	4.98 d (9.7)	
2′			3.70 m	4.67[g]	3.45 m	
3′			3.44 dd (10.2, 3.8)	4.17 t (11.4)	3.47 dd (8.7, 10.9)	
4′			3.33 m	4.67[g]	3.32 m	
5′			3.31 m	4.41[h]	3.31 m	
6′			3.65 dd (12.0, 6.8)	4.67[g]	3.63 dd (11.8, 4.7)	
			3.81 brd (12.0, 1.7)	4.41[h]	3.82 brd (11.8)	
8′			1.98 s	2.15 s	1.98 s	
2″			–	1.27 m	7.88 d (8.7)	7.97 d (8.5)
3‴			–	3.72 m	6.83 d (8.7)	6.83 d (8.5)
4″			7.11 s	7.51 s	6.83 d (8.7)	7.97 d (8.5)
7″			–	2.90 t (6.9)	7.88 d (8.7)	6.83 d (8.5)
8″			3.85 m	4.23 t (6.9)		
OCH_3	3.67 s	3.67 s				

[a] Data were recorded in CD_3OD.
[b] Data were recorded in CD_5N.
[c] Overlapped with each other.
[d] Overlapped with each other.
[e] Overlapped with each other.
[f] Overlapped with each other.
[g] Overlapped with each other.
[h] Overlapped with each other.
[i] Overlapped with each other.

absolute configuration of **5** (deposition No. CCDC-1587954) (Fig. 5) as 1*S*, 3*S*, 5R, 6*S*, 7*R*, 8*S*, 10*S*, 11*R* [the Flack parameter is 0.01(2)] (Fig. 4). The chemical shift of C-8 in **5** was δ_C 102.4. Thereof, the chemical shift of C-8 (δ_C 103.2 in **2**) allowed unambiguous assignment of the absolute configuration of **2** as 1*S*,3*S*,5*R*,6*S*,7*R*,8*S*,10*S*,11*R*, which was an epimer of **1** and named phyacidusin B. The biosynthetic origin of **1** and **2** could be tracked back to farnesyl pyrophosphate (FPP) as shown in Fig. 8.

HRESI(+)MS analysis of **3** revealed a pseudomolecular ion (*m/z* 689.2797 [M - H]$^-$) indicative of a molecular formula ($C_{30}H_{46}N_2O_{16}$, Δmmu 2.2). The NMR (MeOH-d_4) data for **1** (Table 1) revealed one methyl (δ_H 0.83, H_3–14) linked by HMBC correlations to methine (δ_C 70.2, C-10), oxymethylene (δ_C 62.7, C-12) and methine (δ_C 33.0, C-11), with COSY correlations extending connectivity from H-14 to the methylene H_2-9 (Fig. 6). Further NMR analysis revealed the methylene (δ_H 1.99, 2.11, H_2-9) with HMBC correlations to C-8 (δ_C 102.2), with COSY correlations extending connectivity from H-11 to the methylenes H_2-10 and H_2-9. HMBC correlations of oxymethylene (δ_H 3.74, H-7) with C-8, oxymethylene (δ_H 4.29, H-5) with C-7 (δ_C 75.8), C-6 (δ_C 76.2) and C-4 (δ_C 27.1), methylene (δ_H 1.56, 1.72, H-2) with C-3 (δ_C 35.8), C-1 (δ_C 72.1) and C-6, methine (δ_C 2.65, H-3) with carbonyl (δ_C 173.0). The observations outlined above (summarized in Fig. 6) confirmed **3** bearing norbisabolane type sesquiterpenoid skeleton with [5,6]-spiroketal as shown. Further analysis of the NMR (MeOH-*d*4) data for **1** revealed one glucosamine unit [δ_C 80.4, C-1′; 56.2, C-2′; 76.2, C-3′; 71.5, C-4′; 79.8, C-5′; 62.6, C-6′; 174.5, C-7′; 22.8, C-8′; 4.93 (d, J = 9.7 Hz, 1H, H-1′); 1.98 (s, 3H, H-8′)]. The proposed structure was further confirmed by HMBC correlations across the five sp3 carbons (C-1′, 2′, 3′, 4′ 5′), oxymethylene (H-2′) and methyl (H-8′) with carbonyl (δ_C 174.5). Furthermore, HMBC correlations of δ_H 4.98 (d, J = 9.7 Hz, 1H, H-1′) with carbonyl (δ_C 173.0) suggested that the remaining glucosamine unit was linked with C-13, which is similar to phllanthacidoid (PA) A isolated from the title plant by us [6]. However, compared with that of phllanthacidoid A (δ_C 103.1), up field shift of anomeric carbon (C-1′, δ_C 80.4) in **3** was observed. Furthermore, HRESIMS data suggested that the structure of **3** contained two nitrogen atoms. The aforementioned data suggested the existence of amide at C-13 of **3** instead of ester of PA. The proposed structure was supported unambiguously by ^{1}H−^{1}H COSY and HMBC experiments. Moreover, NMR (MeOH-*d*4) data suggested that an olefinic acid unit existed in **3**. However, it is difficult to assign it due to some signals overlapping. Further analysis of the NMR (CD_5N) data for **1** showed that two carbonyls (δ_C 171.6 and 167.3), two olefinic carbons (δ_C 136.3 and 132.7), an oxymethine (δ_C 61.1), and three methines (δ_C 34.2, 31.4 and 29.7). The HMBC and TOCSY correlations constructed the structure as (*E*)-2-(2-hydroxyethyl)hex-2-enedioic acid, supported by the ROESY correlation of δ_H 3.70 (H-3″) with δ_H 2.91 (H-7″) (Fig. 6). The unit linking with C-10 was confirmed by HMBC correlation of δ_H 2.90 ($H-10$) with carbonyl (δ_C 167.3). Thus, the planar structure of **1** was assigned as shown in Fig. 1. The relative configuration was established on the basis of coupling constant analysis and ROESY correlations (Fig. 6). The J values of H-3 (tt, J = 13.3, 8.0 Hz) and H-1 (dd, J = 11.6, 3.1) proposed the axial orientations of H-1 and H-3. The H-5 showed triplet peak at ^{1}H NMR data with J values of (dd, 4.4, 3.3 Hz), which supported equatorial orientation of H-5. The aforementioned data proposed that ring A had a chair conformation. The small coupling constants of $J_{H\text{-}11,\ H\text{-}12}$ showed

Table 2
^{13}C NMR spectroscopic data for compounds 1–4 at 150 MHz (δ in ppm).

No	1[a]	2[a]	3a[a]	3b[b]	4[a]	5[a]
1	67.2, CH	72.1, CH	72.1, CH	72.0, CH	79.2, CH	72.6, CH
2	31.7, CH_2	29.2, CH_2	30.1, CH_2	29.7, CH_2	30.6, CH_2	30.3, CH_2
3	37.9, CH	34.5, CH	35.8, CH	35.4, CH	37.6, CH	36.9, CH
4	28.1, CH_2	27.6, CH_2	27.1, CH_2	27.5, CH_2	32.6, CH_2	28.8, CH_2
5	88.7, CH	82.5, CH	82.4, CH	82.2, CH	84.0, CH	83.3, CH
6	78.2, C	76.3, C	76.2, C	76.6, C	81.7, C	77.2, C
7	76.6, CH	75.9, CH	75.8, CH	80.1, CH	209.9, C	75.9, CH
8	108.0, C	103.2, C	102.2, C	101.5, C	105.4, C	102.4, C
9	35.2, CH_2	38.5, CH_2	35.5, CH_2	35.5, CH_2	32.8, CH_2	36.2, CH_2
10	70.0, CH	68.8, CH	70.2, CH	70.2, CH	71.1, CH	71.3, CH
11	36.2, CH	35.3, CH	33.0, CH	33.1, CH	33.4, CH	34.2, CH
12	66.3, CH_2	62.4, CH_2	62.7, CH_2	61.8, CH_2	65.6, CH_2	62.9, CH_2
13	176.8, C	178.1, C	179.2, C	177.2, C	176.1, C	–
14	9.8, CH_3	13.2, CH_3	13.0, CH_3	12.6, CH_3	10.4, CH_3	13.0, CH_3
1′			80.4, CH	80.8, CH	80.4, CH	
2′			56.2, CH	56.2, CH	56.4, CH	
3′			76.2, CH	76.1, CH	76.3, CH	
4′			71.5, CH	71.9, CH	71.6, CH	
5′			79.8, CH	75.6, CH	79.9, CH	
6′			62.6, CH_2	62.3, CH_2	62.6, CH_2	
7′			174.5, C	171.0, C	174.1, C	
8′			22.8, CH_3	23.1, CH_3	22.8, CH_3	
1″			168.5, C	167.3, C	167.1, C	168.1, C
2″			31.6, CH_2	31.4, CH_2	122.3, C	123.0, C
3″			–	29.7, CH_2	132.7, CH	133.0, CH
4″			–	34.2, CH_2	116.2, CH	116.1, CH
5″			137.4, CH	136.3, CH	163.7, C	163.4, C
6″			133.1, C	132.7, C	116.2, CH	116.1, CH
7″			62.6, CH_2	61.1, CH_2	132.7, CH	133.0, CH
8″			173.0, C	171.6, C		
OCH_3	52.3	52.4				

[a] Data were recorded in CD_3OD.
[b] Data were recorded in CD_5N.

an equatorial orientation of H-11, while the large coupling constants of H-10 (ddd, J = 12.2, 5.1, 2.2 Hz) suggested axial orientation of H-10. Additionally, the ROESY correlations of H-3 and H-1 allowed the construction of the relative configuration of **3**. Furthermore, the chemical shift of C-8 (δ_C 102.2 in **3**) allowed the unambiguous assignment of the absolute configuration of **3** as 1*S*,3*S*,5*R*,6*S*, 7R,8*S*,10*S*,11*R* and named phllanthacidoid A1.

HRESI(+)MS analysis of **4** revealed a pseudomolecular ion (m/z 637.2263 [M - H]$^-$) indicative of a molecular formula ($C_{29}H_{38}N_2O_{14}$, Δmmu 1.3). The NMR (MeOH-d_4) data for **4** (Tables 1 and 2) revealed one methyl (δ_H 1.15, H_3–14) linked by HMBC correlations to methine (δ_C 33.4, C-11), oxymethylene (δ_C 65.6, C-12) and oxymethine (δ_C 71.1, C-10), with COSY correlations extending connectivity from H-14 to the methylene H_2–9 (Fig. 2). Further NMR analysis revealed the methylene (δ_H 1.99, 2.23, H_2–9) with HMBC correlations to C-8 (δ_C 105.4) and C-7 (δ_C 209.9), with HSQC-TOCSY correlations of H-1-H_2–2-H-3-H_2–4-H-5 and H_3–14-H-11-H-10-H_2-9 (Fig. 7). The observations outlined above (summarized in Fig. 2) confirmed that **4** was C-7 keto norbisabolane

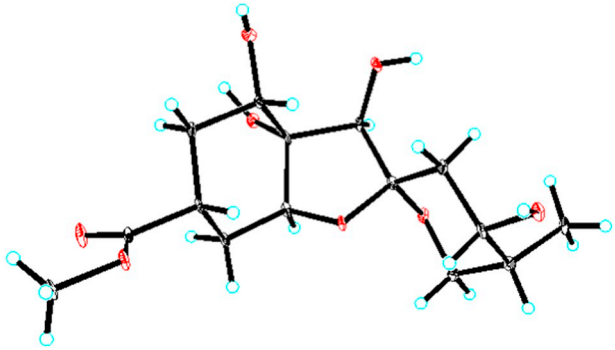

Fig. 3. Single crystal X-ray structure of **1**.

type sesquiterpenoid as shown. Further analysis of the NMR (MeOH-*d*4) data for **4** suggested one glucosamine unit [δ_C 80.4, C-1′; 56.4, C-2′; 76.3, C-3′; 71.6, C-4′; 79.9, C-5′; 62.6, C-6′; 174.1, C-7′; 22.8, C-8′; 4.98 (d, J = 9.7 Hz, 1H, H-1′); 1.98 (s, 3H, H-8′)] (Table 2). The glucosamine unit linked with C-13 by the amide bond, which is same as **3**. The proposal was supported unambiguously by $^1H-^1H$ COSY, HSQC-TOCSY and HMBC correlations (Fig. 7). Further analysis of the NMR (MeOH-*d*4) data for **1** showed the presence of a benzoyl unit [δ_H 7.88 (2H, brd, J = 8.7 Hz) and 6.83 (2H, brd, J = 8.7 Hz), δ_C 167.13, 163.7, 132.74, 122.3 and 116.23]. The HMBC correlations of carbonyl (δ_C 167.1) with H-10 (δ_H 5.45) assigned the benzoyl to C-10. Thus, the planar structure of **4** was assigned as showed in Fig. 1. The relative configuration was established on the basis of coupling constant analysis and ROESY correlations. The J values of H-3 (dd, 12.3, 3.0 Hz) indicated the axial orientations of H-3. The $J_{H\text{-}4,\ H\text{-}5}$ (dd, 3.0, 3.3 Hz) determined the equatorial orientation of H-5. The aforementioned data suggested that ring A had a boat conformation. The small coupling constants of $J_{H\text{-}11,\ H\text{-}12}$ showed an equatorial orientation of H-11, while the large coupling constants of H-10 (dt, J = 10.1 and 5.1 Hz) suggested axial orientation of H-10. Additionally, the ROESY correlations of H-3 and H-1 allowed the construction of the relative configuration of **4**. Furthermore, the chemical shift of C-8 (δ_C 105.4 in **4**) and C-14 (δ_C 10.4 in **4**) allowed the unambiguous assignment of the absolute configuration of **4** as 1*R*,3*S*,5*R*,6*R*,8*R*,10*S*,11*R* and was named phllanthacidoid N1.

The anti-HBV activities of compounds **1–3** were measured by the inhibition of HBsAg and HBeAg by ELISA with HBV infected HepG2.2.2.15 cells assay. The results indicated that compound **2** showed effect to HBsAg with an IC_{50} value 11.2 ± 0.01 μM, while no effect was found HBeAg at 100 μM. However, compound **3** inhibited HBeAg secretion with an IC_{50} value of 57.1 ± 0.02 μM, and no influence was shown on HBsAg at 100 μM. The results enriched the diversity of anti-HBV norbisabolane sesquiterpenes. It is noted that the esterification of C-14 should be the key pharmacophore unit of norbisabolane sesquiterpenes from *Phyllanthus spp*. The aforementioned data provide insight for further structural modification.

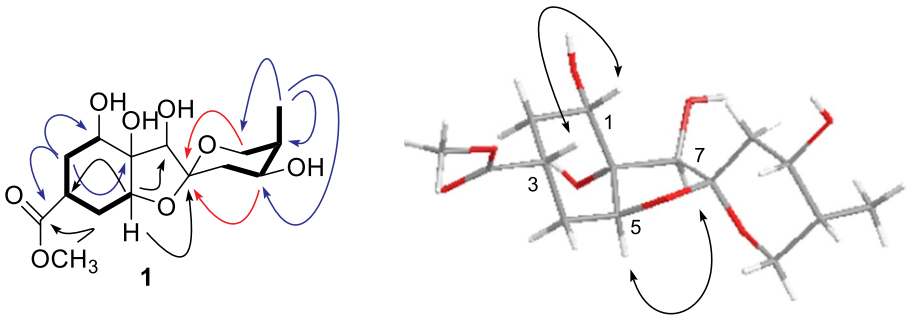

Fig. 2. Key $^1H-^1H$-COSY (—), HMBC (→) and ROESY (↔) correlations of **1**.

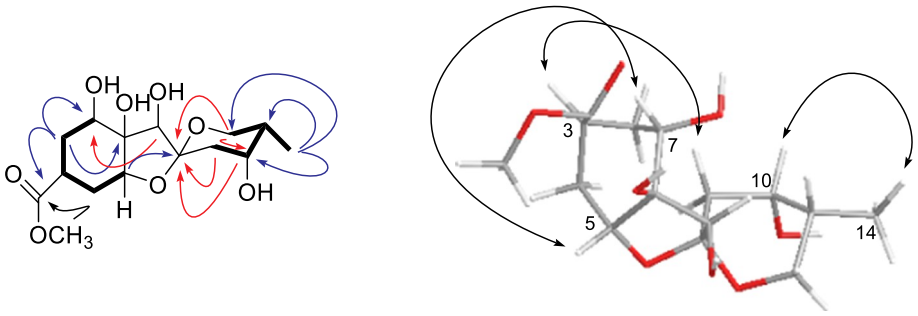

Fig. 4. Key ^{1}H–^{1}H-COSY (—), HMBC (→) and ROESY (↔) correlations of **2**.

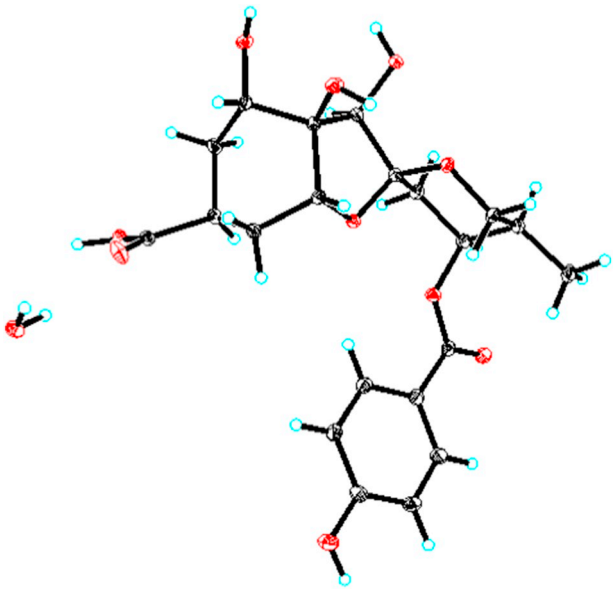

Fig. 5. Single crystal X-ray structure of **5**.

3. Experimental section

3.1. General experimental procedures

Optical rotations were recorded on a Jasco P-1020 digital polarimeter. UV spectra were measured on a Shimadzu UV2401PC spectrometer. CD spectra were measured on an Applied Photophysics Chirascan instrument. 1D- and 2D-NMR spectra were recorded on Bruker AV-600 and AV-800 spectrometers operating at 600 and 800 MHz, respectively, for ^{1}H NMR spectra, and 150 and 200 MHz, respectively, for ^{13}C NMR spectra. Coupling constants are expressed in Hertz and chemical shifts are given on a ppm scale with reference to the solvent signals. X-ray diffraction was realized on a Bruker SMART APEX CCD crystallography system. ESIMS were performed on Waters Xevo TQ-S. HRESIMS were recorded on an API Qstar Pulsa LC/TOF spectrometer. Semi-preparative HPLC were performed on a Waters 1525. Column chromatography (CC) was performed with silica gel (200–300 mesh, Qingdao Hailang Chemical Co., Ltd.), Lichroprep RP-18 (40–63 μm, Merck), Sephadex LH-20 (25–100 μm, Pharmacia Fine Chemical Co., Ltd.) and MCI-gel CHP20P (75–150 μm, Mitsubishi Chemical Co., Ltd.). TLC was performed on precoated TLC plates (0.2–0.25 mm thickness, GF254 Silica gel, Qingdao Hailang Chemical Co., Ltd.) with compounds visualized by spraying the dried plates with 10% aqueous H_2SO_4 followed by heating until the plate was dry.

3.2. Plant material

The stems of *Phyllanthus acidus* was collected from Xishuangbanna County, Yunnan Province, People's Republic of China, in Spe. 15th, 2016. Voucher specimens (KMUST_00001) were deposited at the Center for Pharmaceutical Sciences, Faculty of Life Science and Technology, Kunming University of Science and Technology, Chenggong Campus, Kunming, and were identified by Prof. Chong-Ren Yang at State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

3.3. Extraction and isolation

The air-dried stem of *P. acidus* (10 kg) was extracted in temperature with methanol solution for three times to give the extract (421 g). The methanol extract was resuspended in MeOH (500 mL) and loaded onto a polyamide gel column (520 g bed volume, preequilibrated with MeOH) to remove pigments and polyphenols. The column was eluted with MeOH (10.5 L), and the eluent evaporated to obtain a crude extract. The crude extract was subjected to passage over a silica gel column (200–300 mesh), eluting with ($CHCl_3$-CH_3OH-H_2O, 9:1:0–7:3:0.5), to afford five major fractions. Fraction 2 (70 g) was chromatographed on Sephadex LH20 (CH_3OH 0–100%) to afford five fractions. The first two fractions (40.3 g) were combined and chromatographed on over MCI gel CHP-20P (MeOH/H_2O, 20% - 100%) to give seven sub-fractions (Fr.A – Fr·I). Fr.A was subjected to

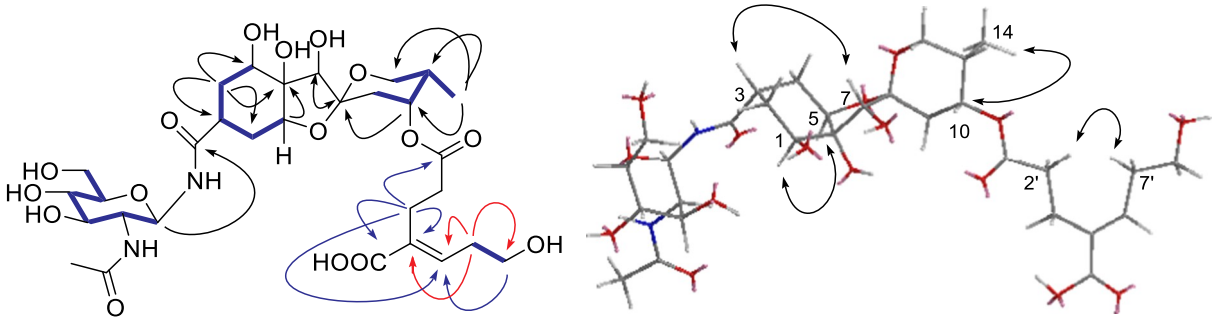

Fig. 6. Key HSQC-TOCSY (▬), HMBC (→) and ROESY (↔) correlations of **3**.

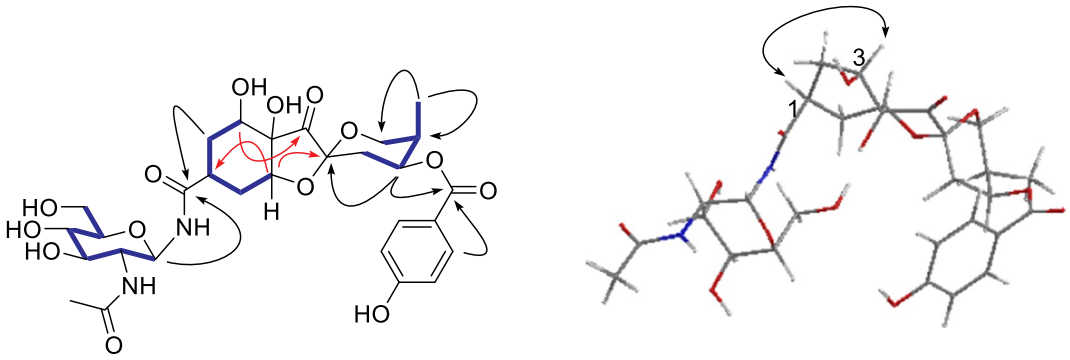

Fig. 7. Key HSQC-TOCSY (▬), HMBC (→) and ROESY (↔) correlations of **4**.

RP-8 (CH_3OH 30% - 80%), and preparative-HPLC with an isocratic flow of CH_3CN/H_2O (10 mL/min, CH_3CN/H_2O 18:82, COSMOSIL Cholester, 21.1 × 250 mm) to afford phyllanthacidoid A (10.2 g, Rt = 21 min), phyllanthacidoid D (488 mg, Rt = 18 min). Fr·B was subjected to RP-8 (CH_3OH 30% - 80%), and preparative-HPLC with an isocratic flow of CH_3CN/H_2O (6 mL/min, CH_3CN/H_2O 19:81, SUNFIRE PraP C18, 10 × 250 mm) to afford phyllanthacidoid B (950 mg, Rt = 16 min) and phyllanthacidoid C (650 mg, Rt = 18 min). Fraction 3 (111.5 g) pass over a Diaion HP20SS column, eluting with CH_3OH/H_2O (0–100%), to afford three major fractions. First fraction (41.6 g) were chromatographed on MCI gel CHP-20P (MeOH/H_2O, 0% - 100%) to give ten sub-fractions (Fr.A – Fr.J). Fr.D was subjected to Carboxyl silica gel (CH_3OH 10% - 100%) to afford four fractions (Fr.D1- Fr.D4), Fr.A1was passage over a silica gel column (500–800 mesh), eluting with (P-EA, 1:1), to afford **2** (5 mg), Fr.D2 was subjected to RP-18 (CH_3OH 30% - 80%), to give 4 fractions (Fr.D2.1- Fr.D2.4), Fr.D2.2 was chromatographed on Sephadex LH20 (CH_3OH 0–100%) to afford **1** (10 mg). Fr.G was subjected to silica gel-COOH (CH_3OH 10% - 100%) to afford 2 fractions (Fr. G1- Fr. G2). Fr. G1 was subjected to RP-18 (CH_3OH 30% - 80%) to give 4 fractions (Fr.G1.1- Fr.G1.4), Fr.G1.3 was chromatographed on MCI gel CHP-20P (MeOH/H_2O, 20% - 100%) to give 4 sub-fractions (Fr.G1.3.1 – Fr.G1.3.4), Fr.G1.3.3 was passage over a silica gel column (200–300 mesh) eluting with ($CHCl_3$-CH_3OH-H_2O, 9:1:0) to give Fr.G1.3.3.1- Fr.G1.3.3.8. Fr.G1.3.3.7 was chromatographed on preparative-HPLC with an isocratic flow of CH_3CN/H_2O (5 mL/min, CH_3CN/H_2O 15:85, COSMOSIL Cholester, 10 × 150 mm) to afford **4** (7 mg, Rt = 8.6 min). Fr.G1.3.4 was passage over a silica gel column (200–300 mesh) eluting with ($CHCl_3$-CH_3OH-H_2O, 9:1:0) to give Fr.G1.3.4.1- Fr.G1.3.4.8. Fr.G1.3.4.6 was chromatographed on preparative-HPLC with an isocratic flow of CH_3CN/H_2O (5 mL/min, CH_3CN/H_2O 15:85, COSMOSIL Cholester, 10 × 150 mm) to afford phyllanthacidoid I (10 mg, Rt = 12 min) and phyllanthacidoid R (4 mg, Rt = 14 min). Fr.G1.3.4.7 was chromatographed on preparative-HPLC with an isocratic flow of CH_3CN/H_2O (5 mL/min, CH_3CN/H_2O 15:85, COSMOSIL Cholester, 10 × 150 mm) to afford phyllanthacidoid J (10 mg, Rt = 14 min) and phyllanthacidoid Q (12 mg, Rt = 17 min). Fr.G1.3.4.8 was chromatographed on preparative-HPLC with an isocratic flow of CH_3CN/H_2O (5 mL/min, CH_3CN/H_2O 15:85, COSMOSIL Cholester, 10 × 150 mm) to afford phyllanthacidoid G (5 mg, Rt = 15 min). Fr·H was passage over a silica gel column (200–300 mesh) eluting with ($CHCl_3$-CH_3OH-H_2O, 9:1:0) to give Fr·H1-Fr. H9. Fr·H7 was subjected to RP-18 (CH_3OH 30% - 80%) to give 2 fractions (Fr.H7.1-Fr.H7.2). Fr.H7.2 was chromatographed on preparative-HPLC with an isocratic flow of CH_3CN/H_2O (5 mL/min, CH_3CN/H_2O 15:85, COSMOSILπNAP, 10 × 150 mm) to afford phyllanthacidoid L (11 mg, Rt = 20 min). Fr·H8 was subjected to RP-18 (CH_3OH 30% - 80%) to give 3 fractions (Fr.H8.1- Fr.H8.3). Fr.H8.3 was chromatographed on preparative-HPLC with an isocratic flow of CH_3CN/H_2O (5 mL/min, CH_3CN/H_2O 18:82, COSMOSILπNAP, 10 × 150 mm) to afford phyllanthacidoid F (4 mg, Rt = 5 min). Fr·I was passage over a silica gel column (200–300 mesh) eluting with ($CHCl_3$-CH_3OH-H_2O, 9:1:0) to give Fr·I1-Fr. I6. Fr·I4 was subjected to RP-18 (CH_3OH 30% - 80%) to give 3 fractions (Fr.I4.1- Fr.I4.3). Fr.I4.3 was chromatographed on preparative-HPLC with an isocratic flow of CH_3CN/H_2O (5 mL/min, CH_3CN/H_2O 20:80, COSMOSILπNAP, 10 × 150 mm) to afford **3** (2 mg, Rt = 19 min).

3.4. Basic hydrolysis of phllanthacidoid A

Phllanthacidoid A (50 mg) was dissolved in 10% K_2CO_3 solution (2 mL) and stirred at 60 °C oil bath for about two hours until the starting material was not observed by TLC. The reaction solution was acidified by 1 M hydrochloric acid (15 mL) at room temperature. The solution was diluted with saturated salt water (4.5 mL) and extracted by EtOAc (20 mL, three times). The organic layer was dried under reduced pressure. The EtOAc extract was resuspended in MeOH (0.5 mL) and chromatographed on preparative-HPLC with an isocratic flow of MeOH/H_2O (10 mL/min, MeOH/H_2O 5:95 to 100:0 in 15 min, COSMOSIL Cholester, 20 × 250 mm) to afford **5** (25.6 mg, Rt = 7.1 min).

Phyacidusin A (**1**): needle white crystal; $[\alpha]_D^{25}$ − 93.5 (*c* 0.32, MeOH); UV (MeOH) λ_{max} (log ε) 203 (2.90), 217 (2.74), 270 (1.93) nm;

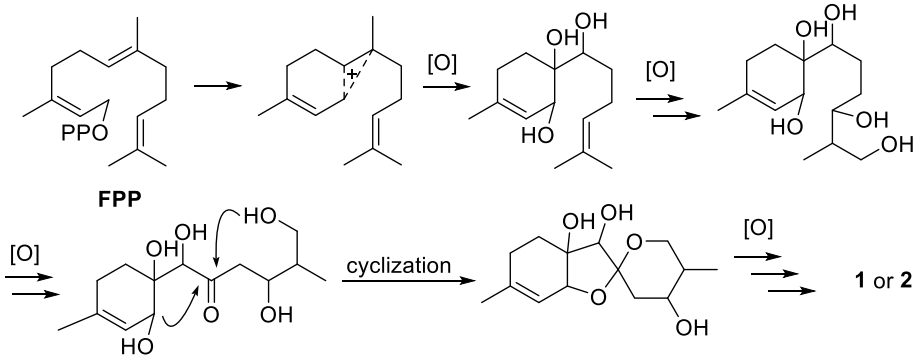

Fig. 8. The possible biosynthesis pathway for **1** and **2**.

CD (in MeOH, λ_{max}[nm], Φ[mdeg]) 202 (1.05), 230 (0.30), 280 (0.05); IR (KBr) υ_{max} 3537, 3429, 3000, 2908, 1724, 1018 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data, see Table 1 and Table 2; ESIMS *m/z* 355 $[M + Na]^+$, HRESIMS *m/z* 355.1365 $[M + Na]^+$ (calcd. For $C_{15}H_{24}O_8Na$, 355.1365).

Phyacidusin B (**2**): Off white amorphous powder; $[\alpha]_D^{25}$ − 28.1 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 195 (1.31), 254 (2.72) nm; IR (KBr) υ_{max} 3441, 2957, 2854, 2074, 1715, 1047 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data, see Table 1 and Table 2; ESIMS *m/z* 355 $[M + Na]^+$, HRESIMS *m/z* 355.1376 $[M + Na]^+$ (calcd for$C_{15}H_{24}O_8Na$, 355.1376).

Phllanthacidoid A1 (**3**) Off white amorphous powder; $[\alpha]_D^{25}$ + 24.2 (*c* 0.23, MeOH); UV (MeOH) λ_{max} (log ε) 203 (3.99), 271 (1.31) nm; CD (in MeOH λ_{max}[nm], Φ[mdeg]) 198 (15.1), 218 (−5.10), 242 (1.5); IR (KBr) υ_{max} 3424, 2932, 2883, 1649, 1542, 1051 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data, see Table 1 and Table 2; ESIMS *m/z* 689 $[M - H]^-$, HRESIMS *m/s* 689.2797 $[M - H]^-$ (calcd for $C_{30}H_{46}N_2O_{16}$, 689.2797).

Phllanthacidoid N1 (**4**): Off white amorphous powder; $[\alpha]_D^{25}$ − 53.4 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.20), 254 (3.83) nm; CD (in MeOH λ_{max}[nm], Φ[mdeg]) 198 (5.2), 218 (−2.0), 252 (1.1), 270 (2.5); IR (KBr) υ_{max} 3432, 2932, 2852, 1645, 1609, 1013 cm^{-1}; ^1H NMR (CD_3OD, 800 MHz) and ^{13}C NMR (CD_3OD, 200 MHz) data, see Table 1 and Table 2; ESIMS m/s 637 $[M - H]^-$, HRESIMS m/s 637.2263 $[M - H]^-$ (calcd for $C_{29}H_{28}N_2O_{14}$, 637.2263).

Aglucon of phllanthacidoid A (**5**): White needle crystal; negative ESI-MS: *m/z* 477 $[M + K]^+$ for $C_{21}H_{26}O_{10}$.

3.5. Virus and cell culture

HepG2.2.2.15 cells used for HBV inhibition were cultured in MEM (Sigma, St. Louis, USA) supplemented with 10% fetal bovine serum (FBS), 0.03% glutamine, 100 unit/ml penicillin, 100 unit/ml streptomycin, and 100 unit/ml kanamycin. The viral titer was tested by the cytopathic end-point assay (Burleson et al., 1992) and expressed as 50% tissue culture infective dose (TCID50). The inhibition of HBsAg and HBeAG was detected by ELISA.

3.6. Cytotoxicity assay

HepG2.2.2.15 cells were plated in 96-well plates in 100 μL medium, in which the test samples were added at varied concentrations. After 48 h incubation, MTT [[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] solution [0.5 mg/mL in phosphate buffered saline (PBS)] was added (20 μL/well), and the incubation was continued for another 4 h to give a formazan product. In each well, 100 μL 20% SDS were added after 100 μL medium were removed, and it was then incubated overnight for the formazan product to dissolve completely. The absorbance of the solution was measured at 570 nm using a Bio-Rad 680. Compound concentrations reducing the viability of MDCK cells by 50% (CC_{50}) were calculated by regression analysis of the dose-response curves.

3.7. Anti-virus assay

HepG2.2.2.15 cells were plated in 96-well plates to form monolayers. 30 TCID50A virus suspensions were added to each well and allowed adsorption to occur for 2 h. Then the viruses were removed and a serial two-fold dilution of the tested compounds at dose below CC_{50} (50 ul per well) each with equal volume were added, and followed by incubation for 72 h. The virus-induced CPE of the tests was expressed as a percentage in comparison with the parallel virus control and cell control. IC_{50} values (the concentration of test compounds required to reduce 50% of CPE) were calculated, and the therapeutic index (TI) was calculated from the ratio CC_{50}/IC_{50}. Lamivudine and Bicyclol were tested as the positive control for anti HBV.

Conflict of interest

We declare no conflict of interest for this study.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2019.04.006.

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