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Two new tirucallane triterpenoids from *Aphanamixis grandifolia*

Xiao-Ying WANG,[a,b] Gui-Hua TANG,[a] Chun-Mao YUAN,[a] Yu ZHANG,[a] Li HOU,[a,b] Qing ZHAO,[b,*] Xiao-Jiang HAO,[a] and Hong-Ping HE[a,*]

[a]State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China

[b]Faculty of Pharmacy, Yunnan University of TCM, Kunming 650500, China

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Abstract: Two new tirucallane triterpenoids, 2α-ethoxy-2,3-secotirucalla-2,29-epoxy-7-ene-23-oxo-3-oic acid (**1**) and (23*E*)-2α-hydroxytirucalla-7,23,25-triene-3-one (**2**), along with the known 2,3-secotirucalla-2,3;2,29-diepoxy-7-ene-3,23-dione (**3**), were isolated from the leaves and twigs of *Aphanamixis grandifolia.* Their structures were elucidated by extensive NMR and MS data, and compound **3** was further confirmed by X-ray crystal diffraction analysis. Antimicrobial activities and insecticidal activities of these three compounds were also evaluated. Compound **1** showed good antimicrobial activity against *Staphylococcus aureus* with the MIC value of 1.56 μg/mL, while compounds **1** and **2** showed insecticidal activity at 100 ppm, with the corrected mortality 79.1% and 60.6%, respectively.

Keywords: Aphanamixis grandifolia, Meliaceae, 2,3-*seco*-tirucallane triterpenoid, bioassays

Introduction

The Meliaceae family is rich in highly complicated structural secondary metabolites with significant bioactivities, which have attracted overwhelming attention in the field of natural products.[1] The genus *Aphanamixis* (Meliaceae), consisting of 25 species, is distributed extensively in the tropical regions of Asia such as Southern China, India, Malaysia, and Indonesia.[2] The roots and leaves of *Aphanamixis grandifolia* Blume have been applied in primitive medicine for the treatment of cold and rheumatic joints pain due to arthritis as well as numbness of limbs owing to wind-cold-dampness.

In recent years, a variety of compounds have been obtained from the genus *Aphanamixis*, such as triterpenes,[3] limonoids,[4] lignans,[5] and alkaloids.[6] As our ongoing research for novel structural and significant bioactive natural products from the Meliaceae family,[7] three triterpenoids (see Fig. 1) including two 2,3-*seco*-tirucallane triterpenoids, 2α-ethoxy-2,3-secotirucalla-2,29-epoxy-7-ene-23-oxo-3-oic acid (**1**) and 2,3-secotirucalla-2,3;2,29-diepoxy-7-ene-3,23-dione (**3**),[8] together with a tirucallane triterpenoid, (23*E*)-2α-hydroxytirucalla-7,23,25-triene-3-one (**2**), were obtained from the leaves and twigs of *A. grandifolia*. Herein, we described the isolation, structural elucidation, antimicrobial activities and the insecticidal bioassays of these three compounds.

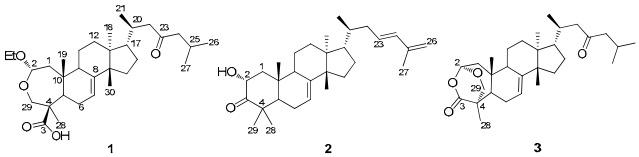

Figure 1. The chemical structures of **1**–**3**

Results and Discussion

Compound **1** was obtained as a colorless oil and its molecular formula was determined to be $C_{32}H_{52}O_5$ with seven degrees of unsaturation due to the positive HREIMS at *m/z* 516.3797 (calcd for 516.3815). The IR spectrum exhibited the existence of hydroxyl (3449 cm^{-1}) and carbonyl (1755 and 1710 cm^{-1}) groups. According to the ^{13}C NMR and DEPT spectra (Table 1), 32 carbon resonances were classified into eight methyls, ten methylenes including two oxygenated carbons (δ_C 64.7 and 63.2), seven methines including an acetal carbon (δ_C 99.9), and seven quaternary carbons including a carbonyl (δ_C 211.2) and an olefinic carbon (δ_C 144.1). Comparison of the NMR data of **1** with those of aphanamgrandin C[8] demonstrated that **1** had an additional ethoxy group [δ_C 63.2 (CH_2, 2-OCH_2CH$_3$) and 15.1 (CH_3, 2-OCH$_2$$CH_3$), δ_H 3.79, 3.47 (each 1H, m, 2-OCH_2CH$_3$) and 1.21 (3H, t, J = 7.0 Hz, 2-OCH$_2$CH_3)], which was located at C-2 through analysis of the HMBC spectrum. As shown in Figure 2. 1a, the seven-membered ring A with a carboxyl (δ_C 175.3, C-3) at C-4 (δ_C 50.0) was established by the HMBC

*To whom correspondence should be addressed. E-mail: hehongping@mail.kib.ac.cn (H.P. He); tempzhao@126.com (Q. Zhao)

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for 1 and 2 in $CDCl_3$ (δ in ppm)

	1		**2**	
no.	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1α	41.2, CH_2	1.71, m	48.3, CH_2	1.20, br. t (13.1)
1β		2.21, m		2.32, dd (13.1, 6.3)
2β	99.9, CH	4.89, dd (9.4, 4.4)	69.2, CH	4.53, dd (13.1, 6.3)
3	175.3, C		216.3, C	
4	50.0, C		47.3, C	
5	54.7, CH	1.56, m	53.2, CH	1.66, m
6α	26.9, CH_2	1.96, m	24.2, CH_2	2.04, m
6β		2.31, m		
7	118.8, CH	5.31, m	117.5, CH	5.24, m
8	144.1, C		145.8, C	
9	47.1, CH	2.30, m	48.7, CH	2.23, m
10	38.1, C		35.6, C	
11	19.6, CH_2	1.48, m; 1.72, m	18.3, CH_2	1.51, m
12α	33.9, CH_2	1.64, m	33.4, CH_2	1.57, m
12β		1.81, m		1.73, m
13	43.2, C		43.6, C	
14	51.6, C		51.2, C	
15	34.3, CH_2	1.49, m	34.1, CH_2	1.42, m
16α	28.4, CH_2	1.26, m	28.2, CH_2	1.24, s
16β		1.91, m		1.91, m
17	52.8, CH	1.48, m	52.8, CH	1.41, m
18	22.3, CH_3	0.85, s	22.0, CH_3	0.73, s
19	11.8, CH_3	0.90, s	13.8, CH_3	1.02, s
20	32.7, CH	1.99, m	36.8, CH	1.41, m
21	19.4, CH_3	0.86, d (6.4)	18.5, CH_3	0.80, m
22a	50.4, CH_2	2.41, dd (16.0, 1.5)	39.5, CH_2	2.20, m
22b		2.13, m		1.73, m
23	211.2, C		129.4, CH	5.56, m
24	52.5, CH_2	2.25, d (7.0)	134.1, CH	6.05, d (15.5)
25	24.5, CH	2.12, m	142.2, C	
26	22.5, CH_3	0.90, d (4.3)	114.0, CH_2	4.78, br. s
27	22.6, CH_3	0.91, d (4.3)	18.7, CH_3	1.77, s
28	23.4, CH_3	1.17, s	24.3, CH_3	1.06, s
29	64.7, CH_2	3.58, d (13.6); 4.12, d (13.6)	21.4, CH_3	1.09, s
30	28.0, CH_3	1.00, s	27.4, CH_3	0.93, s
2-OEt	63.2, CH_2	3.47, m; 3.79, m		
	15.1, CH_3	1.21, t (7.0)		

correlations of Me-19 (δ_H 0.90, s)/C-1 (δ_C 41.2), C-9 (δ_C 47.1), and C-10 (δ_C 38.1), Me-28/C-3, C-4, C-5, and C-29 (δ_C 64.7), H-1 (δ_H 2.21, m)/C-5, and H-2 (δ_H 4.89, dd, J = 9.4, 4.4 H_Z)/C-29, together with ^{1}H-^{1}H COSY correlation of H-1/H-2. The structure of rings B–D and the side chain moiety with a carbonyl (δ_C 211.2) at C-23 were identical to aphanamgrandin C[8]. Then, the planar structure of compound **1** was established.

The relative configuration of compound **1** was deduced from the ROESY correlations. As shown in Figure 2. 1b, observed ROESY correlations of Me-19/H-2, Me-30/H-17, and H-17/Me-21 indicated that they were cofacial and were arbitrarily assigned as β-configuration. Whereas, the ROESY cross-peaks of Me-28/H-5, H-5/H-9, and H-9/Me-18 revealed their α-orientation. Moreover, the key correlation in the NOESY spectrum between H-2/Me-19 suggested 2-OCH_2CH_3 were α-orientated (see Fig 2. 1b). Subsequently, the structure of **1** was determined as 2α-ethoxy-2,3-secotirucalla-2,29-epoxy-7-ene-23-oxo-3-oic acid, namely, 2α-ethoxy-aphanamgrandin C.

Compound **2**, a colorless oil, possessed a molecular formula of $C_{30}H_{46}O_2$ corresponding to its positive HREIMS at *m/z* 438.3501 (calcd for 438.3498). The similarity of chemical shifts of **2** and 3β,25-dihydroxytirucalla-7,23-diene[9] indicated that both compounds shared the same rings system with the differences at ring A and the side-chain. The key HMBC correlations of Me-28 (δ_H 1.06, s) and Me-29 (δ_H 1.09, s) to a carbonyl (δ_C 216.3) confirmed the carbonyl at C-3. The methine [δ_H 4.53, dd (13.1, 6.3), δ_C 69.2] was assigned at C-2 by the ^{1}H-^{1}H COSY cross-peak of δ_H 4.53/H-1. The C-17 side chain with conjugated Δ^{23} and Δ^{25} double bonds was established by the HMBC correlations of Me-27/C-24, C-25, and C-26, H-24/C-22, C-25, C-26, and C-27, and Me-21/C-17, C-20, and C-22. The $\Delta^{23(24)}$ double bond was assigned as *E*-configuration by the coupling constant of H-24 (δ_H 6.05, d, J = 15.5 Hz) together with the ROESY correlations of H-23/Me-27 and H-24/H_2-26.

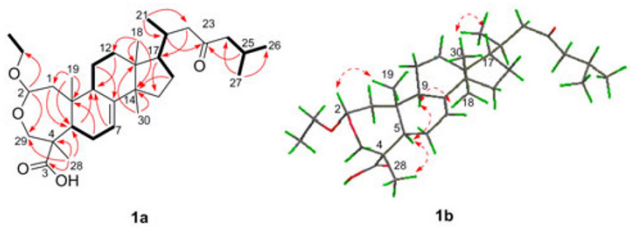

Figure 2. (1a) Selected ^{1}H-^{1}H COSY (▬) and HMBC (H → C); (1b) key ROESY (H ⇠⇢ H) correlations of compound **1**

As shown in Figure 3. 2b, the relative stereochemistry at the chiral centers of carbons C-5, C-9, C-10, C-13, C-14, C-17, and C-20 in **2** was determined as the same with 3β,25-dihydroxytirucalla-7,23-diene[9] by the cross-peaks observed in its ROESY spectrum. The ROESY correlations of H-2/Me-19, Me-30/Me-19, and H-17/Me-21 indicated they were cofacial

and designated as β-configuration. Naturally, the OH group at C-2 was α-configuration because of the β-configuration of H-2. Meanwhile, the ROESY cross-peaks of H-5/H-9 and H-9/Me-18 indicated that these protons were α-orientation. Thus, compound **2** was determined as (23*E*)-2α-hydroxytirucalla-7,23,25-triene-3-one.

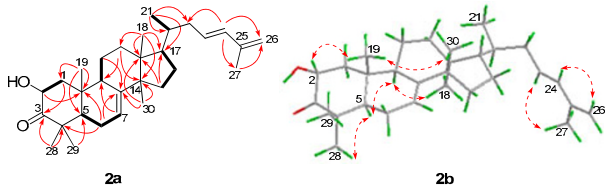

Figure 3. (2a) Selected ^{1}H-^{1}H COSY (▬) and HMBC (H → C); (2b) key ROESY (H ⇠⇢ H) correlations of compound **2**

The structure of compound **3** was determined by analysis of its 2D NMR spectra which was identical to 2,3-secotirucalla-2,3;2,29-diepoxy-7-ene-3,23-dione.[8] As shown in Figure 4, H-2, H-17, Me-19, Me-21, and Me-30 were β-oriented, and H-5, H-9, Me-18, and Me-28 were assigned as α-orientation. However, the structure of compound **3** should be the form in Figure 1 according to its X-ray crystal diffraction analysis in Figure 4 and in the literature.[8]

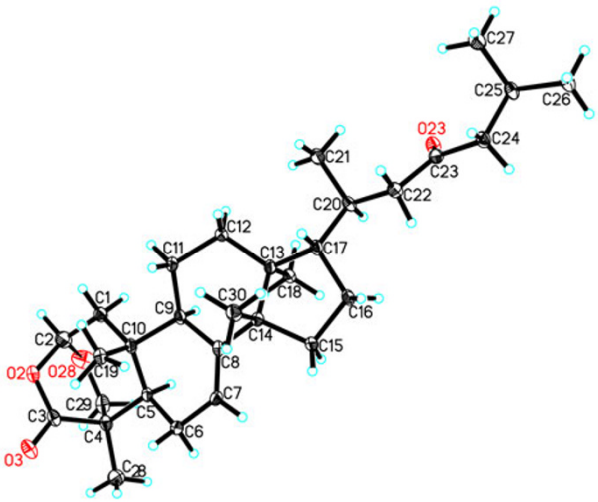

Figure 4. Single-crystal X-ray structure of compound **3**

Compounds **1**–**3** were tested for the antimicrobial activities using a 2-fold dilution method against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, MRSA (methicillin-resistant *S. aureus*) 92#, and MRSA 98# with vancomycin hydrochloride (Eli Lilly Japan K. K, Seishin Laboratories, purity ≥ 98%) as positive control (with the MIC value of 0.78 μg/mL to the test strains). The results revealed that compound **1** exhibited good activity against *S. aureus* (MIC value: 1.56 μg/mL) and weak activities against *P. aeruginosa*, MRSA 92#, and MRSA 98# (MIC value: 25 μg/mL for each). Additionally, compound **2** showed weak antimicrobial activities against all the four bacteria with the MIC values of 25 μg/mL, while compound **3** showed weak antimicrobial activities against *S. aureus* and MRSA 98# with the MIC values of 25 μg/mL, and against *P. aeruginosa* and MRSA 92# with the MIC values of 50 μg/mL.

Furthermore, compound **1**–**3** were assayed in vitro for insecticidal activity against *Artemia salina* L. (brine shrimp) with the Microwell method.[10] At the concentration of 100 mg/L, compounds **1** and **2** displayed higher corrected mortality 79.1% and 60.6%, respectively (Table 2), while compound **3** had the lowest corrected mortality 26.0%.

Table 2. Corrected mortality[a,b] of compounds 1–3 against brine shrimp at 100 mg/L

compound	corrected mortality (%)
1	79.1
2	60.6
3	26.0
toosendanin	100.00

[a]Corrected mortality = $(M_t - M_c)/(1 - M_c) \times 100\%$. [b]The mean mortality of control group was 4.73%. M_t: mortality of treatment group, M_c: mortality of control group.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO P-1020 digital polarimeter. UV spectra were determined on a Shimadzu UV-2401 PC spectro-photometer. IR spectra were detected on a Bruker Tensor-27 infrared spectrometer with a KBr disk. CD spectra were obtained on a JASCO J-810 spectro-photometer. Brucker HCT/E squire and Waters Autospec Premier P776 spectrum were respectively used for measuring ESIMS and HREIMS spectra. 1D NMR and 2D NMR spectra were recorded on a Bruker AM-400 and Bruker DRX-500 spectrometer, using TMS as an internal standard. Column chromatography was performed on silica gel (200−300 and 300−400 mesh, Qingdao Marine Chemical Inc.), MCI gel CHP 20P (75−150 μm, Mitsubishi Chemical Corporation, Tokyo), Sephadex LH-20 (40–70 μm, Amersham Pharmacia Biotech AB, Uppsala, Sweden), and Chromatorex RP-C_{18} gel (20–45 μm, Merck, Darmstadt, Germany). Heating thin-layer chromatography sprayed with 10% H_2SO_4 visualized spots, which were monitored by TLC in the form of precoated plates.

Plant Materal. The leaves and twigs of *A. grandifolia* were collected from Xishuangbanna, Yunnan Province, China in August, 2011, which were identified by Mr. Yu Chen, Kunming Institute of Botany, Chinese Academy of Sciences (CAS). The voucher specimen (No. H20110802) was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, CAS.

Extraction and Isolation. The leaves and twigs of *A. grandifolia* (9.0 kg) were percolated with 95% EtOH three times. The EtOH distillate was concentrated in vacuum to obtain a crude residue, which was partitioned with PE (petroleum ether), EtOAc, and *n*-BuOH, successively. The PE fraction (133.6 g) was submitted to column chromatography (CC) over silica gel (100–200 mesh) using PE/acetone (100:1 → 1:1) to give five fractions 1–5. Fr. 2 was separated over a whole series of CC on silica gel, MCI, RP-C_{18} gel, and Sephadex LH-20 to yield compounds **1** (6.0 mg), **2** (11.6 mg), and **3** (24.5 mg).

2α-Ethoxy-2,3-secotirucalla-2,29-epoxy-7-ene-23-oxo-3-oic acid (1): colorless oil; $[\alpha]_D^{26}$ – 7.0 (*c* 0.16, MeOH); UV (MeOH) λ_{max} (log ε) 203 (2.75) nm; IR (KBr) ν_{max} 3449, 2957, 2927, 1755, 1710 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; ESIMS *m/z* 539 $[M + Na]^+$, 1055 $[2M + Na]^+$; HREIMS *m/z* 516.3797 (calcd for $C_{32}H_{52}O_5$ $[M]^+$, 516.3815).

(23*E*)-2α-Hydroxytirucalla-7,23,25-triene-3-one (2): colorless oil; $[\alpha]_D^{28}$ – 60.2 (*c* 0.06, $CHCl_3$–MeOH); UV (MeOH) λ_{max} (log ε) 205 (3.10) nm; CD (MeOH) λ ($\Delta\varepsilon$) 285 (– 0.89), 254 (– 0.87), 208 (– 10.46) nm; IR (KBr) ν_{max} 3447, 2970, 2951, 2881, 1709, 1463, 755 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; ESIMS *m/z* 899 $[2M + Na]^+$; HREIMS *m/z* 438.3501 (calcd for $C_{30}H_{46}O_2$ $[M]^+$, 438.3498).

2,3-Secotirucalla-2,3;2,29-diepoxy-7-ene-3,23-dione (3)[11]**:** colorless crystal; $[\alpha]_D^{23}$ – 44.8 (*c* 0.21, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 273 (2.48), 239 (2.43) nm; CD (MeOH) λ ($\Delta\varepsilon$) 290 (+ 1.57), 212 (– 11.85) nm; IR (KBr) ν_{max} 3432, 2957, 2925, 2870, 1754, 1712 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; ESIMS *m/z* 493 $[M + Na]^+$, 963 $[2M + Na]^+$; HREIMS *m/z* 470.3388 (calcd for $C_{30}H_{46}O_4$ $[M]^+$, 470.3396).

Antimicrobial Bioassays. Compounds **1**–**3** were evaluated their antimicrobial activities against *S. aureus*, *P. aeruginosa*, MRSA 92#, and MRSA 98# by the 2-fold dilution method.[12] The strains used in antimicrobial tests were obtained from the Research Center of Natural Medicine, Clinical School of Kunming General Hospital of Chengdu Military Command. The protocols of antimicrobial tests were described previously.[13]

Insecticidal Bioassays[7c,7d,14]**.** Compounds **1**–**3** were dissolved in DMSO and then diluted with artificial seawater to the final concentrations of 100, 50, 10 ppm (mg/L), which were added to 96-well plates with each well of 15–25 *Artemia salina*. After 48 hours of incubation at 28 °C, the numbers of the dead (non-motile was considered dead) nauplii in each well were counted under a microscope. Each concentration was repeated in triplicate with toosendanin (Shanghai Standard Biotech Co. Ltd., purity ≥ 98%) as the positive control, which was treated in the same way without samples. The corrected mortality was calculated by the Abbot formula.

Corrected mortality = (the mortality of the *A. salina* with sample – the mortality of the *A. salina* of control group) / (1 – the mortality of the *A. salina* of control group) × 100%

Electronic Supplementary Material

Supplementary material is available in the online version of this article at http://dx.doi.org/ 10.1007/s13659-012-0059-3 and is accessible for authorized users.

Acknowledgments

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[11] X-ray crystallographic analysis of **3**: a colorless cylinder crystal (in Acetone–H_2O), $C_{30}H_{46}O_4$, MW = 470.7. All measurements were made on a Bruker APEX DUO detector employing graphite monochromated Cu Ka radiation λ = 1.54178 Å at 100 K, and operating in the Φ/ω scan mode. Space group P 2_1 with a = 8.2806 (13) Å, b = 10.4107 (17) Å, c = 15.285 (3) Å, $\alpha = \gamma = 90°$, β = 99.515(8)°. Crystal dimensions 0.28 × 0.07 × 0.07 mm, V = 1299.6 （4） Å· Z = 2, dx = 1.203 mg/m^3, F (0 0 0) = 516. The structure was solved by direct methods using Shelxs97 and full-matrix least-squares calculations. The total number of independent reflections measured was 3980, of which 3823 were observed. Final indices ($|F|^2 \geq 2\sigma|F|^2$): And the final agreement factors were R_1 = 0.0712, ω R_2 = 0.1961 (w = $1/\sigma|F|^2$), S = 1.092, Flack x = – 0.7 (4). The crystallographic data for **3** has been

deposited at the Cambridge Crystallographic Data Centre with the deposition number CCDC 895143. These data can be obtained free of charge from the Cambridge Crystallographic Data centre via https://www.ccdc.cam.ac.uk/services/structure_deposit/

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