

Article

# Tetranortriterpenes and Limonoids from the Roots of *Aphanamixis polystachya*

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**Abstract:** Phytochemical investigation of the acetone extract from the roots of *Aphanamixis polystachya* resulted in isolation of four new tetranortriterpenes (1–4) in addition to one protolimonoid (methyl-1 $\xi$ ,7R-diacetoxy-23R,25-dihydroxy-20S,24R-21,24-epoxy-3,4-seco-apotirucall-4(28),14(15)-diene-3-oate (5)), five known limonoids (rohituka 3 (6), rohituka 7 (7), nymania 1 (8), rubrin G (9), prieurianin (10)) and a steroid (2,3-dihydroxy-5-pregnan-16-one (11)). Their structures were determined by spectroscopic analyses, including 2D-NMR (COSY, HMQC, HMBC, and NOESY) and high-resolution electrospray ionization mass spectrometry (HRESIMS). Cytotoxic and anti-inflammatory activities of these compounds were evaluated. Compounds 4 and 5 showed significant inhibition against superoxide generation and elastase release by human neutrophils in response to (formyl-L-methionyl-L-leucyl-L-phenylalanine/cytochalasin B) (FMLP/CB).

**Keywords:** *Aphanamixis polystachya*; apotirucallane-type triterpenoid; limonoid; cytotoxicity; anti-inflammatory activity

## 1. Introduction

*Aphanamixis polystachya* (Wall.) R.N. Parker (Meliaceae) is a tropical neem tree native to Asia, especially China and India. According to previous studies, the meliaceaeous plants are famous for the limonoid-producing source and the pest control function in agriculture [1,2]. Phytochemical investigations of this plant have resulted in isolation of many limonoids with diverse skeletons [3–7]. Other structures such as guaiane sesquiterpenes from the seeds [8], lignans from the stem barks [9], chromone, and flavonoid glycosides from the roots [10] were also reported. In biological studies, it has been used as traditional Bangladesh medicine for the treatment of cancers, diabetes, and liver diseases [11] in addition to insecticide applications. Besides, the leaf extract showed CNS (central

nervous system) depressant and analgesic activity in vivo [12], as well as antimicrobial, antioxidant, cytotoxic, and thrombolytic activities in vitro [13]. Currently, some limonoids from *A. polystachya* attract much attention due to their interesting anticancer [14], antifungal [15], and antifeedant activities [16,17].

Continuing our phytochemical investigation on the new anti-cancer and anti-inflammatory agents from terrestrial source, the crude extract from the roots of *A. polystachya* showed significant inhibition on superoxide anion generation and elastase release by human neutrophils in response to FMLP/CB at 10 µg/mL. Fractionation of the active components from the roots of this species was thus initiated. Herein, we report the isolation and structural elucidation of four new and six known triterpenoids, together with one known steroid from the roots of *A. polystachya*. The evaluation against human tumor cell lines and inhibitory activities on superoxide anion generation and elastase release are also discussed.

## 2. Materials and Methods

### 2.1. General Experimental Procedures

Optical rotations were recorded on a JASCO DIP-1000 polarimeter (JASCO, Tokyo, Japan). IR spectra were taken on a HORIBA FT-720 spectrophotometer (HORIBA, Kyoto, Japan). The  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectra as well as 2D NMR spectra (COSY, HMQC, HMBC, and NOESY) were recorded in  $\text{CDCl}_3$  on a Bruker AVX NMR spectrometer (Bruker, Karlsruhe, Germany) operating at 400 MHz for  $^1\text{H}$  and 100 MHz for  $^{13}\text{C}$  using the  $\text{CDCl}_3$  solvent peak as internal standard ( $\delta_{\text{H}}$  7.265,  $\delta_{\text{C}}$  77.0 ppm). Low-resolution ESIMS were recorded on a VG Quattro 5022 mass spectrometer (VG Instruments, Altrincham, UK). HRESIMS were measured on a JEOL HX 110 mass spectrometer (JEOL, Peabody, MA, USA). LiChrospher<sup>®</sup> Si 60 (5 µm, 250-10, Merck, Darmstadt, Germany) and LiChrospher<sup>®</sup> 100 RP-18e (5 µm, 250-10, Merck) were used for NP-HPLC and RP-HPLC (Hitachi, L-6250, Kyoto, Japan; flow rate 2 mL/min, UV detection at 254 nm), respectively. HepG 2, A549 and MCF-7 cancer cells were purchased from Bioresource Collection and Research Center, Food Industry Research and Development Institute (Hsinchu, Taiwan), and Hep-2 was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA).

### 2.2. Plant Material

The roots of *Aphanamixis polystachya* (Wall.) R.N. Parker were collected in Ping-Tong County, Taiwan, in April 2012. The plant material was identified by one of the authors (C.T.C.). A voucher specimen (code No. TP 98-1) has been deposited at the School of Pharmacy, National Taiwan University, Taipei, Taiwan.

### 2.3. Extraction and Isolation

The air-dried roots (6.6 kg) of *A. polystachya* were ground and extracted thrice with acetone at room temperature and concentrated under reduced pressure to obtain a crude extract (250 g). The extract was partitioned between EtOAc:H<sub>2</sub>O (1:1) to give an EtOAc-soluble layer. After evaporating the organic solvent, the EtOAc residue (90 g) was partitioned between *n*-hexane:MeOH:H<sub>2</sub>O (4:3:1) to afford a MeOH/H<sub>2</sub>O extract. The MeOH/H<sub>2</sub>O extract (60 g) was subjected to pass over a Sephadex LH-20 column eluted with MeOH to afford fractions A–F. Fraction C (38 g) was separated on a Si gel (63–200 µm, 90 × 120 mm) column (*n*-hexane:EtOAc:MeOH, 100:0:0 to 0:0:100, 1 L for each gradient solvent) to afford fraction C-14 (7.1 g), which was further chromatographed on Si gel (<63 µm, 65 × 600 mm) column (*n*-hexane:EtOAc, 1:1, 5 L) to furnish three fractions, C-14-C, C-14-D, and C-14-E. Fraction C-14-C (1.7 g) was separated with a Si gel column and eluted with *n*-hexane:EtOAc:CH<sub>2</sub>Cl<sub>2</sub> (1:1:1, 2.5 L) to give three subfractions, C-14-C-4, C-14-C-5 and C-14-C-6. Subfraction C-14-C-4 (385 mg) was subjected to RP-HPLC (5 µm, 10 mm × 250 mm) (MeOH:H<sub>2</sub>O, 7:1) to give **1** (7.6 mg, Rt = 20.3 min) and **2** (11.5 mg, Rt = 16.4 min). Subfraction C-14-C-6 (802 mg) was washed with MeOH and filtrated to yield **10** (443 mg). Fraction C-14-D (1.8 g) was separated by a reverse-phase column (20–50 µm,

35 × 35 mm) and eluted with MeOH/H<sub>2</sub>O (7:3, 2 L) to afford ten subfractions, C-14-D-1 to C-14-D-10. Subfraction C-14-D-6 (174 mg) was separated with an RP-HPLC (CH<sub>3</sub>CN/H<sub>2</sub>O, 55:45) to yield methyl-1ξ,7R-diacetoxy-23R,25-dihydroxy-20S,24R-21,24-epoxy-3,4-*seco*-apotirucall-4(28),14(15)-diene-3-oate (**5**, 15.6 mg, Rt = 35.4 min). Separation of fraction C-14-E (1.1 g) by a Si gel (<63 μm, 35 cm × 30 mm) column (*n*-hexane–acetone, 2:1, 2 L) obtained three subfractions, C-14-E-3, C-14-E-4 and C-14-E-6. Subfraction C-14-E-3 (236 mg) was applied on an RP-HPLC (CH<sub>3</sub>CN–H<sub>2</sub>O, 3:2) to yield **3** (18.1 mg, Rt = 39.1 min) and 2β,3β-dihydroxy-5α-pragnan-16-one (**11**, 17.8 mg, Rt = 22.2 min). Subfraction C-14-E-4 (187 mg) was purified by reverse-phase HPLC (MeOH–H<sub>2</sub>O, 7:1) to obtain **4** (26 mg, Rt = 11.1 min). Subfraction C-14-E-6 (358 mg) was separated by RP-HPLC (MeOH:H<sub>2</sub>O, 3:2) to furnish rohituka **3** (**6**, 30.7 mg, Rt = 37.7 min), rohituka **7** (**7**, 20.8 mg, Rt = 33.6 min), nymania **1** (**8**, 19.8 mg, Rt = 55.8 min), and rubrin G (**9**, 11.5 mg, Rt = 76.7 min).

### 3. Spectral Data

*Aphataiwanin A* (**1**). Colorless amorphous solid;  $[\alpha]_D^{25} -60$  (*c* 0.1, MeOH); IR (CH<sub>2</sub>Cl<sub>2</sub>)  $\nu_{\max}$  3479, 3078, 1740, 1645, 977, 900 cm<sup>-1</sup>; <sup>1</sup>H-NMR (CDCl<sub>3</sub>) and <sup>13</sup>C-NMR (CDCl<sub>3</sub>) spectroscopic data, see Tables 1 and 2, respectively; HRESIMS *m/z* 653.3684 [M + Na]<sup>+</sup> (calcd for C<sub>36</sub>H<sub>54</sub>O<sub>9</sub>Na, 653.3660).

*Aphataiwanin B* (**2**). Colorless amorphous solid;  $[\alpha]_D^{25} -90$  (*c* 0.1, MeOH); IR (CH<sub>2</sub>Cl<sub>2</sub>)  $\nu_{\max}$  3484, 3071, 1740, 1643, 983, 902 cm<sup>-1</sup>; <sup>1</sup>H-NMR (CDCl<sub>3</sub>) and <sup>13</sup>C-NMR (CDCl<sub>3</sub>) spectroscopic data, see Tables 1 and 2, respectively; HRESIMS *m/z* 653.3681 [M + Na]<sup>+</sup> (calcd for C<sub>36</sub>H<sub>54</sub>O<sub>9</sub>Na, 653.3660).

**Table 1.** <sup>1</sup>H-NMR spectroscopic data (400 MHz) of compounds **1–4** <sup>a</sup>.

Position	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
1	5.49 d (10.8)	5.49 d (10.8)	5.49 d (10.8)	5.48 d (10.8)
2α	2.47 dd (13.8, 10.8)	2.46 dd (13.8, 10.8)	2.46 dd (13.8, 10.8)	2.46 dd (13.8, 10.8)
2β	2.81 d (13.8)	2.81 d (13.8)	2.81 d (13.8)	2.81 d (13.8)
5	2.44 d (3.0)	2.45 d (3.0)	2.44 overlap	2.44 d (3.6)
6α	1.63 dt (14.4, 3.0)	1.62 dt (14.4, 3.0)	1.63 d (13.8)	1.63 dd (13.8, 3.6)
6β	2.14 d (14.4)	2.14 d (14.4)	2.14 d (13.8)	2.15 d (13.8)
7	5.16 s	5.15 s	5.15 s	5.14 s
9	2.18 m	2.20 dd (12.0, 6.6)	2.19 dd (12.0, 6.6)	2.19 dd (12.0, 6.6)
11α	1.89 m	1.92 dd (12.0, 3.0)	1.94 d (12.0)	1.90 overlap
11β	1.67 m	1.66 m	1.70 m	1.67 dd (15.0, 12.0)
12α	1.65 m	1.86 dd (10.2, 3.0)	1.66 d (13.2)	1.81 dd (10.8, 9.0)
12β	1.54 dt (13.2, 9.6)	1.48 d (10.2)	1.55 dd (13.2, 9.6)	1.46 dd (10.8, 8.4)
15	5.27 d (3.0)	5.25 d (2.4)	5.27 d (2.4)	5.25 s
16α	1.93 dd (13.8, 7.8)	1.93 overlap	1.99 d (11.4)	1.92 d (15.6)
16β	2.10 ddd (13.8, 7.8, 3.0)	2.1 ddd (15.6, 7.2, 3.6)	2.10 m	2.08 dd (15.6, 7.8)
17	1.92 m	1.70 dd (7.2, 3.6)	1.97 m	1.71 dd (10.2, 7.8)
18	1.00 s	1.03 s	1.00 s	1.02 s
19	0.96 s	0.96 s	0.95 s	0.95 s
20	2.21 m	2.35 m	2.19 overlap	2.31 ddd (10.2, 9.6, 3.6)
21	4.78 d (4.2)	4.84 d (3.6)	4.76 d (4.8)	4.80 d (3.6)
22α	1.87 m	1.89 dd (12.6, 10.8)	1.89 overlap	1.87 m
22β	1.70 m	1.35 dd (12.6, 5.4)	1.86 dd (12.0, 7.8)	1.79 overlap
23	4.23 ddd (9.0, 6.6, 4.8)	4.08 ddd (10.8, 6.0, 5.4)	4.43 t (7.8)	4.25 dd (10.2, 4.2)
24	3.83 d (4.8)	3.92 d (6.0)	3.17 s	3.24 s
26	1.78 s	1.78 s	1.27 s	1.27s
27	5.05 s	5.00 s	1.26 s	1.26 s
27	4.93 s	4.91 s		
28	1.78 s	1.78 s	1.78 s	1.77 s
29	4.85 s	4.85 s	4.84 s	4.84 s
29	5.02 s	5.02 s	5.02 s	5.01 s
30	1.14 s	1.14 s	1.14 s	1.13 s
1-OAc	2.04 s	2.04 s	2.04 s	2.03 s
7-OAc	1.96 s	1.96 s	1.96 s	1.95 s
3-OCH <sub>3</sub>	3.66 s	3.66 s	3.65 s	3.65 s
21-OCH <sub>3</sub>	3.39 s	3.41 s	3.38 s	3.39 s

<sup>a</sup> Chemical shifts are in ppm; *J* values in Hz are in parentheses; Measured in CDCl<sub>3</sub>.

**Table 2.**  $^{13}\text{C}$ -NMR spectroscopic data (100 MHz) of compounds 1–4 <sup>a</sup>.

Position	1	2	3	4
1	77.2 CH	76.9 CH	76.8 CH	76.9 CH
2	35.5 CH <sub>2</sub>	35.5 CH <sub>2</sub>	35.5 CH <sub>2</sub>	34.9 CH <sub>2</sub>
3	172.0 C	172.1 C	172.0 C	172.1 C
4	145.1 C	145.0 C	145.1 C	145.0 C
5	44.3 CH	44.3 CH	44.3 CH	44.3 CH
6	29.3 CH <sub>2</sub>	29.3 CH <sub>2</sub>	29.3 CH <sub>2</sub>	29.3 CH <sub>2</sub>
7	74.7 CH	74.6 CH	74.7 CH	74.6 CH
8	42.5 C	42.5 C	42.4 C	42.4 C
9	34.5 CH	34.5 CH	34.4 CH	34.0 CH
10	44.3 C	44.3 C	44.3 C	44.3 C
11	18.5 CH <sub>2</sub>	18.4 CH <sub>2</sub>	18.5 CH <sub>2</sub>	18.4 CH <sub>2</sub>
12	33.5 CH <sub>2</sub>	33.7 CH <sub>2</sub>	33.5 CH <sub>2</sub>	33.4 CH <sub>2</sub>
13	46.3 C	46.6 C	46.2 C	46.6 C
14	159.2 C	159.5 C	159.1 C	159.4 C
15	119.1 CH	118.7 CH	119.2 CH	118.7 CH
16	35.2 CH <sub>2</sub>	35.0 CH <sub>2</sub>	35.2 CH <sub>2</sub>	34.4 CH <sub>2</sub>
17	52.9 CH	58.1 CH	52.7 CH	57.9 CH
18	20.8 CH <sub>3</sub>	20.2 CH <sub>3</sub>	20.8 CH <sub>3</sub>	20.2 CH <sub>3</sub>
19	15.2 CH <sub>3</sub>	15.2 CH <sub>3</sub>	15.2 CH <sub>3</sub>	15.2 CH <sub>3</sub>
20	45.8 CH	47.0 CH	45.0 CH	46.1 CH
21	104.6 CH	109.3 CH	104.9 CH	109.7 CH
22	31.0 CH <sub>2</sub>	34.2 CH <sub>2</sub>	31.6 CH <sub>2</sub>	33.6 CH <sub>2</sub>
23	80.7 CH	79.0 CH	79.0 CH	76.9 CH
24	78.1 CH	78.4 CH	76.8 CH	75.7 CH
25	144.9 C	144.6 C	73.1 C	73.3 C
26	18.7 CH <sub>3</sub>	18.4 CH <sub>3</sub>	26.6 CH <sub>3</sub>	26.6 CH <sub>3</sub>
27	112.8 CH <sub>2</sub>	113.6 CH <sub>2</sub>	26.5 CH <sub>3</sub>	26.5 CH <sub>3</sub>
28	23.0 CH <sub>3</sub>	23.0 CH <sub>3</sub>	23.0 CH <sub>3</sub>	23.0 CH <sub>3</sub>
29	116.6 CH <sub>2</sub>	116.6 CH <sub>2</sub>	116.6 CH <sub>2</sub>	116.6 CH <sub>2</sub>
30	26.9 CH <sub>3</sub>	26.9 CH <sub>3</sub>	26.9 CH <sub>3</sub>	26.9 CH <sub>3</sub>
1-OAc	170.5 C	170.5 C	170.5 C	170.5 C
	21.2 CH <sub>3</sub>	21.2 CH <sub>3</sub>	21.2 CH <sub>3</sub>	21.2 CH <sub>3</sub>
7-OAc	170.2 C	170.2 C	170.2 C	170.2 C
	21.5 CH <sub>2</sub>	21.5 CH <sub>2</sub>	21.5 CH <sub>2</sub>	21.4 CH <sub>2</sub>
3-OCH <sub>3</sub>	52.3 CH <sub>3</sub>	52.3 CH <sub>3</sub>	52.2 CH <sub>3</sub>	52.3 CH <sub>3</sub>
21-OCH <sub>3</sub>	54.9 CH <sub>3</sub>	55.7 CH <sub>3</sub>	55.4 CH <sub>3</sub>	55.9 CH <sub>3</sub>

<sup>a</sup> Assignments were made using HMQC and HMBC techniques; Measured in CDCl<sub>3</sub>.

*Aphataiwanin C* (**3**). Colorless amorphous solid;  $[\alpha]_{\text{D}}^{25} -60$  (*c* 0.1, MeOH); IR (CH<sub>2</sub>Cl<sub>2</sub>)  $\nu_{\text{max}}$  3510, 2930, 1740, 1650, 972, 900 cm<sup>-1</sup>; <sup>1</sup>H-NMR (CDCl<sub>3</sub>) and <sup>13</sup>C-NMR (CDCl<sub>3</sub>) spectroscopic data, see Tables 1 and 2, respectively; HRESIMS *m/z* 671.3785 [M + Na]<sup>+</sup> (calcd for C<sub>36</sub>H<sub>56</sub>O<sub>10</sub>Na, 671.3765).

*Aphataiwanin D* (**4**). Colorless amorphous solid;  $[\alpha]_{\text{D}}^{25} -170$  (*c* 0.1, MeOH); IR (CH<sub>2</sub>Cl<sub>2</sub>)  $\nu_{\text{max}}$  3491, 2932, 1741, 1642, 900 cm<sup>-1</sup>; <sup>1</sup>H-NMR (CDCl<sub>3</sub>) and <sup>13</sup>C-NMR (CDCl<sub>3</sub>) spectroscopic data, see Tables 1 and 2, respectively; HRESIMS *m/z* 671.3763 [M + Na]<sup>+</sup> (calcd for C<sub>36</sub>H<sub>56</sub>O<sub>10</sub>Na, 671.3765).

## 4. Biological Activities

### 4.1. Cytotoxicity Assay

Cytotoxicity was tested against human Hep-G2 (hepatocellular carcinoma), HEp-2 (laryngeal carcinoma), A549 (lung carcinoma) and MCF-7 (breast adenocarcinoma) tumor cell lines. The assay procedure using MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) was carried out as previously described [18,19]. The cells were cultured in RPMI-1640 medium. After seeding of cells in a 96-well microplate for 4 h, 20 μL of sample was placed in each well and incubated at 37 °C for 3 days, and then 20 μL MTT was added for 5 h. After removing the medium and putting DMSO (200 μL/well) into the microplate with shaking for 10 min, the formazan crystals were redissolved and

their absorbance was measured on a microtiter plate reader (MR 7000, Dynatech, Missouri City, TX, USA) at a wavelength of 550 nm. Mitomycin C was used as a positive control.

#### 4.2. Anti-Inflammatory Assays, Inhibitory Effect on Superoxide Anion Generation and Elastase Release by Human Neutrophils

Neutrophils were obtained by means of dextran sedimentation and Ficoll centrifugation. Superoxide generation and elastase release were carried out according to a procedure described previously [20]. Superoxide anion production was assayed by monitoring the superoxide dismutase-inhibitable reduction of ferricytochrome *c*. Elastase release experiments were performed using MeO-Suc-Ala-Ala-Pro-Valp-nitroanilide as the elastase substrate. Genistein was used as a standard compound, which showed inhibition of  $65.0 \pm 5.7$  and  $51.6 \pm 5.9$  at 10  $\mu\text{g/mL}$ , respectively, on superoxide anion generation and elastase release.

### 5. Results and Discussion

Sephadex LH-20 and extensive column chromatography furnished four new apotirucallane-type triterpenoids 1–4 (Figure 1), together with a protolimonoid, methyl-1 $\xi$ ,7 $R$ -diacetoxy-23 $R$ ,25-dihydroxy-20 $S$ ,24 $R$ -21,24-epoxy-3,4-*seco*-apotirucall-4(28),14(15)-diene-3-oate (5) [21]; five limonoids, rohituka 3 (6) [22], rohituka 7 (7) [22], nymania 1 (8) [23], rubrin G (9) [24], prieurianin (10) [25,26]; and one steroid, 2 $\beta$ ,3 $\beta$ -dihydroxy-5 $\alpha$ -pragnan-16-one (11) [27,28]. The structures of the new compounds were established by interpretation of their spectroscopic data, especially 2D NMR. The relative configurations of compounds 1–4 were determined by NOESY and comparison of NMR data with those published in reference papers.

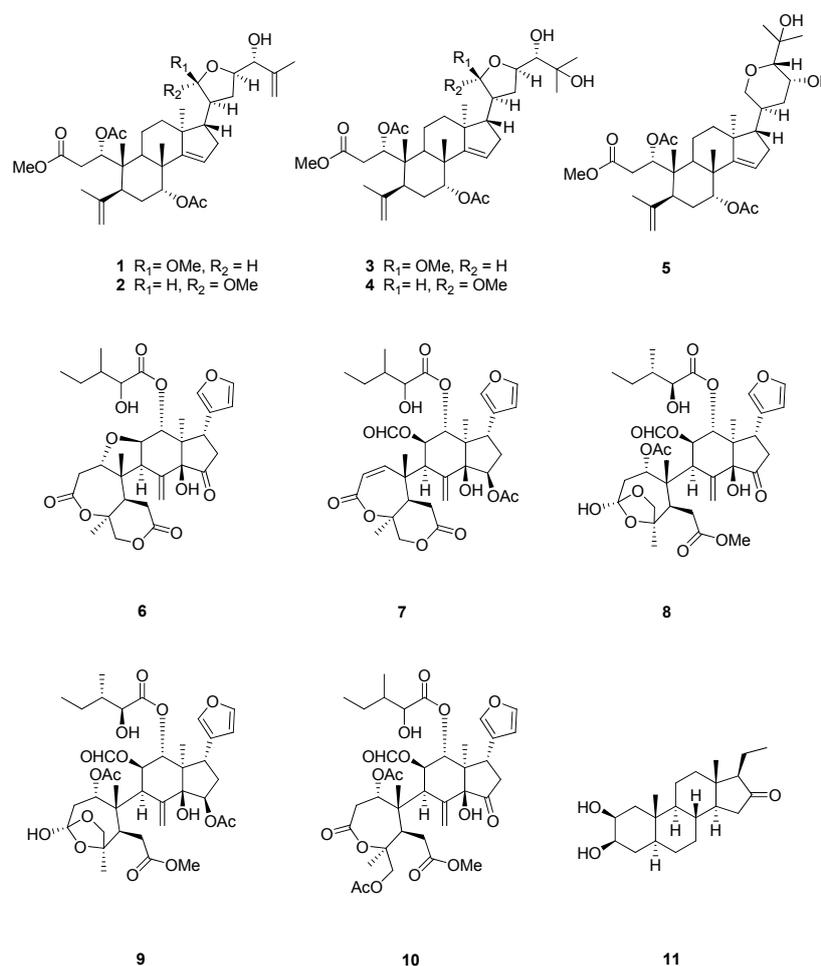


Figure 1. Chemical structures of compounds 1–11.

Compound **1**,  $[\alpha]_D^{25} -60$  (MeOH), had the molecular formula of  $C_{36}H_{54}O_9$  and 10 degrees of unsaturation, as deduced from the HRESIMS ( $m/z$  653.3684  $[M + Na]^+$ ) and  $^{13}C$ -NMR/DEPT spectra. The IR absorption bands revealed the presence of hydroxy ( $3479\text{ cm}^{-1}$ ), ester ( $1740\text{ cm}^{-1}$ ), and double bond ( $1645\text{ cm}^{-1}$ ) functionalities in **1**. The  $^1H$ -NMR data of **1** (Table 1) exhibited seven methyl singlets ( $\delta_H$  0.96, 1.00, 1.14, 1.78, 1.79, 1.96 and 2.04), two methoxy singlets ( $\delta_H$  3.39, 3.66), exomethylene ( $\delta_H$  4.85, 4.93, 5.02 and 5.05), an olefinic methine doublet ( $\delta_H$  5.27,  $J = 3.0$  Hz), and five oxygen-bearing methine signals ( $\delta_H$  3.83, 4.23, 4.78, 5.16 and 5.49). The  $^{13}C$ -NMR (Table 2) and DEPT spectra of **1** showed 36 carbon signals, consisting of three ester carbonyls ( $\delta_C$  170.2, 170.5, and 172.0), two olefinic methylenes ( $\delta_C$  112.8, 116.6), an olefinic methine ( $\delta_C$  119.1), three olefinic quaternary carbons ( $\delta_C$  144.9, 145.1, and 159.2), five oxymethines ( $\delta_C$  74.7, 77.2, 78.1, 80.7 and 104.6), four aliphatic methines ( $\delta_C$  34.5, 44.3, 45.8, and 52.9), six aliphatic methylenes ( $\delta_C$  18.5, 29.3, 31.0, 33.5, 35.2, and 35.5), two methoxyls ( $\delta_C$  52.3, 54.9), and seven methyls ( $\delta_C$  15.2, 18.7, 20.8, 21.2, 21.5, 23.0, and 26.9). This accounted for 6 of the 10 degrees of unsaturation, indicating that **1** is a tetracyclic triterpenoid with two acetyl and two methoxyl moieties.

The COSY spectrum (Figure 2) of **1** exhibited four proton spin systems of H-1 ( $\delta_H$  5.49)/H<sub>2</sub>-2 ( $\delta_H$  2.47, 2.81) and H-5 ( $\delta_H$  2.44)/H<sub>2</sub>-6 ( $\delta_H$  1.63, 2.14)/H-7 ( $\delta_H$  5.16); H-9 ( $\delta_H$  2.18)/H<sub>2</sub>-11 ( $\delta_H$  1.67, and 1.89)/H<sub>2</sub>-12 ( $\delta_H$  1.54, and 1.65); and a proton sequence between H-15 ( $\delta_H$  5.27)/H<sub>2</sub>-16 ( $\delta_H$  1.93, 2.10)/H-17 ( $\delta_H$  1.92)/H-20 ( $\delta_H$  2.21)/H-21 ( $\delta_H$  4.78) and between H-20/H<sub>2</sub>-22 ( $\delta_H$  1.70, 1.87)/H-23 ( $\delta_H$  4.23) /H-24 ( $\delta_H$  3.83). In the HMBC spectrum (Figure 2), the H<sub>3</sub>-19 signal showed correlations to C-1 ( $\delta_C$  77.2), C-5 ( $\delta_C$  44.3), C-9 ( $\delta_C$  34.5) and C-10 ( $\delta_C$  44.3), and the H<sub>2</sub>-29 had correlations with C-4 ( $\delta_C$  145.1), C-5 and C-28 ( $\delta_C$  23.0) suggesting the ring B. In addition, the HMBC correlations of H<sub>3</sub>-18 ( $\delta_H$  1.00) with C-13 ( $\delta_C$  46.3), C-12 ( $\delta_C$  33.5), C-14 ( $\delta_C$  159.2), C-17 ( $\delta_C$  52.9), and of H<sub>3</sub>-30 ( $\delta_H$  1.14) with C-7 ( $\delta_C$  74.7), C-8 ( $\delta_C$  42.5), C-9 ( $\delta_C$  34.5) and C-14 ( $\delta_C$  159.2) established rings C and D. Moreover, the HMBC correlations of both H<sub>3</sub>-26 ( $\delta_H$  1.78) and H<sub>2</sub>-27 ( $\delta_H$  4.93, 5.05) with C-24 ( $\delta_C$  78.1) and C-25 ( $\delta_C$  144.9) indicated a methyl vinyl group to be attached at the oxygenated C-24. The HMBC correlations of the methoxy protons ( $\delta_C$  3.39) with C-21 ( $\delta_C$  104.6) and of H-21 ( $\delta_H$  4.78) with C-23 ( $\delta_C$  80.7) revealed the tetrahydrofuran ring with a methoxy group at C-21. The remaining methoxy ( $\delta_H$  3.36) group was found to be connected with C-3 ( $\delta_C$  172.0), and the H-1 ( $\delta_H$  5.49) and H-7 ( $\delta_H$  5.16) were connected with the acetyl carbonyl carbons ( $\delta_C$  170.5, 170.2) indicated that the acetyl groups were attached at C-1 and C-7, respectively. This 2D NMR spectroscopic analysis was used to identify **1** as an A-seco apo-tirucallane triterpenoid [15].

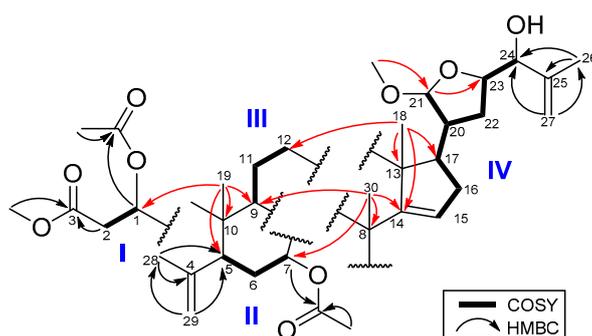


Figure 2. COSY and HMBC of **1**.

The relative configuration of **1** was determined by the analysis of NOESY correlations (Figure 3). Assuming that H-5 of **1** was  $\alpha$ -oriented similar to that of the A-seco apotirucallane tetranortriterpenes [15,29], the NOESY correlations between H-6 $\alpha$ /H-5/H-9/H<sub>3</sub>-18/H-16 $\alpha$  revealed that these protons were on the  $\alpha$ -face. The NOESY correlations of H<sub>3</sub>-19 with H-1, H-6 $\beta$  and H-11 $\beta$ , as well as Me-30 with H-7 indicating these protons were on the  $\beta$ -face. In addition, NOESY correlations of H-20/H-21/H-22 $\alpha$ /H-23 suggested that these protons were all in  $\alpha$ -face and the C-21 methoxy was  $\beta$ -oriented. On comparing the  $^1H$ - and  $^{13}C$ -NMR spectra of **1** with those of chisopanin C [30] and



suggesting that compound **4** was an epimer of **3**. The configuration at C-21 was determined by NOESY experiment. NOESY correlations between H-21/H-22 $\beta$ , H-20/H-22 $\alpha$  and H-22 $\alpha$ /H-23 indicated that the methoxy group was  $\alpha$ -oriented. Other NOESY correlations were the same as those of **3**. Thus, the structure of compound **4** was elucidated and the name aphataiwanin D was given.

The 11 isolates were evaluated for their cytotoxic activities against human hepatocellular carcinoma (Hep-G2), lung carcinoma (A549), epithelial type 2 (HEp-2), and breast adenocarcinoma (MCF-7) cell lines in vitro. Among these compounds, **5** possessed significant activity (ED<sub>50</sub> value of  $6.83 \pm 0.63$   $\mu\text{g/mL}$ ) against MCF-7 cells and mild activity against Hep-G2 and A549 cells (ED<sub>50</sub> value of  $11.38 \pm 0.98$  and  $15.49 \pm 0.76$   $\mu\text{g/mL}$ ). Simultaneously, compounds **1** and **10** also showed mild activities against Hep-G2 and HEp-2, respectively, with IC<sub>50</sub> values of 16–17  $\mu\text{g/mL}$  (Table 3). In terms of anti-inflammation, compounds **1–11** were tested on superoxide anion generation and elastase release by human neutrophils in the presence of FMLP/CB (Table 4). Only compounds **4** and **5** showed significant anti-inflammatory activity, as tested on superoxide anion generation with IC<sub>50</sub> at  $5.79 \pm 0.88$  and  $1.25 \pm 0.17$   $\mu\text{g/mL}$ , as well as the significant inhibition on elastase release with IC<sub>50</sub> at  $5.22 \pm 0.24$  and  $2.26 \pm 0.05$   $\mu\text{g/mL}$ , respectively.

**Table 3.** Cytotoxic activities of compounds **1–11** <sup>a</sup>.

Compound	ED <sub>50</sub> ( $\mu\text{g/mL}$ )			
	HEp-2	Hep-G2	A549	MCF-7
<b>1</b>	$28.12 \pm 0.60$	$16.02 \pm 0.41$	$33.56 \pm 0.92$	>40
<b>2</b>	$36.05 \pm 2.78$	$24.86 \pm 1.70$	>40	>40
<b>3</b>	$37.78 \pm 0.43$	$30.34 \pm 0.51$	>40	>40
<b>4</b>	$37.72 \pm 0.94$	>40	>40	>40
<b>5</b>	$11.38 \pm 0.98$	$26.72 \pm 1.01$	$15.49 \pm 0.76$	$6.83 \pm 0.63$
<b>6</b>	>40	>40	>40	$33.78 \pm 1.66$
<b>7</b>	>40	>40	>40	$37.49 \pm 1.98$
<b>8</b>	$31.06 \pm 2.89$	>40	>40	>40
<b>9</b>	$36.69 \pm 2.30$	$34.36 \pm 3.43$	>40	>40
<b>10</b>	$16.77 \pm 2.36$	>40	>40	>40
<b>11</b>	>40	>40	>40	>40
mitomycin	$0.16 \pm 0.01$	$0.19 \pm 0.01$	$0.19 \pm 0.01$	$0.18 \pm 0.01$

<sup>a</sup> Results are presented as mean  $\pm$  S.E.M. ( $n = 3$ ).

**Table 4.** Anti-inflammatory data of compounds **1–11** <sup>a</sup>.

Compound	Superoxide Anion		Elastase Release	
	IC <sub>50</sub> ( $\mu\text{g/mL}$ ) <sup>b</sup>	Inh % <sup>c</sup>	IC <sub>50</sub> ( $\mu\text{g/mL}$ ) <sup>b</sup>	Inh % <sup>c</sup>
<b>1</b>	>10	$41.94 \pm 2.39$	>10	$41.72 \pm 4.47$
<b>2</b>	>10	$43.00 \pm 4.46$	>10	$44.83 \pm 5.06$
<b>3</b>	>10	$33.20 \pm 6.23$	>10	$16.54 \pm 3.36$
<b>4</b>	$5.79 \pm 0.88$	$74.39 \pm 2.90$	$5.22 \pm 0.24$	$78.36 \pm 5.74$
<b>5</b>	$1.25 \pm 0.17$	$86.65 \pm 1.75$	$2.26 \pm 0.05$	$78.19 \pm 0.56$
<b>6</b>	>10	$18.40 \pm 0.84$	>10	$7.93 \pm 3.27$
<b>7</b>	>10	$13.71 \pm 3.60$	>10	$18.85 \pm 2.47$
<b>8</b>	>10	$45.60 \pm 3.63$	>10	$21.39 \pm 3.77$
<b>9</b>	>10	$43.92 \pm 7.10$	>10	$8.49 \pm 1.05$
<b>10</b>	>10	$49.20 \pm 2.20$	>10	$39.36 \pm 4.48$
<b>11</b>	>10	$44.85 \pm 1.63$	>10	$33.64 \pm 8.34$

<sup>a</sup> Results are presented as mean  $\pm$  S.E.M. ( $n = 3$ ); <sup>b</sup> Concentration necessary for 50% inhibition (IC<sub>50</sub>); <sup>c</sup> Percentage of inhibition (Inh %) at 10  $\mu\text{g/mL}$  concentration.

## 6. Conclusions

In the present study, 10 tetranortriterpenoids, including four new compounds and a steroid, were isolated; compounds 1–5 belong to a group of protolimonoids, ring A-seco apotirucallol, while 6–10 can be classified as rings A,B-seco prierianins. Among these secondary metabolites, liaphataiwanins A (1) and D (4), 5, and prierianin (10) were found to have mild cytotoxicities against cancer cells and moderate to potent anti-inflammatory activities. Compound 5, which has the moiety of six-member ether ring, showed the best biological function. The chemical constituents and the evaluation of cytotoxicities against human cancer cells and anti-inflammatory activities reported herein may provide beneficial information for further phytotherapy research.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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