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Four new diterpenes from *Aphanamixis polystachya*

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ABSTRACT

Four novel diterpenes possessing rare five-membered peroxide ring, aphanaperoxides E–H (**1**–**4**), were isolated from stem bark of *Aphanamixis polystachya*. Their structures including the absolute configuration were elucidated by spectroscopic data and CD analysis. The cytotoxicities of the isolated compounds against a panel of human cancer cell lines were evaluated.

1. Introduction

Aphanamixis polystachya (Wall.) J. N. Barker (syn. *Amoora rohituka* Wight & Arn) (Meliaceae), an evergreen tree mainly growing in the tropical area of Asia [1], is traditionally used as herbal medicine for cancer, liver, and spleen diseases [2,3]. Previous investigations on this species have led to the isolation of limonoids [4–9], sesquiterpenes [10], a diterpene [11], a flavone glycoside [12], a lignan [13], an alkaloid [14], a saponin [15], and a keto fatty acid [16]. As a part of our ongoing program towards the discovery of novel bioactive constituents, four new diterpenes possessing rare five-membered peroxide ring in their skeletons, namely aphanaperoxides E–H (**1**–**4**) (Fig. 1), were isolated from the stem bark of *A. polystachya*. Among them, aphanaperoxides G (**3**) and H (**4**) were obtained as a mixture with a ratio of 2:1. Until now, a limonoid containing seven-membered-ring peroxide, was only obtained from *Walsura robusta* (Meliaceae) [17]. Herein, the isolation, structure characterization, and *in vitro* cytotoxic evaluation of the new isolates against human hepatoma (HepG2), human breast adenocarcinoma (MCF-7), human lung carcinoma (A-549), and human gastric carcinoma (AGS) cancer cell lines are reported.

2. Experimental

2.1. General

Optical rotations were obtained on a Perkin-Elmer 341 digital polarimeter. UV spectra were recorded on a Shimadzu UV2550 spectrometer. CD spectra were recorded on a JASCO J-815 spectropolarimeter. IR spectra were measured on a FTIR-8400S spectrometer. 1D- (^{1}H, ^{13}C-APT) and 2D- (^{1}H-^{1}H COSY, HSQC, HMBC) NMR experiments were performed on Bruker AV III 600 spectrometers operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C, respectively (TMS an internal standard). Chemical shifts are expressed in δ (ppm) referenced to solvent peaks at δ_H 3.31 and δ_C 49.2 for CD_3OD and δ_H 2.50 for DMSO-d_6, respectively, and coupling constants are in Hz. HRESIMS spectra were obtained from a Thermo Scientific LTQ-Obitrap XL instrument (Thermo Scientific, Bremen, Germany). Elemental

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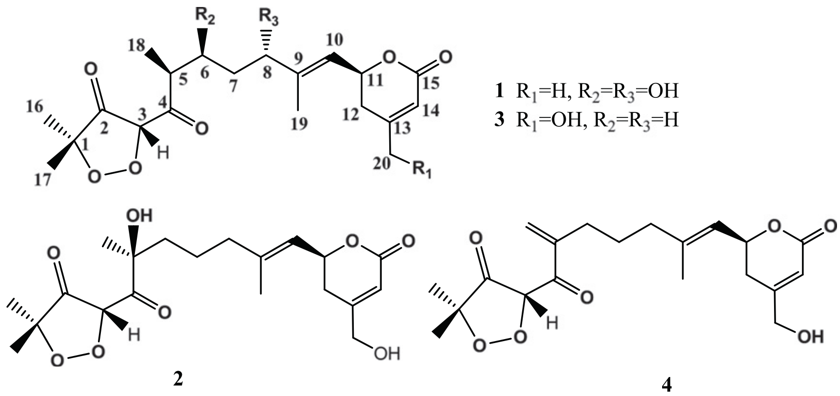

Fig. 1. Structures of compounds **1**–**4**.

analysis was recorded on an EA 1108, CHNS-O (Fisons). Silica gel (100–200 mesh, Qingdao Marine Chemical, Inc.) and Sephadex LH-20 (Amersham Pharmacia Biotech AB) were used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F_{254}, 0.25 mm) were used for TLC analyses. Preparative HPLC was performed on a Lumtech K1001 analytic LC equipped with two pumps of K-501, a UV detector of K-2600, and an Kromasil (250 × 10 mm) semi-preparative column packed with C_{18} (5 μm). Preparative MPLC was carried out on a Biotage SP1 flash chromatography instrument with a dual wavelength UV detector using an ODS column (50 μm, 400 × 40 mm i. d.; Merck). Mixtures of MeOH/H_2O were used as the eluents. All solvents used were of analytical grade (Beijing Chemical Works, Beijing, China).

2.2. Plant material

The stem bark of *A. polystachya* was collected in July 2012 from Tunchang County, Hainan Province, China, and was identified by Prof. Jianpin Tian, School of Pharmaceutical Science, Hainan Medical University. A voucher specimen (No. AP21020720) was deposited in the Herbarium of Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College.

2.3. Extraction and isolation

The air-dried stem bark of *A. polystachya* (1.5 kg) was extracted with 95% EtOH (3 × 10.0 L) under reflux for 3 × 2 h. The ethanol extract was filtered and concentrated under reduced pressure to yield a crude extract (120.0 g), which was suspended in distilled H_2O (2.0 L) and then successively partitioned with hexane (3 × 2.0 L) and $CHCl_3$ (3 × 2.0 L) to afford $CHCl_3$-souble fraction (30.0 g). The $CHCl_3$-souble fraction was subjected to silica gel chromatography (150 g, 100–200 mesh, 10 × 120 cm) eluting with a gradient of EtOAc in hexane (0:1, 20:1, 10:1, 5:1, 2:1, v/v) to give six fractions (Fr. A–F) monitored by TLC. Fr. E (2.8 g) was subjected to a column chromatography of Sephadex LH-20 (2.5 × 120 cm) eluting with CH_3OH to give three fractions (Fr 5A–5C). A mixture (10.0 mg) of **3** and **4** was obtained from Fr. 5C (220.0 mg) by further purification using preparative HPLC chromatography eluting with MeOH–H_2O (42:58, v/v, flow rate 2.0 mL/min, t_R = 32.5 min). Fr. F (2.2 g) was purified over a column chromatography of Sephadex LH-20 (2.5 × 150 cm) eluting with CH_3OH followed by preparative HPLC chromatography eluting with MeOH–H_2O (38:62, v/v, flow rate 2.0 mL/min) to yield compounds **1** (10.0 mg, t_R = 27.4 min) and **2** (12.0 mg, t_R = 23.5 min).

2.3.1. Aphanaperoxide E (**1**)

Colorless oil (MeOH); [α]25 D− 48.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 201 (2.36) nm, 260 (2.18) nm; CD (MeOH, $\Delta\varepsilon$) λ_{max} 255 (−20.9), 316 (+2.62) nm; [$Mo_2(OAc)_4$]-induced CD (DMSO-d_6, $\Delta\varepsilon$) λ_{max} 386.5 (−0.0426) nm; IR (KBr) ν_{max} 3445, 2976, 2934, 1718, 1688, 1587, 1384, 1248, 1173 cm^{-1}; (+)-HRESIMS *m/z* 387.1785 [M–O_2+Na]$^+$ (calcd for $C_{20}H_{28}O_6Na$, 387.1784); *anal.* found C 60.55 %, H 7.18 %, calcd for $C_{20}H_{28}O_8$, C 60.59 %, H 7.12%, and O 32.29 %; ^{1}H NMR (CD_3OD, 600 MHz), see Table 1, ^{13}C APT (CD_3OD, 150 MHz) data, see Table 2.

2.3.2. Aphanaperoxide F (**2**)

Colorless oil (MeOH); [α]25 D −32.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 201 (2.25) nm, 260 (2.08) nm; CD (MeOH, $\Delta\varepsilon$) λ_{max} 255 (−12.6), 313 (+1.54) nm; $Rh_2(OCOCF_3)_4$–induced CD ($CDCl_3$, $\Delta\varepsilon$) λ_{max} 345.5 (+0.0435) nm; IR (KBr) ν_{max} 3442, 2976, 2933, 1720, 1690, 1585, 1381, 1248, 1175 cm^{-1}; (+)-HRESIMS *m/z* 387.1784 [M–O_2+Na]$^+$ (calcd for $C_{20}H_{28}O_6Na$, 387.1784); ^{1}H NMR (CD_3OD, 600 MHz), see Table 1, ^{13}C APT (CD_3OD, 150 MHz) data, see Table 2.

2.3.3. A mixture of Aphanaperoxides G (**3**) and H (**4**)

Colorless oil (MeOH); [α]25 D −26.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 201 (2.57) nm, 262 (2.23) nm; CD (MeOH, $\Delta\varepsilon$) λ_{max} 250 (−21.5), 315 (+3.01) nm; IR (KBr) ν_{max} 3378, 2974, 2934, 1718, 1694, 1581, 1381, 1177 cm^{-1}; (+)-HRESIMS *m/z* 371.1833 [M–O_2+Na]$^+$ (calcd for $C_{20}H_{28}O_5Na$, 371.1834); (+)-HRESIMS *m/z* 369.1678 [M–O_2+Na]$^+$ (calcd for $C_{20}H_{26}O_5Na$, 369.1678); ^{1}H NMR (CD_3OD, 600 MHz), see Table 1, ^{13}C APT (CD_3OD, 150 MHz) data, see Table 2.

2.4. Cytotoxic assay

The cytotoxic assays were measured on HepG2 (human hepatoma), A-549 (human lung carcinoma), AGS (human gastric carcinoma), and MCF-7 (human breast adenocarcinoma)

Table 1
^{1}H NMR Data for Aphanaperoxides E–H (**1–4**) (600 MHz, CD_3OD).

Position	1	2	3	4
3	5.49, s	5.61, s	5.47, s	5.47, s
5	2.82, quint (7.2)	–	2.76, m	–
6	3.81, m (4.8)	1.64, dd (13.2, 3.6) 1.75, td (13.2, 5.4)	1.53, m	1.67, m
7	1.71, m	1.39–1.43, m	1.53, m 1.72, m	1.53, m 1.72, m
8	4.26, t (7.2)	2.07, m	2.13, dd (7.2, 3.0)	2.17, t (7.2)
10	5.62, d (8.4)	5.37, d, (8.4)	5.40, d (8.4)	5.42, dd (8.4, 2.0)
11	5.26, ddd (12.0, 9.0, 4.8)	5.23, ddd (12.0, 9.0, 4.8)	5.26, ddd (12.0, 9.0, 4.8)	5.25, ddd (12.0, 9.0, 4.8)
12	2.37, d (18.0, 6.0) 2.45, d (18.0, 10.8)	2.35, m	2.37, d (18.0, 6.0) 2.41, d (18.0, 12.0)	2.34, d (18.0, 5.4) 2.43, d (18.0, 12.0)
14	5.80, s	6.00, s	6.02, s	6.02, s
16	1.36, s	1.37, s	1.37, s	1.42, s
17	1.36, s	1.37, s	1.37, s	1.42, s
18	1.25, d (7.2)	1.43, s	1.26, d (7.2)	5.59, s 6.10, s
19	1.72, d (1.2)	1.73, d (1.2)	1.76, d (1.2)	1.78, d (1.2)
20	2.03, s	4.22, q (18.0)	4.24, q (18.0)	4.24, q (18.0)

(obtained from the Institute of Materia Medica of Chinese Academy of Medical Sciences) using the MTT assay method. Cells were plated in the appropriate media on 96-well plates in a 100 μL total volume at a density of 1×10^5 cells/mL. Each tumor cell line was exposed to the tested compounds at various concentrations in triplicates and incubated at 37 °C and 5% CO_2 for 48 h. Cell viability was determined based on the mitochondrial conversion of MTT to formazan. Doxorubicin (Sigma, St. Louis, MO, USA) was used as positive control. After treatment, cell viability was measured and the cell growth curve was plotted. Half maximal inhibitory (IC_{50}) values were calculated by the Reed and Muench method [18].

3. Results and discussion

Compound **1** was obtained as an optically active colorless oil with an [α]25 D −48.0 (*c* 0.1, MeOH). Its molecular formula was deduced as $C_{20}H_{28}O_8$ based on the pseudo-molecular ion peak at 387.1785 $[M–O_2+Na]^+$ (calcd for $C_{20}H_{28}O_6Na$, 387.1784) in the positive HRESIMS and elemental analysis, requiring seven double-bond equivalents. The loss of O_2 from the sodium adduct ion peak evidenced the existence of a peroxide group in **1** [19]. IR spectrum displayed characteristic hydroxyl absorption (3445 cm^{-1}) and carbonyl absorptions (1718 and 1688 cm^{-1}). The ^{1}H and ^{13}C NMR spectra of **1** (Tables 1 and 2) exhibited signals due to five methyl groups (one secondary at δ_H 1.25, two tertiary at δ_H 1.36, and two vinylic at δ_H 1.72, 2.03), two methylene groups (δ_C 35.7, 39.8), five methine groups at δ_C 43.6, 72.5, 75.8, 75.9, 102.6 (four oxygenated), two ketone groups (δ_C 210.4, 196.7), two trisubstituted double bonds at δ_C 116.7 (protonated), 124.8 (protonated), 144.9, 161.2, one oxygenated quaternary carbon at δ_C 90.0, and one ester carbon (δ_C 168.0). Additionally, the olefinic protons attached to the double bonds are grouped into one singlet at δ 5.80 (s) and one doublet at δ 5.62 (d, $J =$ 8.4 Hz), while the oxygenated methine protons were categorized into one triplet at δ 4.26 (t), singlet at δ 5.49 (s) and one tridoublet at δ 5.26 (ddd, $J =$ 12.0, 9.0, 4.8 Hz). Moreover, the downfield chemical shifts of C-1 (δ_C 90.0) and C-3 (δ_C 102.6) fully agreed with the presence of the five-membered ring peroxide which was located across C-1 and C-3 in **1** as shown in Fig. 1. On basis of these data and chemotaxonomic considerations, compound **1** was presumed to be a diterpene featuring highly oxygenated framework with a peroxide group.

Close interpretation of the COSY data for **1** led to the establishment of the two partial structural units as shown in boldface in Fig. 2. The complete assignment of the linkages from C-1 to C-5 was determined by observed long-range correlations in the HBMC spectrum, mainly between δ_H 1.36 (H_3-16, H_3-17) and δ_C 90.0 (C-1), δ_C 210.4 (C-2); δ_H 5.49 (H-3) and δ_C 90.0 (C-1), 210.4 (C-2), 196.7 (C-4), 43.6 (C-5); δ_H 2.82 (H-5) and δ_C 102.6 (C-3), 196.7 (C-4). The HMBC spectrum also defined the attachment from C-12 to C-15, since the methyl group (δ_H 2.03, H_3-20) showed correlations to δ_C 35.7 (C-12) and 116.7 (C-14), and the olefinic proton (δ_H 5.80, H-14) displayed a cross peak to δ_C 168.0 (C-15). One hydroxyl group was attached to C-8, which was demonstrated by observed HMBC correlations from H-10 (δ_H 5.62), H-19 (δ_H 1.72) to C-8 (δ_C 75.9), and from H-8 (δ_H 4.27) to C-7 (δ_C 39.8), C-9 (δ_C 144.9) and C-10 (δ_C 124.8).

Table 2
^{13}C NMR Data for Aphanaperoxides E–H (**1–4**) (150 MHz, CD_3OD).

Position	1	2	3	4
1	90.0s	91.3s	90.0s	90.0s
2	210.4s	210.2s	210.5s	210.4s
3	102.6d	100.9d	100.9d	100.7d
4	196.7s	199.3s	199.0s	186.2s
5	43.6d	73.4s	36.9d	140.2s
6	72.5d	40.8t	34.8t	32.8t
7	39.8t	22.8t	26.1t	27.7t
8	75.9d	40.4t	40.4t	39.8t
9	144.9s	144.0s	144.0s	143.9s
10	124.8d	123.8d	123.8d	124.0d
11	75.8d	76.4d	76.4d	76.4d
12	35.7t	31.6t	31.5t	31.5t
13	161.2s	163.8s	163.8s	163.8s
14	116.7d	114.0d	113.8d	113.8d
15	168.0s	168.2s	168.2s	168.2s
16	23.2q	23.2q	23.2q	23.2q
17	23.2q	23.2q	23.2q	23.2q
18	14.2t	27.0t	18.3t	127.7t
19	12.2q	16.8q	16.7q	16.8q
20	23.1t	64.3t	64.2t	64.2t

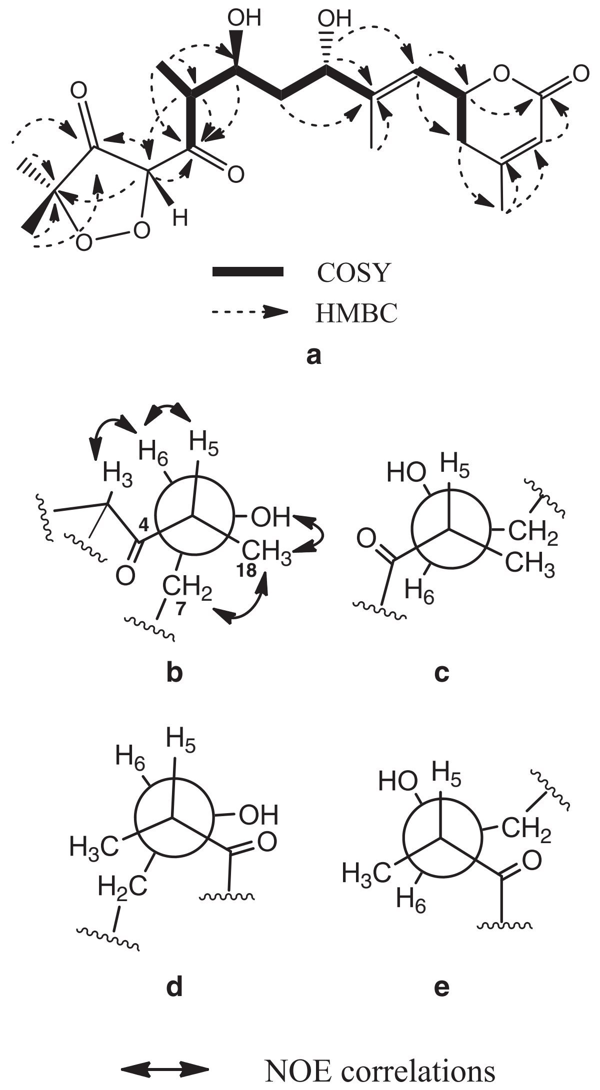

Fig. 2. Selected COSY, HMBC, NOESY correlations of **1** and the Newman projections of **1** viewed along C-5/C-6 (**2b**–**2e**).

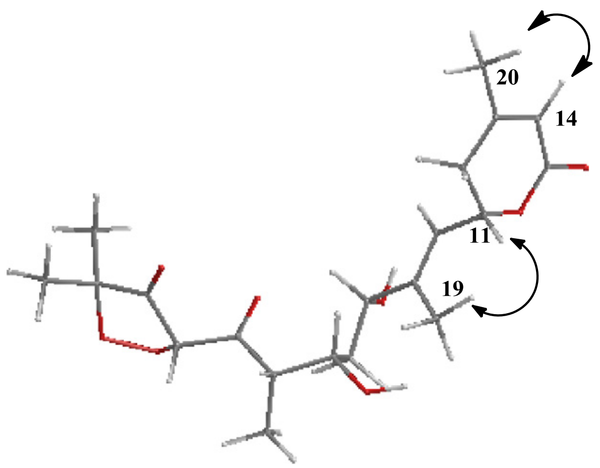

Fig. 3. Selected NOESY correlations of **1** (↔).

Connectivity of the five methyl groups to the diterpene skeleton and the linkage of C-8 and C-9 still relied on the observed HMBC correlations as shown in Fig. 2, respectively. In particular, the presence of α, β-unsaturated-δ-lactone ring was disclosed on basis of HMBC correlation between δ 5.62 (H-10) and δ 168.2 (C-15). The olefinic geometries were determined as 9*E* and 13*Z* according to the NOE interactions between H_3-19 and H-11, H_3-20 and H-14 as shown in Fig. 3 in association with the chemical shifts of C-19 (<20 ppm) and C-20 (>20 ppm) [20]. The absolute configurations of C-3 and C-11 stereogenic centers in **1** were assigned by CD analysis because the crystals were not available. Its CD spectrum, measured in MeOH, revealed a positive Cotton effect at 316 nm (Δε +2.62) and a negative Cotton effect at 255 nm (Δε −20.9) (Fig. 4). The negative Cotton effect observed at 255 nm corresponding to the n–π* transition of α, β-unsaturated-δ-lactone chromophore indicated an 11*S* configuration by comparing with model compounds [21]. The positive Cotton effect observed at 316 nm allowed the assignment of the absolute configuration of 3*S* according to the octant rule of five-membered ketone ring [22]. In order to further confirm these assignments, we calculated ECD spectrum by time dependent density functional theory (TDDFT) [23,24] and compared the result with the experimental CD data of **1**. The conformational analysis was performed by means of the semi-empirical PM3 method, as implemented in the program package Gaussian 09, starting from pre-optimized geometries generated by the MM2 force field in Chem 3D software 12.0 overlaid with key correlations observed in the NOESY spectrum. The corresponding minimum geometries were further optimized by DFT calculations at the B3LYP/6-31G (d) level. The calculated ECD of **1** matched the experimental result well, confirming the absolute configurations of 3*S* and 11*S* as depicted in Fig. 5. The absolute configuration of chiral centers of C-6 and C-8 in **1** was assigned by a further step using the CD data of the *in situ* formed $[Mo_2(OAc)_4]$ complex, with the inherent contribution subtracted (Figs. 5 and 6). The Mo-complex of **1** displayed a negative CE at λ_{max} 386.5 nm, correlating with 6*S* and 8*S* absolute configuration according to the rules established by Frelek and coworkers [25,26]. Then, the coupling constant of $J = 7.2$ Hz between H-5 and H-6 observed from the ^{1}H NMR spectrum suggested the dihedral angle φ of H-5/H-6 was near 0° or 150° as depicted in the Newman projections viewed along C-5/C-6 in Fig. 2 [27]. Together with the observed NOE correlations between H-6 (δ_H 3.81) and H-5 (δ_H 2.82), H-3 (δ_H 5.49) and H-6 (δ_H 3.81), and H_3-18 (δ_H 1.25) and H-7 (δ_H 1.71), as well as 6-OH (δ_H 4.87) and H_3-18 (δ_H 1.25) (in DMSO-d_6), these data revealed the favored conformation as shown in Fig. 2b, suggesting a 5*S* absolute configuration. Accordingly, the absolute configuration of **1** was determined to be 3*S*, 5*S*, and 11*S* (Fig. 1) and its structure was unequivocally assigned as depicted. Therefore, compound **1** was determined as a new diterpene and has been named Aphanaperoxide E.

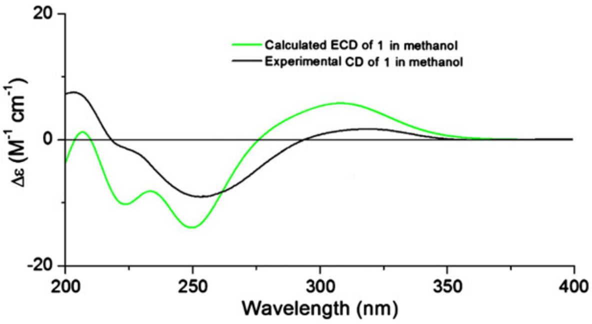

Fig. 5. Comparison of experimental CD spectrum of **1** with the calculated ECD spectrum of (3*S*, 11*S*)-**1**.

Compound **2** was isolated as colorless oil with molecular formular $C_{20}H_{28}O_8$. According to its molecular formula and NMR spectroscopic data, compound **2** was determined to have the same carbon framework as **1**. Its NMR spectrometric data were very similar to those of **1** except that the methyl group of C-20 (δ_C 23.1) in **1** was replaced by a hydroxymethyl moiety in **2** (δ_C 64.3), that signals due to 6-OH and 8-OH disappeared and that methine of C-5 in **1** was assigned as an oxygenated quaternary carbon in **2**, as confirmed by the HMBC cross peaks from H-3 (δ_H 5.61), H-6 (δ_H 1.64, 1.75), and H_3-18 (δ_H 1.43) to C-5 (δ_C 73.4). HMBC correlations between H-20 (δ_H 4.22), and C-12 (δ_C 31.6), C-13 (δ_C 163.8), and C-14 (δ_C 114.0) allowed the assignment of the location of C-20. Further detailed 2D NMR

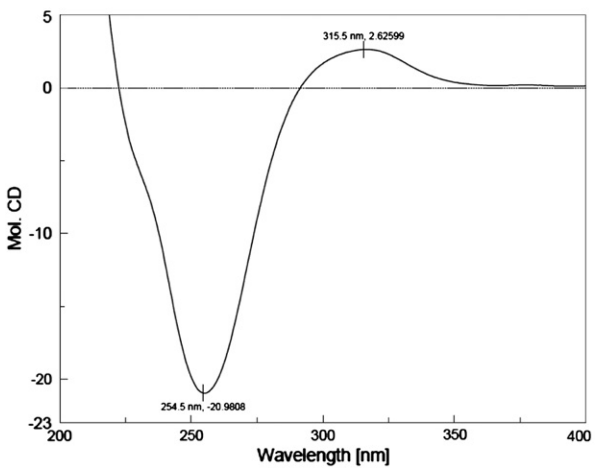

Fig. 4. CD spectrum (MeOH) of **1**.

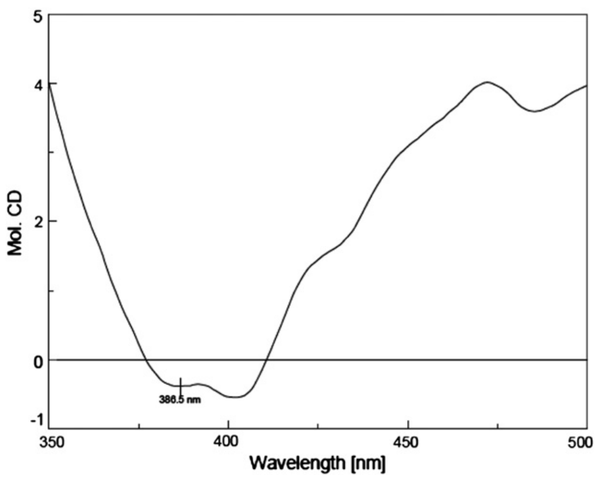

Fig. 6. CD spectrum of Mo complex of **1** with the inherent CD spectrum substracted.

spectroscopic analysis consequently confirmed the structure of **2**. Similarly, the olefinic geometries and the absolute configuration of C-3 and C-11 were established in the same manner as for **1**. Furthermore, the Rh-complex of **2** displayed a positive CE at λ_{max} 345.5 nm, correlating with a 5*S* absolute configuration [28,29]. Compound **2** was determined as a new diterpene and has been named Aphanaperoxide F.

Compounds **3** and **4** were obtained as a colorless oil in the form of a mixture with an approximately ratio of the amounts of 2:1 by integration of certain clearly discernible paired protons in their ^{1}H NMR spectrum. However, the two compounds could not be completely separated by any chromatographic methods used in present study. Fortunately, their spectroscopic data were considered to be sufficient for the structural elucidation described below. The elemental formula of **3** and **4** was determined to be $C_{20}H_{28}O_7$ and $C_{20}H_{26}O_7$, respectively, as deduced from their HRESIMS and NMR spectra, respectively. In comparison with **1**, the ^{1}H and ^{13}C NMR data of **3** revealed that both compounds **1** and **3** shared the same backbone, with the only differences being the presence of hydroxymethyl at C-20, and the disappearance of signals due to 6-OH and 8-OH. The connectivity of the hydroxymethyl moiety was confirmed by HMBC correlation of H-20 (δ_H 4.24) with C-12 (δ_C 31.5), C-13 (δ_C 163.8), and C-14 (δ_C 113.8). The spectroscopic data of **4** were closely comparable to those of **3**, except that the methyl group of C-18 in **3** was replaced by a terminal double bond in **4**. This was demonstrated by both NMR spectrometric data and the HMBC correlations between H-18 (δ_H 5.59, 6.10) and C-3 (δ_C 100.7), C-4 (δ_C 186.2), and C-5 (δ_C 140.2). The absolute configurations of the two compounds were established also based on their CD spectra. Compounds **3** and **4** were determined as new diterpenes and have been named Aphanaperoxides G and H, respectively.

The new isolates were tested for their cytotoxicities against four human cancer cell lines (HepG2, MCF-7, A-549, and AGS) using MTT method with Doxorubicin as the positive control. None of compounds showed cytotoxicity against tested cell lines (IC_{50} >20 μM) [4].

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

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