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Three new diterpenoids from *Euphorbia peplus*

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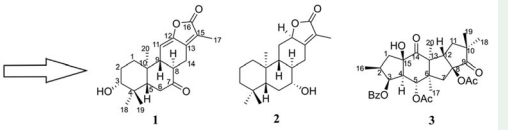
ABSTRACT

Three new diterpenoids (**1–3**) (two abietane type diterpenoids and a paralianone type diterpenoid), together with four known compounds (**4–7**) were isolated from the whole plants of *Euphorbia peplus*. Their structures were elucidated through spectroscopic analysis and physicochemical characteristics. The cytotoxic activities of compounds **1–7** against five human tumour cell lines were evaluated, however, they were inactive at the concentration of 40 μM. The compound **3** enhanced lysosomal biogenesis with Lyso Tracker staining intensity of 132.6%.



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1. Introduction

The annual herbaceous plant *Euphorbia peplus* (Euphorbiaceae) is native to Mediterranean coast, and was introduced into Yunnan province of China (Hohmann et al. 1999). It is used in folk medicine to treat asthma, catarrh and internal tumours, and recently applied in the treatment of warts, corns, waxy growths and skin cancers (Vasas et al. 2012; Ogbourne and Parsons 2014; Jian et al. 2018,). The characteristic diterpenoids in this plant show high structural diversity and broad biological activities, which attracts the attention of natural product and medicinal chemists (Gao et al.

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2007; Li et al. 2011; Tang, He, et al. 2012; Tang, Zhang, Gu, et al. 2012; Tang, Zhang, Yuan, et al. 2012; Li et al. 2013; Frezza et al. 2018; Adelakun, et al. 2019). Previously, we discovered 20-deoxyingenol and its analogues, which are typical diterpenoids from *E. peplus*, that could enhance lysosome biogenesis, and are capable of amyloid-β clearance from mice's brain (Li et al. 2016), suggesting that such compounds have the potential of being developed as drugs for the treatment of some neurodegenerative diseases. To find new and potentially bioactive diterpenoids from *E. peplus*, we investigated the chemical constituents of this plant and obtained three new diterpenoids namely 11,12-didehydro-8α,14-dihydro-7-oxo-helioscopinolide A (**1**), 7α-hydroxy-8α,14-dihydro jolkinolide E (**2**), 8β-acetyl-paralianone D (**3**) and four known ones (**4–7**). Their structures were elucidated based on extensive NMR and MS analyses. Herein, the isolation, structure elucidation, and *in vitro* cytotoxic activity of isolated compounds are reported.

2. Results and discussion

Compound **1** was obtained as a white powder. Its molecular formula was established to be $C_{20}H_{26}O_4$ by positive HR-ESI-MS (*m/z* 353.1726 $[M+Na]^+$, calcd. 353.1723), with 8 degrees of unsaturation. Its spectrum showed absorption bands at 3436, 1709 and 1767 cm^{-1}, indicating the presence of a hydroxyl group, a carbonyl group, and a lactone group, respectively. The ^1H NMR spectrum of **1** displayed signals for four methyl groups (δ_H 1.90, H_3-17; 0.86, H_3-18; 1.00, H_3-19; 1.09, H_3-20), one oxymethine proton (δ_H 3.30, dd, $J = 11.6$, 4.5 Hz, H-3), an olefinic proton (δ_H 5.68, d, $J = 2.0$ Hz, H-11), and a series of aliphatic groups. The ^{13}C NMR and DEPT spectra (Table S1, Figure S7–S12) of compound **1** showed 20 carbon signals, which were recognised as four methyls, four methylenes, five methines and four quaternary carbons, including two olefinic (150.9, C-13; 121.4, C-15), and two sp^3 quaternary carbons (δ_C 39.3, C-4; 37.0, C-10). The above information suggested structure of **1** was similar to that of the known 8α,14-dihydro-7-oxo-helioscopinolide A (Appendino et al. 1998), and the differences could be rationalised in terms of the presence of an additional double bond (δ_H 5.68, br s, δ_C 106.6; δ_C 146.5) and the absence of signals corresponding to C-11 methylene and C-12 methine of the known compound. ^1H– ^1H COSY correlation of H-9 (δ_H 2.21) to the proton (δ_H 5.68) as well as HMBC correlations of H-14 (δ_H 2.43; 3.06) to the sp^2 quaternary carbon (δ_C 146.5) and the additional proton (δ_H 5.68) to C-13 (δ_C 150.9) and C-10 (δ_C 37.0) established the location of the double bond between C-11 and C-12. Accordingly, the structure of **1** was established as shown in Figure 1. Detailed 2D NMR analyses (HSQC, ^1H– ^1H COSY, and HMBC) supported the planar structure and relative configuration (ROESY) of **1** (Supplementary information, Figures S1–S2).

Compound **2** was isolated as a white amorphous powder. HR-ESI mass spectrum of **2** showed ion peak at *m/z* 341.2095 $[M+Na]^+$ (calcd. for $C_{20}H_{30}NaO_3$: 341.2087). The ^1H, ^{13}C, DEPT and 2D NMR spectra (Table S1, Figures S13–S18) of compound **2** were closely similar to those of a previously reported compound 7β-hydroxy-8α,14-dihydro jolkinolide E (Marco et al. 1999), except for signals of γ-position carbons to 7-OH (δ_C 52.1, C-5; 47.5, C-9 for compound **2** and δ_C 46.8, C-5; 42.5, C-9 for 7β-hydroxy-8α,14-dihydro jolkinolide E) that were significantly downfield shifted. Clearly, the axial 7β-OH

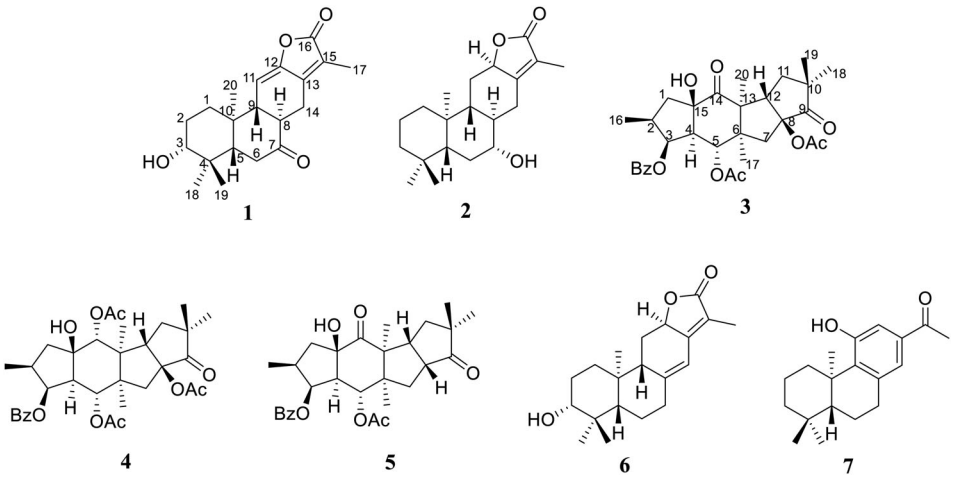

Figure 1. Molecular structures of **1–7** isolated from *Euphorbia peplus*.

in the molecule of 7β-hydroxy-8α,14-dihydro jolkinolide E shields C-5 and C-9 through the syn-orientated H-5 and H-9, whilst the equatorial 7α-OH in **2** does not (Wang et al. 2018). Besides, albeit overlapping of the signals of H-9(δH 1.11) and H-3(δH 1.13), cross-peak of δH 1.11/δH 3.28 could only be assigned to H-9/H-7, in that H-3 is located on ring A and is far away from H-7. Based on these analyses, an α- instead of β-orientation for the hydroxyl group at C-7 in compound **2** was determined (Figures S3–S4). Therefore, the structure of compound **2** was elucidated as shown in Figure 1.

Compound **3** was obtained as a white solid. The molecular formula $C_{31}H_{38}O_9$ was determined for compound **3** from a sodium adduct ion in HR-ESI-MS data at *m/z* 557.2412 $[M+Na]^+$ (calcd. for $C_{31}H_{38}NaO_9$: 557.2408), with 13 degrees of unsaturation. Its ^{1}H- and ^{13}C-NMR spectra (Table S2, Figures S19–S24) showed signals for two acetoxy groups, a benzoyloxy group, five methyls (one secondary and four tertiary), three methylenes, five methines (two oxygenated at δ_H 5.83, m, H-3; 6.33, d, $J = 12.0$ Hz H-5), three quaternary carbons, and two carbonyls (δ_C 216.9, C-9; 207.5, C-14). These functionalities accounted for 9 degrees of unsaturation, and thus, compound **3** should be a tetra-cyclic diterpenoid. The NMR data of **3** were similar to those of paralianone D (Wan et al. 2016), except for the presence of signals responsible for an acetoxy group, an additional oxygenated quaternary carbon (δ_C 91.9), and the absence of the proton signal at C-8, indicating that the acetoxy group was substituted at C-8. HMBC correlation of H-11 and H-12 with the additional oxygenated quaternary carbon further supported this possibility. Thus, the structure of compound **3** was finally elucidated as 8β-acetyl- paralianone D (Figure 1).

The known compounds, helioscopinolide A (**4**) (Borghi et al. 1991), 11-hydroxy-*ent*-abieta-8,11,13-trien-15-one (**5**) (Haba et al. 2009), paralianone (**6**) (Jakupovic et al. 1998), paralianone D (**7**), were identified by comparison of their spectroscopic data with those reported in the literature.

Compounds **1–7** were evaluated for cytotoxic activities against five human cell lines, (Leukaemia HL-60, lung cancer A-549, liver cancer SMMC-7721, breast cancer MCF-7, and colon cancer SW480) with paclitaxel and cisplatin as two positive controls.

Unfortunately, the results showed that these compounds were inactive at the concentration of 40 μM. The new compounds **1–3** were assessed for their activity to enhance lysosomal biogenesis through Lyso Tracker Red staining (Li et al. 2016; Zhao et al. 2018). Our results showed that compound **3** could increase the Lyso Tracker staining intensity of 132.6% (Figure S37), while compounds **1** and **2** have no obvious effect.

3. Experimental

3.1. General experimental

Optical rotation measurements were conducted with a Jasco P-1020 automatic polarimeter. CD spectra were determined on the Applied Photophysics circular dichroism spectrometer (Applied Photophysics, Leatherhead, Surrey, UK). IR spectra were recorded on a NICOLET iS107 Mid-infrared spectrometer. NMR spectra were measured on AVANCE III 500 MHz NMR spectrometers with TMS as the internal standard. High-resolution MS data were recorded on an Agilent 1290 UPLC/6540 Q-TOF mass spectrometer in positive mode. An Agilent 1260 series instrument equipped with a SunFire-C_{18} column (5 μm, 10 mm × 250 mm) and XSelect HSS T3(5 μm, 10 mm × 150 mm) were used for high-performance liquid chromatography (HPLC) Semipreparation. Silica gel (100 – 200, 200 – 300, 300 – 400) mesh (Qingdao Marine Chemical, Inc.), NH MB 100-40/75 Silica gel (FUJI SILYSIA CHEMICAL LTD.), Lichroprep RP-18 (40 – 63 μm, Fuji), and Sephadex LH-20 (20 – 150 μm, Pharmacia) was used for CC.

3.2. Plant material

The whole plant parts of *E. peplus* were collected in July 2018 from Kunming Botanical Garden, Yunnan Province, People's Republic of China. The plant was identified by Prof. Hu Shi-Jun (Kunming Institute of Botany, Chinese Academy of Sciences). A voucher specimen (no. kep-09-13) has been deposited in the herbarium of the Kunming Institute of Botany, Chinese Academy of Science.

3.3. Extraction and isolation

The air-dried whole plant parts of *E. peplus* (29 kg) were extracted with methanol (room temperature) thrice. The crude extract was obtained by reflux. After suspension in water, the combined extract was successively partitioned with petroleum ether, and ethyl acetate. The ethyl acetate extract (660 g) was then subjected to a silica gel column and eluted with a gradient of petroleum ether/ethyl acetate (100:0 to 0:100, v/v) to obtain 10 fractions, F1 – F10. Fraction F8 (79 g) was further subjected to MCI gel with MeOH-H_2O (40:60 to 100:0) to give 11 subfractions, F8-1 – F8-11. F8-8 (7.9 g) was further subjected to Sephadex LH-20 (MeOH/CH_2Cl_2 50:50) to obtain 6 subfractions, F8-8-1 – F8-8-6. F8-8-3 (2.1 g) was purified by silica gel column chromatography with petroleum ether/ethyl acetate (20:1 to 1:1, v/v) to give 8-8-3-6 (51.2 mg) and 8-8-3-7 (10.1 mg), and then by preparative HPLC (CH_3CN/H_2O, 45:55) to afford compounds **1** (6.7 mg), **2** (2.5 mg), **4** (11.9 mg), and **5** (8.3 mg). Following the same separation

method, fraction F7 (58 g) yielded F7-7-2 (65.8 mg), which was purified by preparative HPLC (CH_3CN/H_2O, 70:30) to afford compounds **3** (20.4 mg), **6** (18.0 mg), and **7** (4.3 mg).

3.4. Cytotoxicity assays

The cytotoxic activity of compounds **1–7** were evaluated using human Leukemia (HL-60), lung cancer (A-549), liver cancer (SMMC-7721), breast cancer (MCF-7), and colon cancer (SW480) cell lines. A single cell suspension was prepared by using a culture solution containing 10% fetal bovine serum (DMEM). The cytotoxicity assay was performed by using MTS assay (a brand new MTT analogue, 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfopheny)-2H-tetrazolium) in 96-well micro-plates. Briefly, adherent cells (100 μl) were seeded into each well of 96-well cell culture plates and allowed to adhere for 12 h before compound addition, while suspended cells were seeded just before compound addition with an initial density of 1×105 cells/mL. Test compounds were dissolved in DMSO and was initially sieved at a concentration of 40 μM then with 8, 1.6, 0.32, 0.0625 gradient to treat each tumour cell line in triplicates for 48 h, with cisplatin (DDP) and paclitaxel (Taxol) as two positive controls. Cell viability was then measured and cell growth curve was plotted.

3.5. Lysosomal biogenesis induction activity assays

The activity to enhance lysosomal biogenesis of compounds **1–3** were evaluated using HeLa cell line, which was cultured at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (HyClone), 100 U/ml penicillin and 100 mg/mL streptomycin. No cell lines used in this study were found in the database of commonly misidentified cell lines that is maintained by ICLAC and NCBI Biosample. HeLa cell was purchased from ATCC. Briefly, HeLa cells with 85% cell density in 96-well plates were treated with individual compounds at 40 μM in triplicate. Three hours later, cells were grown in fresh medium containing Lyso Tracker Red DND-99 (0.2 μM) for 30 min. Then, medium was changed to Lyso Tracker-free medium and images were taken with ArrayScan Infinity (Cellomics, ArrayScan VTI HCS). Positive compounds were subjected to validation by treating HeLa cells with two different concentrations (20 and 40 μM) and at 3 h in triplicate and staining with Lyso Tracker Red DND-99.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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