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Subnudatones A and B, new *trans*-decalin polyketides from the cultured lichen mycobionts of *Pseudopyrenula subnudata*

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ABSTRACT

Chemical investigation of the cultured polyspore-derived mycobionts of a *Pseudopyrenula subnudata* lichen led to the isolation of two new compounds, subnudatones A and B (**1** and **2**), together with four known compounds, 1-(2-hydroxy-1,2,6-trimethyl-1,2,4a,5,6,7,8,8a-octahydronaphthalen-1-yl)ethanone (**3**), libertalide C (**4**), aspermytin A (**5**), and 6,7-dimethoxy-4-hydroxymellin (**6**). Their chemical structures were elucidated by extensive 1D and 2D NMR analysis and high resolution mass spectroscopy, and comparisons were made with the literature. The absolute configuration of **1** was defined unambiguously using single crystal X-ray crystallography. Compound **1** represents the first dimeric decalin polyketide to be found in nature. The *in vitro* cytotoxicity of **1** against two cancer cell lines (K562 and MCF-7) was evaluated. Compound **1** showed moderate cytotoxic activity with IC_{50} values of 23.5 ± 1.0 and 51.9 ± 1.4 μM, respectively.

1. Introduction

Lichens are symbiotic associations of an algal or cyanobacterial photobiont and a fungal mycobiont, and produce a range of bioactive metabolites. However, the metabolites that are extracted from cultured lichen mycobionts are not detectable in natural lichens under stressed conditions. Phytochemical investigation of mycobionts cultured from Vietnamese lichens identified bioactive compounds with unique scaffolds [1–3]. In our search for metabolites of crustose lichen mycobionts, the mycobionts of *Pseudopyrenula subnudata* lichen were isolated and cultivated on MY10 at 18oC in the dark over several months. The colonies were then harvested and extracted with EtOAc. The extract was separated by a combination of chromatographic procedures to afford two new compounds, subnadatones A and B (**1** and **2**), along with four known compounds, 1-(2-hydroxy-1,2,6-trimethyl-1,2,4a,5,6,7,8,8a-octahydronaphthalen-1-yl)ethanone (**3**) [4], libertalide C (**4**) [5], aspermytin A (**5**) [6], and 6,7-dimethoxy-4-hydroxymellin (**6**) [7]. The structures of these compounds were determined by extensive spectroscopic analysis (1D, 2D-NMR, and HRESIMS) and single-crystal X-ray crystallography. Compound **1** was assessed for its *in vitro* cytotoxicity against the MCF-7 (breast cancer) and K562 (chronic myelogenous leukemia) cell lines.

2. Experimental

2.1. General experimental procedures

NMR spectra were measured on Bruker Avance II (500 MHz for ^{1}H

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NMR and 125 MHz for ^{13}C NMR) and Varian Mercury-400 Plus NMR (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR) spectrometers with TMS as internal standard. Chemical shifts were expressed in ppm with reference to the residual protonated solvent signals (chloroform-d_1 with δ_H 7.26 and δ_C 77.16, and dimethylsulfoxide-d_6 with δ_H 2.50 and δ_C 39.52). Crystallographic analyses of suitable single crystals were performed using an automatic diffractometer (Bruker Model D8 Quest) with graphite-monochromated Mo-*Kα* radiation (λ = 0.71073 A) at 100 K. The HRESIMS were recorded on a HRESIMS Bruker MicroTOF. TLC was carried out on precoated silica gel 60 F_{254} or Sephadex LH-20 and spots were visualized using UV_{254nm} and UV_{365nm} lamps. Gravity column chromatography was performed with silica gel 60 (0.040–0.063 mm).

2.2. Fungal isolation, cultivation, and identification

Specimens of *P. subnudata* Müll. Arg. were collected from tree bark in Quang Ngai City (15.10437 N; 108.80656 E), Vietnam (approx. 9 m alt.), in March 2017 by D.H. Le. The voucher specimens were identified by Dr. Vo Thi Phi Giao (University of Sciences, Ho Chi Minh City, Vietnam) and deposited at the University of Sciences, Ho Chi Minh City, Vietnam (registration No. QNg170301). Mycobionts were obtained from the spores discharged from the apothecia of a thallus, and were cultivated in test tubes containing modified MY10 medium (malt extract 10 g, yeast extract 4 g, sucrose 100 g, agar 15 g, H_2O 1 L, pH 7) at 18 °C in the dark. After cultivation for 4–8 months, the colonies were harvested.

2.3. Extraction and isolation

The harvested colonies (dry weight 31.5 g) were extracted with EtOAc (3 × 100 mL each) at room temperature, and the combined extracts were concentrated under reduced pressure to give a 608.8 mg residue. The EtOAc extracts were subjected to silica gel column chromatography (CC) and eluted by the solvent system *n*-hexane-EtOAc with increasing ethyl acetate ratios to yield eighteen fractions: EA1 to EA18. Fraction EA3 (12 mg) was subjected to reversed-phase CC using solvent system of MeOH-H_2O (10:1, *v*/v) as a mobile phase to afford **2** (2 mg), **3** (5.3 mg), and **4** (1.8 mg). Fraction EA9 (173 mg) was used for further isolation through reversed-phase CC using a solvent system of MeOH-H_2O (10:1, v/v), yielding **1** (44 mg). This was crystallized in MeOH, to obtain a single purified crystal. Fraction EA14 (109 mg) was subjected to a silica gel CC eluted with *n*-hexane-$CHCl_3$-EtOAc-acetone-AcOH (100:20:8:5:2) to give five subfractions (EA14.1-EA14.5). Fraction EA14.3 (25 mg) was selected for reversed-phase CC using MeOH-H_2O (5:1). Three subfractions (EA14.3.1-EA14.3.3) were obtained. Compound **5** was isolated from subfraction EA14.3.1 by crystallization in MeOH. Finally, subfraction EA14.3.3 (5 mg) was purified by a Sephadex LH-20 with MeOH-CH_2Cl_2 (1:1, *v*/v) as an eluent to afford **6** (1.2 mg).

2.3.1. Subnudatone A (1)

Colorless crystal; $[\alpha]_D^{22}$ + 115 (c 0.1, MeOH); UV (EtOH) λ_{max} (log *ε*) 204 (2.9), 285 (1.9) nm; ^{1}H ($CDCl_3$, 500 MHz) and ^{13}C ($CDCl_3$, 125 MHz) NMR data, see Table 1; HRESIMS *m/z* 493. 3321 $[M + H]^+$ (calcd for $C_{32}H_{45}O_4$, 493.33129); 515.3015 $[M + Na]^+$ (calcd for $C_{32}H_{44}O_4Na$, 515.3137).

2.3.1.1. X-ray crystallographic analysis of ***1***. Single crystals of **1** were obtained by vapor diffusion of MeOH, in the form of colorless crystals. Crystal analysis was conducted using a D8 Quest Diffractometer with Mo-Kα radiation (λ = 0.71073 Å) at 100.0 [2] K. The structures were solved by the intrinsic phasing method (ShelXT) and refined using a full-matrix least-squares difference Fourier technique (ShelXL). The positions of hydrogen atoms were determined using 'riding' mode. The crystallographic data for Compound 1 has been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: 44-(0)1223–336,033, or e-mail: deposit@ccdc.cam.ac.uk). Crystal data of 1:$C_{32}H_{44}O_4$, Mr. = 492.67, orthorhombic, a = 6.238 [1] Å, b = 12.317 [1] Å, c = 36.844 [2] Å, α = 90.00, β = 90.00, γ = 90.00, V = 2830.6 [3] Å^3, space group $P2_12_12_1$, Z = 4, D_{calcd} = 1.156 g/cm^3, μ = 0.074 mm^{-1}, and F (000) = 1072.0. Crystal dimensions: 0.25 × 0.25 × 0.10 mm^3. Independent reflections: 70333 (R_{int} = 0.0561). The final R1 values were 0.0482, ωR2 = 0.0876 ($I > 2\sigma(I)$). The goodness of fit on F2 was 1.066. Flack parameter: 0.2 [3]. CCDC number: 1954725.

2.3.2. Subnudatone B (2)

Colorless oil; $[\alpha]_D^{22}$ + 315 (c 0.1, MeOH); UV (EtOH) λ_{max} (log *ε*) 204 (2.5), 283 (1.7) nm; ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 1; HRESIMS *m/z*: $[M + Na]^+$ 269.1514 (calcd for $C_{16}H_{22}O_2Na$, 269.1517).

2.4. Cytotoxicity assay

The cytotoxicity of **1** was evaluated against the MCF-7 (breast cancer) and K562 (chronic myelogenous leukemia) tumor cell lines, cultured in RPMI 1640 medium and in DMEM medium, respectively. The cultures were supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycin, and maintained at 37 °C, 5% CO_2, and 95% humidity. Viable cells were counted and inoculated into a 96-well plate at densities of 10^4 cells/100 μL/well for MCF-7 and 10^5 cells/100 μL/well for K562. After 24 h, the cells were treated with the two compounds, using doxorubicin as a positive control. They were diluted in the culture media at concentrations of 100, 50, 25, 12.5, 6.25, 3.125, and 0 μg/mL, containing 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, and 0% dimethyl sulfoxide (DMSO), respectively. The DMSO was used as a negative control, and culture medium without cells was used as a blank. All experiments were done in triplicate. The plates were incubated at 5% CO_2, 95% humidity, and 37 °C for 72 h. 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL stock solution) was added to each well and incubated at 37 °C in 5% CO_2 for 3.5 h. 70 μL of Detergent Reagent (10% SDS) was added to each well and the plate was maintained at 37 °C for 16 h. The optical density of each well was read by a scanning multiwall spectrophotometer (Sunrise) at a wavelength of 595 nm. Cell survival was measured by comparing the percentage absorbance with that of the negative control.

3. Results and discussion

Compound **1** was isolated as a colorless crystal. The molecular formula was established to be $C_{32}H_{44}O_4$ on the basis of the positive-ion mode HRESIMS data with a sodiated ion peak at *m/z* 515.3015 $[M + Na]^+$ (calcd. 515.3137 for $C_{32}H_{44}O_4Na$). The ^{1}H NMR and HSQC spectra showed seven olefinic protons (δ_H 7.50, 7.45, 7.02, 5.67, 5.46, 5.37, and 5.37, each 1H), four tertiary methyl protons (δ_H 1.42, 1.22, 1.26, and 1.17, each 3H), two secondary methyls (δ_H 0.91 and 0.93, each 3H), and high field protons in the range 0.85 to 2.83 ppm. The ^{13}C and DEPT NMR spectra revealed 32 carbons, including two ketone carbons (δ_C 204.2 and 197.8), seven olefinic methine carbons (δ_C 162.6, 135.3, 133.2, 132.4, 130.6, 128.0, and 123.5), five quaternary carbons (δ_C 114.4, 87.8, 74.2, 56.2, and 48.3) of which two (δ_C 87.8 and 74.2) were oxygenated, six methyl carbons (δ_C 28.2, 23.0, 22.6, 22.5, 13.9, and 12.1), six sp^3 methylene carbons (δ_C 41.8, 41.2, 35.7, 35.6, 28.2, and 27.7), and six sp^3 methine carbons (δ_C 43.7, 42.7, 41.3, 39.1, 33.7, and 33.6). The chemical features were similar to those of 16-carbon polyketides, having a *trans*-fused decalin ring that is common in marine-fungal and lichen-fungal sources [8–11].

The HMBC correlations identified the first spin system, later referred to as subunit A. The HMBC correlation of protons at H-1 (δ_H

Table 1
^{1}H and ^{13}C NMR data for compounds **1** and **2** ($CDCl_3$).

	1[a]					**2**[b]		
Position	**Subunit A** δ_H, mult (*J*, Hz)	δ_C	Position	**Subunit B** δ_H, mult (*J*, Hz)	δ_C	Position	δ_H, mult (*J*, Hz)	δ_C
1	7.35, 1H, s	162.6	1’	7.50, 1H, d (15.5)	135.3	1	6.98, 1H, d (5.6)	157.5
2		114.4	2’	7.02, 1H, d (15.5)	123.5	2	5.22, 1H, d (6.0)	106.6
3		197.8	3’		204.2	3		200.6
4		48.3	4’		56.2	4		48.5
5	1.64, 1H, td (12.0, 2.0)	42.7	5’	1.83, 1H, m	43.7	5	1.63, 1H, td (12.0, 2.5)	42.7
6	2.82, 1H, dq (12.0, 2.5) 1.18, 1H, m	27.7	6’	1.71, 1H, m 1.07, 1H, m	28.2	6	2.82, 1H, dq (12.0, 3.2) 1.17, 1H, m	27.6
7	1.77, 1H, m 1.78, 1H, m	35.6	7’	1.79, 1H, m 0.83, 1H, m	35.7	7	1.77, 2H, m	35.7
8	1.50, 1H, m	33.6	8’	1.50, 1H, m	33.7	8	1.49, 1H, m	33.6
9	1.80, 1H, m 0.85, 1H, m	41.8	9 ‘	1.80, 1H, m 0.85, 1H, m	41.2	9	1.80, 1H, m 0.85, 1H, m	41.2
10	1.93, 1H, m	41.3	10’	1.87, 1H, m	39.1	10	1.92, 1H, m	41.1
11	5.46, 1H, dd (10.0, 2.5)	132.4	11’	5.37, 1H, brs	130.6	11	5.75, 1H, dd (10.0, 2.8)	131.8
12	5.66, 1H, dd (10.0, 2.0)	128.0	12’	5.37, 1H, brs	133.2	12	5.42, 1H, dd (10.0, 2.0)	128.7
13		87.8	13’		74.2	13		86.3
14	1.42, 3H, s	23.0	14’	1.17, 3H, s	28.2	14	1.44, 3H, s	22.6
15	1.22, 3H, s	13.9	15’	1.26, 3H, s	12.1	15	1.28, 3H, s	14.3
16	0.91, 3H, d (6.5)	22.5	16’	0.93, 3H, d (6.5)	22.6	16	0.91, 3H, d (6.8)	22.2

[a] ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR.
[b] ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR.

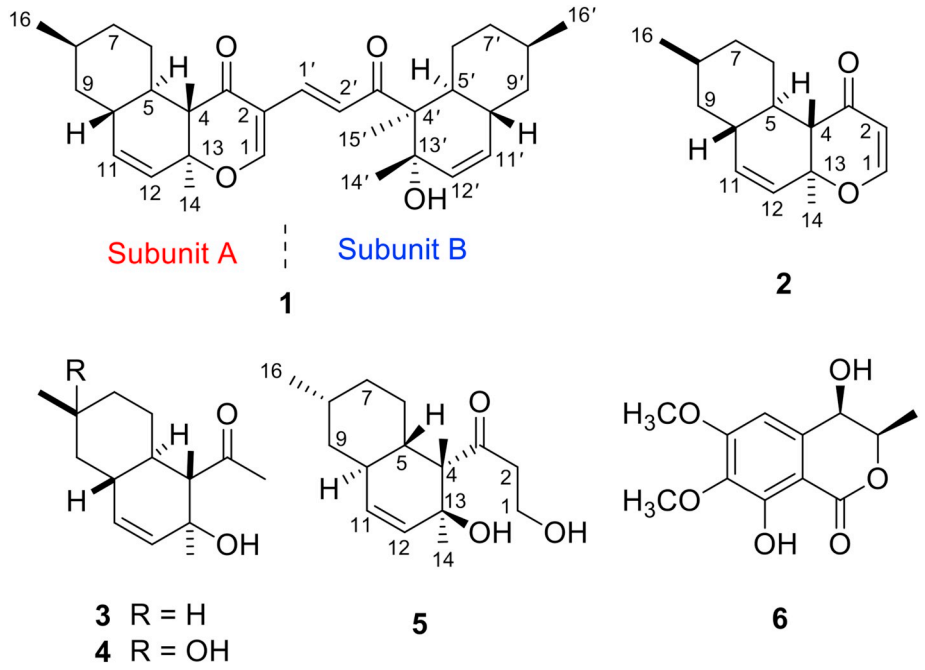

Fig. 1. Chemical structures of compounds **1–6**.

7.35) to carbon at C-3 (δ_C 197.8) and C-2 (δ_C 114.4); H_3–15 (δ_H 1.22) to C-3 (δ_C 197.8) and C-4 (δ_C 48.3); and H_3–14 (δ_H 1.42) to C-4 (δ_C 48.3) and C-13 (δ_C 87.8) defined the connectivity as C-1-C-2-C-3-C-4-C-13. The downfield ^{13}C chemical shift of C-1, together with the key HMBC correlation of H-1 to C-13, indicated ether linkage between C-1 and C-13. Furthermore, HMBC correlations of H_3–14 to C-12 (δ_C 128.0), and of both olefinic protons H-11 (δ_H 5.46) and H-12 (δ_H 5.66) to C-13, established the vicinity of these groups. The rest of the subunit A could be defined from $^{1}H-^{1}H$ COSY correlations of H-11/H-10/H-7/H-9/H-8/H_3–16 and H-8/H-7/H-5. Finally, HMBC correlations of H-5 (δ_H 1.64) to C-4 and C-3 validated the C-3-C-4-C-5 connectivity. The large coupling constant of H-5 (12.0 Hz) confirmed the *trans*-decalin conformation of subunit A, further supported by the NOESY correlations. NOESY correlations of H_3–14 (δ_H 1.42)/H-5 (δ_H 1.64) and H_3–15 (δ_H 1.22)/H-10 (δ_H 1.93) were observed, while the absence of NOESY correlations between the two methyls indicated the *syn*-orientation of H-5/H_3–14 and H-10/H_3–15. The NMR data on subunit A were strongly reminiscent of those of versiol [8] with the exception of an additional double bond at C-1 and C-2. Accordingly, subunit A was characterized as shown in Fig. 1.

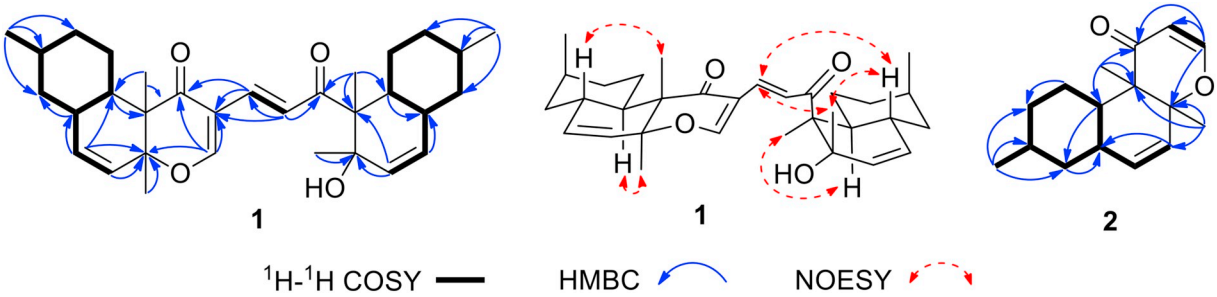

Fig. 2. Key ^{1}H–^{1}H COSY, HMBC, and NOESY correlations of compounds **1** and **2**.

In subunit B, two *trans* coupled olefinic protons at δ_H 7.50 (H-1′) and 7.02 (H-2′) gave HMBC correlation to C-2. Conversely, proton H-1 (δ_H 7.35) showed HMBC correlation to C-1′, indicating connectivity between subunits A and B at C-2. HMBC cross peaks of H-1′ (δ_H 7.50) and H_3–15′ (δ_H 1.26) to C-3 and C-4, and H_3–14′ (δ_H 1.17) to C-4′ (δ_C 56.2), suggested connectivity through C-1′ to C-4′. Moreover, the methyl H_3–14′ gave an HMBC cross peak to C-12′ (δ_C 133.2), indicating a vicinal C-11′ and C-12′ double bond. COSY correlations from H-5′ to H-12′ validated the remaining signals of subunit B. In this subunit, NOESY correlations of H-5′/H-1′/H_3–14′ defined their co-facial side. Likewise, a NOESY cross peak between methyl H_3–15′ and H-5′ indicated that they were in the same orientation. These chemical features were very similar to those of aspermytin A (**5**), with the exception of an additional double bond at C-1 and C-2.

The absolute stereochemistry of **1** was determined using single-crystal X-ray crystallography. A qualified single crystal was successfully obtained and an ORTEP diagram identified a *trans*-decalin conformation in both subunits A and B as well as other chiral centers (see Fig. 3). The chemical structure of **1** was elucidated as that of subnudatone A, and is shown as Fig. 1.

Compound **2** was obtained as colorless oil. Its molecular formula was determined as $C_{16}H_{22}O_2$ by the positive HRESIMS measurement through the ion peak at *m/z* 269.1514 (calcd. 269.1517 for $C_{16}H_{22}O_2Na$). The ^{1}H NMR and HSQC spectra showed two singlet methyl groups [δ_H 1.44 (3H, s, H_3–14) and 1.28 (3H, s, H_3–15)], four olefinic protons [δ_H 6.98 (1H, d, J = 5.6 Hz, H-1), 5.65 (1H, dd, J = 10.0, 2.8 Hz, H-12), 5.42 (1H, dd, J = 10.0, 2.0 Hz, H-11), 5.22 (1H, d, J = 6.0 Hz, H-2), one secondary methyl group [δ_H 0.88 (3H, d, J = 6.8 Hz, H_3–16)], and protons in the range 1.00 to 3.11 ppm. The ^{13}C and DEPT NMR spectra revealed 16 carbon resonances attributable to one ketone carbonyl group (δ_C 200.6), three methyl carbons (δ_C 22.8, 14.5, and 14.3), three methylene carbons (δ_C 41.4, 35.7, and 27.6), four sp^2 methine carbons (δ_C 157.5, 131.8, 128.7, and 106.6), three sp^3 methine carbons (δ_C 42.7, 41.2, and 33.6), and two quaternary carbons (δ_C 86.3 and 48.5) of which one (δ_C 86.3) was oxygenated. NMR data of **2** were highly similar to those of the subunit A of **1**. Indeed, the HMBC correlations of protons at H-1 (δ_H 6.98) to carbon at C-3 (δ_C 200.6), C-2 (δ_C 106.6); H_3–15 (δ_H 1.28) to C-3 and C-4 (δ_C 48.5); H_3–14 (δ_H 1.44) to C-4 (δ_C 48.5) and C-13 (δ_C 86.3) defined the connectivity through C-1-C-2-C-3-C-4-C-13. The relative configuration of **2** was defined due to the analysis of the coupling constant values and NOESY correlations. Particularly, the *trans*-decalin conformation of **2** was determined by the large coupling constant of H-5 (12.0 Hz) and further backed up by NOESY data (see Fig. S14). Accordingly, the structure of **2** was established and named as subnudatone B.

To date, over 40 *trans*-decalin polyketides with similar scaffolds [6,8–11] have been isolated. Very rare decalins having a C-1-C-2 double bond have been reported, but no dimeric compounds were found. The linkage between monomers A and B made our skeleton unique. To the best of our knowledge, subnudatone A (**1**) is the first example of a dimer comprising two *trans*-fused decalin moieties. Subnudatone A (**1**) bears striking similarities to co-isolated compounds, subnudatone B (**2**) and aspermytin A (**5**), proposing that **1** should be a C-acylated product of two monomers. First, compound **5** was dehydrated to form the monomer B. Then, this activated monomer enables the cross-coupling reaction with **2** (subunit A) to provide **1** [12]. Compound **3** was synthesized previously by Inoue and coworkers when these authors conducted the total synthesis of aspermytin A [4]. Therefore, this compound was found as a new natural compound.

The cytotoxicity against K562 and MCF-7 cell lines of **1** was investigated. Activity against these two cancer cell lines was moderate, with IC_{50} values of 23.5 ± 1.0 and 51.9 ± 1.4 μM, respectively.

4. Conclusions

In this study, six compounds (**1–6**) were isolated from the cultured lichen mycobionts of *P. subnudata* and their structures were elucidated as subnudatones A and B (**1** and **2**), 1-(2-hydroxy-1,2,6-trimethyl-1,2,4a,5,6,7,8,8a-octahydronaphthalen-1-yl)ethanone (**3**), libertalide C (**4**), aspermytin A (**5**), and 6,7-dimethoxy-4-hydroxymellin (**6**). To the best of our knowledge, compound **2** is a new compound and represents the first example of *trans*-decalin polyketide with a C-1-C-2 double bond, while compound **1** is the first example of a novel dimer comprising two *trans*-fused decalin skeletons. On the other hand, compound **3** is a new natural compound. Compound **1** showed moderate cytotoxic activity against K562 (IC_{50} 23.5 ± 1.0 μM) and MCF-7 (IC_{50} 51.9 ± 1.4 μM) cell lines Fig. 2.

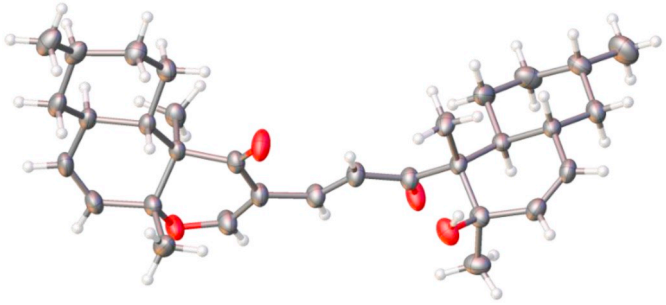

Fig. 3. ORTEP drawing of compound **1**.

Declaration of Competing Interest

No potential conflict of interest was declared by the authors.

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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.2020.104512.

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