DOI 10.1007/s10600-022-03592-2
Chemistry of Natural Compounds, Vol. 58, No. 1, January, 2022

SESQUITERPENOIDS FROM THE EDIBLE MUSHROOM *Pleurotus nebrodensis*

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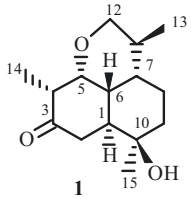
*Pleunebron A (**1**), a new cadinane sesquiterpenoid, together with three known drimane sesquiterpenoids (**2–4**), was isolated from cultures of the edible mushroom* Pleurotus nebrodensis*. Their structures were elucidated on the basis of extensive spectroscopic methods. Compounds **1** and **2** were tested for their cytotoxicities against five human cancer cell lines and for their inhibitory activity against isozymes of 11*β*-hydroxysteroid dehydrogenases (11*β*-HSD).*

Keywords: *Pleurotus nebrodensis*, sesquiterpenoids, 11*β*-hydroxysteroid dehydrogenases.

The fungus *Pleurotus nebrodensis* only grows on limestone and is an edible mushroom belonging to the order *Agaricales*. The polysaccharide of *P. nebrodensis* was reported to exhibit anticancer properties and immune-stimulating activity [1–3] and offer cardiac protective properties [4]. In our ongoing investigation on structurally interesting and biologically active natural products from higher fungi [5–8], an EtOAc extract of cultures of the edible mushroom *P. nebrodensis* was subjected to investigation, which resulted in the isolation of a new cadinane sesquiterpenoid, named pleunebron A (**1**), together with three known drimane sesquiterpenoids, 3*β*-hydroxydrimenol (**2**) [9], 3*β*-hydroxydihydroconfertifolin (**3**), and 3*β*-hydroxycinnamolide (**4**) [10]. Their structures were established by extensive spectroscopic data analysis. The cytotoxicity of compounds **1** and **2** against five human cancer cell lines and their inhibitory activity against isozymes of 11*β*-hydroxysteroid dehydrogenases (11*β*-HSD) were evaluated.

Compound **1**, a colorless oil, gave a molecular formula of $C_{15}H_{24}O_3$ by HR-ESI-MS at *m/z* 252.1723 $[M]^+$ (calcd 252.1725), corresponding to four degrees of unsaturation. The IR spectrum showed absorption bands attributable to hydroxy (3447 cm^{-1}) and carbonyl (1706 cm^{-1}) groups. The ^{13}C NMR and DEPT experiments displayed 15 carbons, including a ketone carbonyl group (δ 211.3), one oxygenated quaternary carbon (δ 71.8), six methines (oxygenated one at δ 80.4), four methylenes (oxygenated one at δ 66.5), one tertiary methyl, and two secondary methyls. The four degrees of unsaturation were accounted for by a ketone carbonyl and the presence of a three-ring system.



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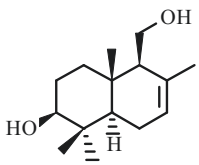

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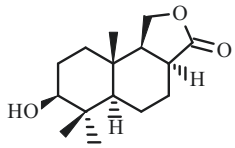

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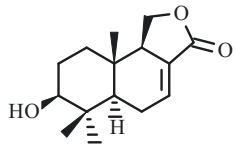

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The ^1H–^1H COSY spectrum indicated partial structures; three fragments [a: -CH(4)-CH(5)-CH(6)-CH(1)-CH_2(2)-; b: -CH(1)-CH(6)-CH(7)-CH_2(8)-CH_2(9)-; c: -CH(5)-CH(6)-CH(7)-CH(11)-CH_2(12)-] were established as shown. The HMBC cross-peaks from H-14, H-4, and H-2 to C-3 (δ 211.3) indicated the presence of a six-membered carbon ring A with a methyl at C-14 (δ 10.0). In the HMBC spectrum, Me-15 showed correlations to C-1 (δ 44.1), C-10 (δ 71.8), and C-9 (δ 34.2).

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TABLE 1. ^{1}H (500 MHz) and ^{13}C (150 MHz) NMR Spectral Data of Compound **1** ($CDCl_3$, δ, ppm, J/Hz)

C atom	δ_H	δ_C	C atom	δ_H	δ_C
1	1.78 (1H, m)	44.1	9	1.71 (1H, m); 1.61 (1H, m)	34.2
2	2.57 (1H, m); 2.38 (1H, m)	38.5	10	–	71.8
3	–	211.3	11	1.55 (1H, m)	36.6
4	3.07 (1H, m)	43.3	12	3.63 (1H, m); 3.27 (1H, m)	66.5
5	3.53 (1H, m)	80.4	13	0.89 (3H, d, J = 5.8)	14.3
6	2.02 (1H, m)	41.3	14	1.10 (3H, d, J = 6.3)	10.0
7	1.55 (1H, m)	35.9	15	1.40 (3H, s)	28.5
8	2.03 (1H, m); 1.08 (1H, m)	27.4			

Meanwhile, H-9 also showed critical HMBC correlations to C-1, which indicated that a six-membered carbon ring B was established by C-1, C-6, C-7, C-8, C-9, and C-10. In addition, the HMBC correlation of H-5 with C-12 (δ 66.5), as well as the requirement of degrees of unsaturation, revealed that C-5, C-6, C-7, C-11, C-12 and an oxygen atom formed a six-membered ring C. In the ROESY spectrum of **1**, the correlations of H-4 and H-5, H-5 and H-6, H-6 and H-7, H-7 and H-4, Me-13 and H-8β, H-1 and Me-15, and H-8α and Me-15 indicated the β-orientations of H-4, H-5, H-6, H-7, and Me-13 and the α-orientations of H-1, H-11, Me-14, and Me-15. Therefore, compound **1** was established to be pleunebron A.

Compounds **1** and **2** were evaluated for their cytotoxicity against five human cancer cell lines using the MTT method as reported previously [11]). Unfortunately, no compound showed significant activity (IC_{50} values > 8 μM). In addition, the inhibitory effects of **1** and **2** on human and mouse 11β-HSD1 were also investigated. The results showed that compound **1** had inhibitory activity against 11β-HSD1 (human IC_{50} = 33.73 μg/mL; mouse IC_{50} = 1.69 μg/mL).

EXPERIMENTAL

General Experimental Procedures. Optical rotations (OR) were recorded on a Jasco P-1020 digital polarimeter. UV data were obtained on a Shimadzu UV-2401A spectrophotometer. Infrared spectroscopy (IR) spectra were obtained on a Bruker Tensor27 FT-IR spectrometer with KBr pellets. NMR spectra were obtained on Bruker AV-400 and DRX-500 instruments and a Bruker Avance III 600 MHz spectrometer with TMS as internal standard. Mass spectra were recorded on a VG Autospec-3000 mass spectrometer and an API QSTAR Pulsar I spectrometer. Silica gel (200–300 mesh, Qingdao Marine Chemical Ltd., China) and Sephadex LH-20 (Amersham Biosciences, Sweden) were used for open column chromatography (CC). Fractions were monitored by TLC. Spots were visualized by heating silica gel plates immersed in vanillin-H_2SO_4 in ethanol.

Fungal Material and Cultivation Conditions. *Pleurotus nebrodensis* was provided and fermented by Zheng-Hui Li, Kunming Institute of Botany. A voucher specimen (No. CGBWSHF00011) was deposited in the Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences. The culture medium consisted of glucose (5%), peptone from porcine meat (0.15%), yeast powder (0.5%), KH_2PO_4 (0.05%), and $MgSO_4$ (0.05%). Fermentation was carried out on a shaker at 160 RPM for 25 days.

Extraction and Isolation. The culture broth (25 L) was filtered, and the filtrate was extracted three times with EtOAc, while the mycelium was extracted three times with $CHCl_3$–MeOH (1:1). The EtOAc layer together with the mycelium extraction was concentrated under reduced pressure to give a crude extract (6.0 g), and this residue was subjected to CC over silica gel (200–300 mesh) eluted with a gradient of $CHCl_3$–MeOH (1:0→0:1) to obtain four fractions (A–D). Fractions C and D eluted with petroleum ether–acetone (5:1) was separated repeatedly by reverse-phased RP-18 (MeOH–H_2O, 1:1) CC, followed by Sephadex LH-20 (Me_2CO) to afford **1** (3.7 mg), **2** (3.0 mg), and a mixture of **3** and **4** (2.0 mg), respectively.

Pleunebron A (1), a colorless oil; $[\alpha]_D^{25}$ +1.4° (*c* 0.30, MeOH). HR-ESI-MS *m/z* 252.1723 $[M]^+$ (calcd for $C_{15}H_{24}O_3$, 252.1725). IR (KBr, ν_{max}, cm^{-1}): 3441, 2935, 1727, 1653, 1667, 1454, 1127. For ^{1}H (500 MHz, $CDCl_3$) (300 K) and ^{13}C NMR (150 MHz, $CDCl_3$) (300 K) spectral data, see Table 1.

Cytotoxicity Assay. Five human cancer cell lines, breast cancer SK-BR-3, hepatocellular carcinoma SMMC-7721, human myeloid leukemia HL-60, pancreatic cancer PANC-1, and lung cancer A-549 cells, were used in the cytotoxic assay. Cells were cultured in RPMI-1640 or in DMEM medium (Hyclone, USA), supplemented with 10% fetal bovine serum (Hyclone, USA) in 5% CO_2 at 37°C. The cytotoxicity assay was performed according to the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide) method in 96-well microplates [11]. Briefly, 100 μL of adherent cells were seeded into each well of 96-well cell culture plates and allowed to adhere for 12 h before addition of test compounds, while suspended cells were seeded just before drug addition with initial density of 1×10^5 cells/mL. Each tumor cell line was exposed to the test compound at concentrations of 0.0625, 0.32, 1.6, and 8 μM in triplicate for 48 h, with cisplatin (Sigma, USA) as positive control. After treatment of the compound, the cell viability was detected, and a cell growth curve was plotted. The IC_{50} values were calculated using Reed and Muench′s method [12].

Inhibition on 11β-HSD1 Activity Assays. The inhibition activity of the compounds on human or mouse 11β-HSD1 enzymatic activities was determined in the scintillation proximity assay (SPA) using microsomes containing 11β-HSD1 as described in previous studies [13]. Briefly, the full-length cDNAs of human or murine 11β-HSD1 were isolated from the cDNA libraries provided by the NIH Mammalian Gene Collection and cloned into a pcDNA3 expression vector. HEK-293 cells were transfected with the pcDNA3-derived expression plasmid and selected after cultivation in the presence of 700 μg/mL of G418. The microsomal fraction overexpressing 11β-HSD1 was prepared from HEK-293 cells stably transfected with 11β-HSD1 and used as the enzyme source for the scintillation proximity assay (SPA). Microsomes containing human or mouse 11β-HSD1 were incubated with NADPH and [^{3}H]cortisone, and then the product [^{3}H]cortisol was specifically captured by a monoclonal antibody coupled to protein A-coated SPA beads. All experiments were done in duplicate with glycyrrhizic acid as positive control. IC_{50} (± S.D., n = 2) values were calculated using Prism Version 4 (GraphPad Software, San Diego, CA, USA). IC_{50} values of glycyrrhizic acid (positive control) are 5.41 and 8.42 nM for mouse 11β-HSD1 and human11β-HSD1, respectively.

ACKNOWLEDGMENT

This project was supported by the Ningxia Normal University Research Grant (NXSFZDA1904), the First-Rate Discipline (Education) Construction Project of the Higher Education Institutions of Ningxia (NXYLXK2017B110, China, the Natural Science Foundation of Ningxia, China (2019AAC03245), the Key Research Foundation of Ningxia, China (2019BDE03009), the "Domestic Talent 312 Program" of Ningxia, China (NDB2018.139), the Guyuan Science and Technology Support Program (2018GYYQ0007 and 2019GKGY039), the National Natural Science Foundation of China (31760090), the Program for Innovation Talents of Science and Technology in Ningxia, China (KJT2015005), Youth Talent Cultivation Project in Ningxia, China (NXRS2017(279)), the Guyuan Talent Support Program (2019GYSZZB1202), and the Engineering and Technology Research Center of Liupanshan Resources Grant (HGZD20-02), Ningxia Normal University, China.

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