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Eryngiolide A, a Cytotoxic Macrocyclic Diterpenoid with an Unusual Cyclododecane Core Skeleton Produced by the Edible Mushroom *Pleurotus eryngii*

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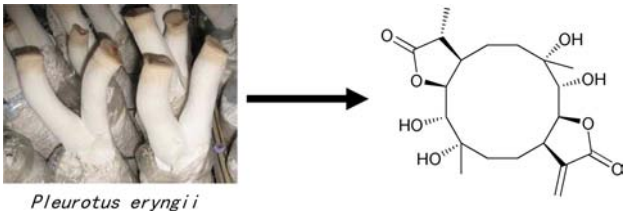
ABSTRACT



Eryngiolide A (1), the first member of C20 diterpenoids with the skeleton deriving from a cyclododecane core fused with two γ-lactone units, was isolated from the solid culture of the edible mushroom *Pleurotus eryngii*. The structure of 1 was elucidated by extensive analysis of NMR spectra. Compound 1 exhibited moderate cytotoxicity against two human cancer lines in vitro.

Mushroom-forming fungi of the genus *Pleurotus* have been known as a source of biologically active secondary metabolites. Examples include the antibacterial mutilin and pleuromutilin derivatives from *P. mutilus*,[1] the cytotoxic and antibacterial pleurotin from *P. griseus*,[2] the cytotoxic illudane sesquiterpenoids from *P. japonicus*,[3] and the nematocidal fatty acid derivatives from *P. pulmonarius*.[4] To search for bioactive metabolites from *Pleurotus*, 60 strains of *Pleurotus* including *P. ostreatus*, *P. sapidus*, *P. cornucopiae*, *P. cystidiosus*, *P. nebrodensis*, *P. citrinopileatus*, and *P. djamor* were collected and cultured by solid state fermentation.

The α,β-unsaturated lactone moiety has been regarded as one of the most significant structural characteristics for many biologically active natural products, such as terpene trilactones from *Ginkgo biloba*,[5] the antitumor macrolides from marine bryozoan *Bugula neritina*,[6] and the cytotoxic and antibacterial cembranolides from soft coral.[7] Through HPLC-DAD on line analysis, the solid culture extract of a strain of *P. eryngii* was found to contain secondary metabolites with the characteristic ultraviolet absorption at 220 nm due to the α,β-unsaturated lactone moiety in structures. To obtain new bioactive metabolites, a chemical investigation was conducted on the ethyl acetate extract of the solid culture of *P. eryngii* fermented on cooked rice. As a result, one novel macrocyclic diterpene possessing one previously undescribed skeleton originating from a cyclododecane core fused with two γ-lactone units (**1**) and two known lactones, 1,2-dihydroxymintlactone (**2**)[8] and

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5-hydroxy-3,4,5-trimethylfuran-2(5*H*)-one (**3**),[9] were obtained (Figure 1). Details of the isolation, structure elucidation, cytotoxicity of these three metabolites, and the possible biosynthetic pathway for **1** and **2** are reported herein.

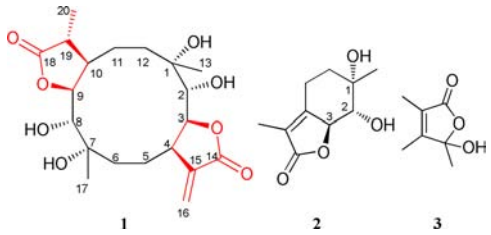

Figure 1. Structures of compounds **1**–**3**.

The molecular formula of eryngiolide A (**1**) was established as $C_{20}H_{30}O_8$ on the basis of high-resolution ESI-MS (found: *m*/*z* 421.1834 $[M + Na]^+$, calcd: *m*/*z* 421.1833) requiring 6 degrees of unsaturation. Analysis of the ^1H and ^{13}C NMR data of **1** in methanol-d_4 with the aid of an HSQC spectrum revealed resonances for three methyls [δ_H 1.19 (d, J = 6.8 Hz), 1.23 (s), and 1.27 (s); δ_C 14.0, 26.8, and 26.8], five methylenes including one olefinic methylene [δ_H 5.64 (d, J = 3.5 Hz), 6.20 (d, J = 3.5 Hz), δ_C 120.2), seven methines including four oxymethines [δ_H 3.04 (d, J = 8.2 Hz), δ_C 78.8; δ_H 3.33 (overlapped with solvent signals), δ_C 77.4; δ_H 4.47 (dd, J = 8.2 Hz), δ_C 84.7; δ_H 4.63 (dd, J = 8.2 Hz), δ_C 84.7], five quaternary carbons including two oxygenated carbons (δ_C 73.1 and 73.5), an olefinic carbon (δ_C 139.5), and two ester carbonyl carbons (δ_C 173.1 and 182.0) (Table 1). These data accounted for all the ^1H and ^{13}C resonances and required **1** to be tricyclic. The HMBC spectrum of **1** showed correlations among H-3/C-2, C-14, and C-15; H-9/C-10, C-18, and C-19; H-10/C-8, C-9, C-11, C-12, and C-20; H_3-13/C-1, C-2, and C-12; H_2-16/C-4, C-14, and C-15; H_3-17/C-6, C-7, and C-8; and H_3-20/C-10, C-18, and C-19 (Figure 2), which in combination with the cross peaks of H-2/H-3, H-3/H-4, H-4/H_2-5, H-5/H_2-6, H-8/H-9, H-9/H-10, H-10/H_2-11, H-10/H-19, H_2-11/-H_2-12, H-19/H_3-20 in the $^1H-^1H$ COSY spectrum established the skeleton of **1** (Figure 2). The downfielded chemical shifts of H/C-3 [δ_H 4.63 (dd, J = 8.2 Hz), δ_C 84.7] and H/C-9 [δ_H 4.47 (dd, J = 8.2 Hz), δ_C 84.7], as well as the unsaturation degree requirement of **1**, also supported the formation of the γ-lactone unit at C-3 and C-9, respectively. The chemical shifts of C-1 (δ_C 73.1), C-2 (δ_C 78.8), C-7 (δ_C 73.5), and C-8 (δ_C 77.4) and the molecular formula of **1** indicated that C-1, C-2, C-7, and C-8 all bear a hydroxyl group to complete the gross structure of **1**. In addition, four hydroxyl protons [δ_H 4.31 (s, 1-OH), 5.14 (d, 7.0, 2-OH), 4.20 (s, 7-OH), and 4.98 (d, 7.0, 8-OH)] were observed and assigned by the ^1H NMR, $^1H-^1H$ COSY, and NOESY spectrum of **1** recorded in DMSO-d_6 (Table 1).

The relative stereochemistry for **1** was established by analysis of its NOESY spectrum recorded in the solvent of MeOH-d_4 and DMSO-d_6, respectively. Key NOEs of H-2/H_3-13, H-2/H-6α, H-3/H-4, H-5β/H_2-16, H-6β/H_3-17, H-8/H_3-17, H-8/H-11β, H-9/H-10, H-11α/H_3-20, and H-11β/H-19 indicated the α-configuration for 1-OH, 2-OH, 3-H, 4-H, 7-OH, 8-OH, 9-H, 10-H, and 20-CH_3 (Figure 2). In Figure 2, the 3D-presentation of **1** originated from the conformation with minimized energy by MM2 calculation using CS Chem 3D software.

The structural assignment and relative stereochemistry of **1** was also supported by comparison of its NMR data with those of the benzofuran-2-one moiety in cheimonophyllon E (**4**) (Figure 3). Compound **4** was first isolated from basidiomycete fungus *Cheimonophyllum candidissimum*, and the coupling constant between H-3 and H-4 in **4** (numbered as H-1 at δ_H 4.35 (dd, J = 8.0, 8.0 Hz) and H-6 at δ_H 2.91 (m) in the literature, respectively) was reported as 8.0 Hz.[10] The structure of **4** was further confirmed by asymmetric total syntheses, and the ^1H NMR data for H-3 (numbered as H-1 in the literature) was observed at δ_H 4.36 (t, J = 8.0 Hz).[11] From comparison with the known compound **4**, as well as the NOE correlation between H-3 and H-4, the syn cofiguration of C-3 and C-4 can be definitely assigned. The dihedral angle of H-3–C-3–C-4–H-4 in the conformation of **1** with miminal energy is calculated as 6.6° (Figure 2), which in part explains the relative larger coupling constant between H-3 and H-4.

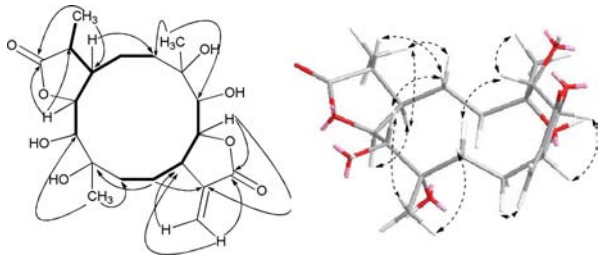

Figure 2. Selected HMBC (H→C), $^1H-^1H$ COSY (bold lines), and NOESY (arrows) correlations of **1**.

The absolute configuration of the secondary alcohol carbon (C-2 and C-8) was determined by application of the modified Mosher method.[12] The ^1H NMR signals of **1a** and **1b** were assigned by analysis of $^1H-^1H$ COSY spectrum. The $\Delta\delta$ values of the (*S*)- and (*R*)-MTPA esters **1a** and **1b**, respectively, indicated the 2*R* and 8*R* configuration (Figure 4). Considering the relative configuration established,

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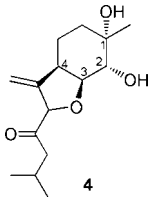
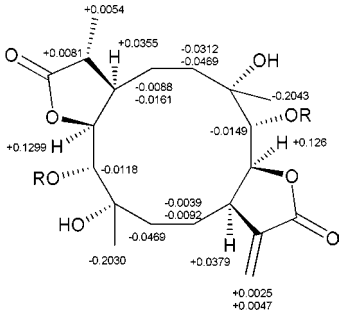


Figure 3. Structure of cheimonophyllon E (**4**) isolated from *Cheimonophyllum candidissimum*.

the absolute configuration of **1** was determined as 1*S*, 2*R*, 3*S*, 4*R*, 7*S*, 8*R*, 9*S*, 10*R*.



1a: R = (*S*)-MTPA ester
1b: R = (*R*)-MTPA ester

Figure 4. $\Delta\delta$ values (in ppm) $=\delta_S - \delta_R$ obtained for (*S*)- and (*R*)-MPTA esters **1a** and **1b**.

Compounds **1**–**3** were tested for their cytotoxic effects against two human cancer cell lines, Hela and HepG2 using the MTT method. Interestingly, **1** showed moderate toxicities against two cell lines with IC_{50} values of 20.6 and 28.6 μM, respectively, while **2** and **3** exhibited no inhibitory activity at the concentration of 100 μM.

The significance of compound **1** can be accounted for by virtue of the unprecedented macrocyclic carbon skeleton and its unique biosynthetic route postulated in this study. Compound **1** represents the first member of C20 diterpenoids with the skeleton deriving from a cyclododecane core fused with two γ-lactone units. Compound **1** is composed of two similar parts, each bearing a resemblance to the structure of **2** in the feature of vicinal dihydroxyl and γ-lactone moieties. Biogenetically, compounds **1** and **2** are secondary metabolites generated via the isoprenoid pathway. 1,2-Dihydroxylmintlactone (**2**) produced by the fungus *P. eryngii* could be synthesized from menthol via a classical monoterpene biosynthetic pathway as those described for mintlactone in *Mentha piperita*.[13] Considering the structural similarity between **1** and **2**, compounds **5** and **6** could be the plausible biosynthetic precursor for **1**.

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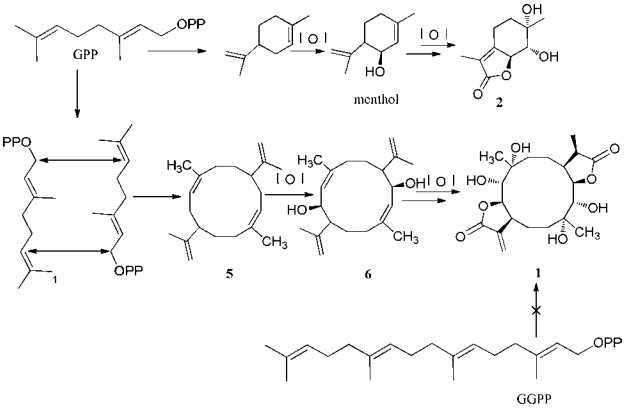
Table 1. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) Data of **1**[a]

	1 (MeOH-d_4)		**1** (DMSO-d_6)
position	δ_C	δ_H (mult; *J* in Hz)	δ_H (mult; *J* in Hz)
1	73.1		
2	78.8	3.04 (d, 8.2)	2.81 (dd, 7.0, 8.2)
3	84.7	4.63 (dd, 8.2, 8.2)	4.46 (dd, 8.2)
4	41.6	3.35 (o)[b]	3.29 (m)
5	20.3	1.91 (m, H-β);	1.80 (m, H-β);
		2.17 (m, H-α)	2.01 (m, H-α)
6	33.5	1.47 (m, H-α);	1.31 (qd,5.3, 16.2, H-α);
		1.62 (m, H-β)	1.45 (m, H-β)
7	73.5		
8	77.4	3.33 (o)[b]	3.18 (dd, 7.0, 8.2)
9	84.7	4.47 (dd, 8.2, 8.2)	4.27 (dd, 8.2)
10	44.8	2.40 (m)	2.28 (m)
11	20.3	1.61 (m, H-β);	1.52 (m, H-β);
		2.08 (m, H-α)	1.91 (m, H-α)
12	33.9	1.61 (m)	1.43 (m)
13	26.8	1.23 (s)	1.08 (s)
14	173.1		
15	139.5		
16	120.2	5.64 (d, 3.5);	5.62 (d, 3.7);
		6.20 (d, 3.5)	6.07 (d, 3.7)
17	26.8	1.27 (s)	1.12 (s)
18	182.6		
19	37.7	2.58 (m)	2.60 (m)
20	14.0	1.19 (d, 6.8)	1.05 (d, 6.8)
1-OH			4.31 (s)
2-OH			5.14 (d, 7.0)
7-OH			4.20 (s)
8-OH			4.98 (d, 7.0)

[a] The coupling pattern of H-4, H-5, H-6, H-10, H-11, H-12, and H-19 was too complex to afford accurate coupling constants. [b] Signals overlapped with the solvent signal of MeOH-d_4.

Compound **5** can be formed by a [6 + 6] cycloaddition from two molecules of geranyl pyrophosphate (GPP) (Scheme 1). It can be postulated that the enzymes responsible for the biosynthesis of **1** and **2** in *P. eryngii* may be the same or share much in common. To confirm this, direct evidence is necessary from biosynthetic studies using

Scheme 1. Plausible Biogenetic Origin of **1** and **2**



purified enzymes isolated from this fungus. To the best of our knowledge, diterpenes reported so far are biogenetically produced from geranylgeranyl pyrophosphate (GGPP). Compound **1** could be the first diterpene not synthesized from GGPP, which indicates a completely new route for diterpene biosynthesis in nature. We believe that the discovery of this novel skeleton will definitely attract interest from synthetic chemists and biosynthetic researchers.

The current research on edible mushroom *P. eryngii* provides evidence that mushroom-forming fungi have great potential to produce natural products with unprecedented structure. The value of mushrooms as a source of bioactive agents deserves further investigation.

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Supporting Information Available. Experimental section, NMR data of eryngiolide A and its Mosher esters (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.