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NOTE

Pleurone, a novel human neutrophil elastase inhibitor from the fruiting bodies of the mushroom *Pleurotus eryngii* var. *ferulae*

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The genus *Pleurotus* (Jacq.: Fr.) Kumm. (Pleurotaceae, higher Basidiomycetes) comprises a diverse group of cultivated mushroom species with high nutritional value and significant pharmacological properties. In the past decade, compounds with medicinal properties, including antiviral,[1] antitumor,[2] antibacterial,[3] antibiotic,[4] anticholesterologenic[5] or immunostimulatory[6] effects, have been isolated from several *Pleurotus* spp.[7] However, the constituents of *Pleurotus eryngii* var. *ferulae*, a mushroom popular in China for its flavor and high nutritive value, have not been thoroughly examined for their potential medicinal properties.

P. eryngii var. *ferulae*, the ferulae mushroom, grows on the medicinal plant *Ferula assa-foetida* in central China. A previous study revealed that a crude extract of *P. eryngii* var. *ferulae* has significant free-radical-scavenging, acetylcholinesterase-inhibitory and brain cell-protective effects.[8] Recently, an ethanol (EtOH) extract of the fruiting bodies of *P. eryngii* var. *ferulae* was reported to show a strong antitumor activity against three human solid carcinomas, a lung carcinoma (A549) and two cervical carcinomas (SiHa and HeLa).[9]

In searching for novel, naturally occurring inhibitors of human neutrophil elastase (HNE), a serine protease found in the azurophilic granules of neutrophils, we found that the EtOAc-soluble fraction of a methanol (MeOH) extract of *P. eryngii* var. *ferulae* sporocarps has considerable HNE-inhibitory activity (IC_{50}, 62.9 $\mu g\, ml^{-1}$). Further investigation of this fraction resulted in the isolation of one new compound (**1**), together with three known compounds (**2**–**4**). This report describes the isolation and structural elucidation of these compounds, as well as the characterization of their HNE-inhibitory effects.

The dried sporocarps of *P. eryngii* var. *ferulae* (4 kg, cultured in Cheonan, Korea) were extracted with MeOH (8 l) at room temperature for 7 days, filtered and concentrated to yield a MeOH extract (110 g). This extract was suspended in H_2O (2 l) and then partitioned successively with *n*-hexane (3 l) and ethyl acetate (EtOAc, 4 l) to yield *n*-hexane- and EtOAc-soluble fractions (20 and 8 g, respectively). The EtOAc-soluble fraction, which significantly inhibited HNE, was subjected to chromatography on a silica gel column. Elution with a gradient solvent system consisting of CH_2Cl_2–MeOH (50:1 → 1:1) yielded four fractions A–C. Fraction A (1.1 g) was applied to a silica gel column and eluted using *n*-hexane–acetone (10:1), yielding compound **2** (25 mg). Fraction B (0.8 g) was applied to the same silica gel column and eluted using a hexane–acetone gradient solvent system (10:1 → 5:1), yielding compound **3** (8 mg). Chromatography of fraction C (1.8 g) on the silica gel column using a CH_2Cl_2–MeOH gradient solvent system (10:1 → 1:1) yielded four subfractions (C1–C4). Subfraction C2 (0.2 g) was further purified by preparative reverse phase–high-performance liquid chromatography on a YMC-pack Pro C_{18} (YMC, Tokyo, Japan) (250 × 10 mm) column (UV detection at 254 nm) using MeOH–H_2O (20:80, v/v) at a flow rate of 2.0 $ml\, min^{-1}$ as the mobile phase. This procedure yielded compounds **1** (5 mg) and **4** (4 mg) with retention times (t_R) of 6.5 and 5.2 min, respectively.

Compound **1**, designated 'pleurone,' was obtained as an amorphous white powder with the following spectral characteristics: UV (MeOH) λ_{max} (log ε): 227 nm (3.2), 258 nm (3.8); IR (KBr) ν_{max}: 1714, 1654 cm^{-1}; ^{1}H-NMR (400 MHz, dimethyl sulfoxide-d_6): δ 5.44 (d, *J*=7.6 Hz, H-3), 7.38 (d, *J*=7.6 Hz, H-4; Supplementary Information); ^{13}C NMR (100 MHz, dimethyl sulfoxide-d_6): δ 151.5 (C-2), 164.3 (C-4), 100.2 (C-5), 142.2 (C-6; Supplementary Information); electron ionization MS *m/z* (rel. int.): 114 $[M]^+$ (2), 112 $[M-2H]^+$ (100), 78 (18), 69 (49), 68 (18), 64 (16), 63 (22); high-resolution electron ionization MS *m/z*: 113.9950 $[M]^+$ (calcd for $C_4H_2O_4$, 113.9953).

The molecular ion peak at *m/z* 114 $[M]^+$ obtained by high-resolution electron ionization MS is in accordance with the molecular

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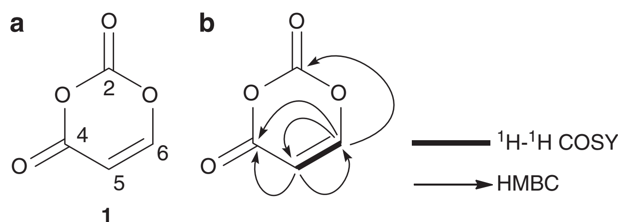

Figure 1 Chemical structure (**a**) and ^{1}H-^{1}H COSY and HMBC correlations (**b**) of pleurone (**1**).

formula $C_4H_2O_4$, indicating that **1** has a six-membered ring structure containing two oxygen atoms and four carbon atoms. Compound **1** also yielded a UV absorption peak at 258 nm and an IR band at 1714 cm^{-1}, suggesting the presence of an α,β-unsaturated lactone system. The ^{1}H-NMR spectrum of **1** contained peaks at δ_H 5.44 (d, J=7.6 Hz) and δ_H 7.38 (d, J=7.6 Hz), indicating two olefinic protons. Analysis by ^{13}C NMR, combined with DEPT, revealed that **1** contains two sp^2 methine carbons (at δ_C 100.2 and 142.2) and two lactone carbonyl carbons (at δ_C 151.5 and 164.3). All protonated carbons and their protons were assigned by ^{1}H-^{1}H COSY and HMQC experiments. The carbonyl carbons at δ_C 151.5 and 164.3 were assigned to C-4 and C-2, respectively, by an HMBC experiment showing long-range correlations between the carbon at δ_C 151.5 and H-6 (δ_H 7.38), as well as between the carbon at δ_C 164.3 and H-5 (δ_H 5.44) and H-6 (δ_H 7.38) (Figure 1). On the basis of the above data, the structure of pleurone was established as 4*H*-1,3-dioxine-2, 4-dione (**1**).

Comparison of the physicochemical and spectral data for compounds **2**, **3** and **4** with those in the literature identified these compounds as ergosterol (**2**),[10] (24*E*)-3β-hydroxycucurbita-5,24-diene-26-oic acid (**3**)[11] and nicotinic acid (**4**).[12] To the best of our knowledge, this is the first report of the constituents of *P. eryngii* var. *ferulae*.

The inhibitory activity of the isolated compounds **1**–**4** on HNE was evaluated according to a previously described procedure.[13] Briefly, 100-μl reactions containing 10 mM Tris-HCl buffer (pH 7.5), 1.4 mM MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide, 0.18 U HNE (EC 3.4.21.37, from Serva, Heidelberg, Germany), and various concentrations of sample were incubated in the wells of a 96-well plate for 2 h at 37 °C in the dark. Each reaction was stopped by the addition of 100 μl of soybean trypsin inhibitor (0.2 mg ml^{-1}), and the absorbance at 405 nm was immediately measured using a microplate reader. Epigallocatechin gallate was used as a positive control. As shown in Table 1, all of the tested compounds except nicotinic acid (**4**) moderately inhibited HNE. The IC_{50} values for **1**, **2**, and **3** ranged from 49.4 to 61.5 μM; in contrast, the IC_{50} value for the positive control, epigallocatechin gallate, was 8.8 ± 0.8 μM. Although pleurone exhibited a relatively low HNE-inhibitory activity compared with epigallocatechin gallate, it might be more useful as a new HNE inhibitor due to its relatively high water solubility and low MW.

To further characterize the HNE-inhibitory behavior of pleurone, kinetic studies were carried out in the same reaction medium in the presence of 0, 30 or 100 μM pleurone at substrate concentrations ranging from 0.25 to 1 mM. Reactions were started by the addition of diluted substrate and recorded over a time interval of 10 min. Estimations of the maximal velocity (V_{max}) and K_m were made according to Eisenthal and Cornish-Bowden.[14] The kinetic data are shown as a Lineweaver–Burk plot in Figure 2. The oxidation of HNE

Table 1 HNE inhibitory activity of compounds isolated from the fruit bodies of *P. eryngii* var. *ferulae*[a]

Compounds	IC_{50} (*μM*)[b]
Pleurone (**1**)	61.5 ± 1.2
Ergosterol (**2**)	49.4 ± 2.3
(24*E*)-3β-Hydroxycucurbita-5,24-diene-26-oic acid (**3**)	59.1 ± 3.5
Nicotinic acid (**4**)	>100
EGCG[c]	8.8 ± 0.8

Abbreviations: EGCG, epigallocatechin gallate; HNE, human neutrophil elastase.
[a]Results are expressed as means ± s.d. (*n*=3).
[b]IC_{50} indicates the concentration (μM) at which the inhibition percentage of HNE activity was 50%, and the values were determined by regression analysis.
[c]Positive control.

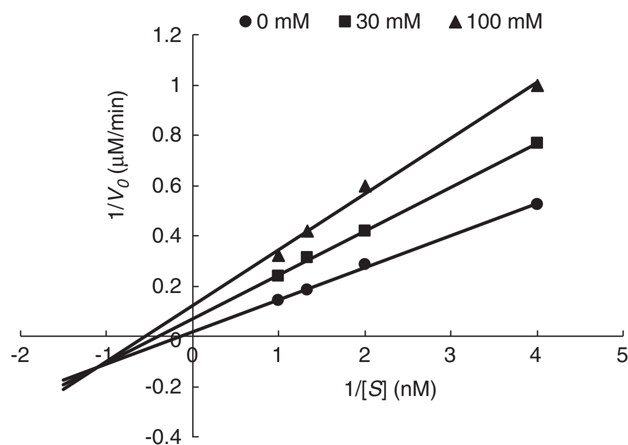

Figure 2 Lineweaver–Burk plot for inhibition of human neutrophil elastase by pleurone. The concentrations of pleurone were 0 (●), 30 (■) and 100 μM (▲).

by pleurone followed Michaelis–Menten kinetics under the experimental conditions used. The intersection of the best-fit lines for the uninhibited enzyme and for the two different concentrations of pleurone occurs to the left of the $1/V_0$ axis and below the $1/[S]$ axis on the Lineweaver–Burk plot, indicating that both K_m and V_{max} decreased with increasing pleurone concentration. Thus, the inhibitory mechanism of pleurone on HNE is a mixed-type, a combination of noncompetitive and uncompetitive inhibition against HNE.

Despite its name, HNE has broad substrate specificity and can cleave not only elastin but also other extracellular matrix proteins, such as collagen, fibronectin, laminin and proteoglycan.[15] Under normal physiological conditions, tissue repair after wounding requires the activity of elastase, which degrades foreign proteins within the extracellular matrix during phagocytosis.[13] Because the pharmacologic inhibition of HNE might prevent the loss of skin elasticity, thus preventing skin sagging during aging, efforts to discover potent inhibitors of HNE have increased considerably in the last several years. These efforts have focused on natural products as a rich source of potential HNE inhibitors. Our findings suggest that *P. eryngii* var. *ferulae* and its components might be beneficial for the prevention or treatment of skin aging.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)