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Clitocybin D, a Novel Human Neutrophil Elastase Inhibitor from the Culture Broth of *Clitocybe aurantiaca*

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Clitocybin D, a novel human neutrophil elastase inhibitor, was isolated from the culture broth of *Clitocybe aurantiaca*. This compound was purified by solvent extraction, silica gel column chromatography, Sephadex LH-20 column chromatography, and preparative HPLC. The compound was determined to be 4-(4,6-dihydroxy-3-methoxy-3H-isoindol-1-yl)-benzoic acid on the basis of 1D and 2D NMRs and MS spectroscopic analysis. Analysis of the human neutrophil elastase (HNE) inhibitory activity of the isolated compound revealed that it showed significant HNE inhibitory activity with an IC_{50} value of 17.8 μM.

Keywords: *Clitocybe aurantiaca*, clitocybin D, human neutrophil elastase

The skin is composed of two tissue layers, the epidermis and the dermis. The epidermis is a thin protective layer that consists primarily of keratinocytes, melanocytes, and Langerhans cells. The dermis is a thick complex layer of connective tissue that is primarily composed of collagen and elastic fibers [11]. Collagen is the major structural protein of skin and is responsible for its tensile strength and toughness. Elastic fibers are crucial to the resilience and elasticity of skin, even though they make up only 1–2 percent of its dry weight [3, 9]. Human neutrophil elastase (HNE), a serine protease primarily located in the azurophil granules of polymorphonuclear leukocytes, has broad substrate specificity. Indeed, HNE is able to break down elastin, an insoluble elastic fibrous protein, as well as connective tissue proteins such as fibronectin, collagen, and cartilage [1]. Biologically, elastase activity significantly increases with age, which results in reduced skin elasticity [7, 12]. Therefore, inhibition of the elastase activity can also be used as a method of protecting against skin aging [10].

In the course of our screening program for HNE inhibitors, we isolated a novel compound, clitocybin D (**1**, Fig. 1), from the culture broth of *Clitocybe aurantiaca* [2, 4, 6]. Here, we report the isolation, structure elucidation, and biological activities of **1**. We were provided with this strain from the staff of the mushroom taxonomy laboratory at the National Institute of Agricultural Science and Technology, Suwon, Republic of Korea (MKACC 53745).

The *C. aurantiaca* strain was maintained in solid potato dextrose agar medium. A piece of the *C. aurantiaca* strain from a mature plate culture was added to a 500-ml Erlenmeyer flask containing 100 ml of medium composed of 2% glucose, 0.5% polypeptone, 0.2 % yeast extract, 0.1% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ (pH 5.7). The strain was cultured on a rotary shaker (140 rpm) at 28°C for 7 days. After 7 days of culture, the entire 3-l culture was harvested, and the culture broth was then separated

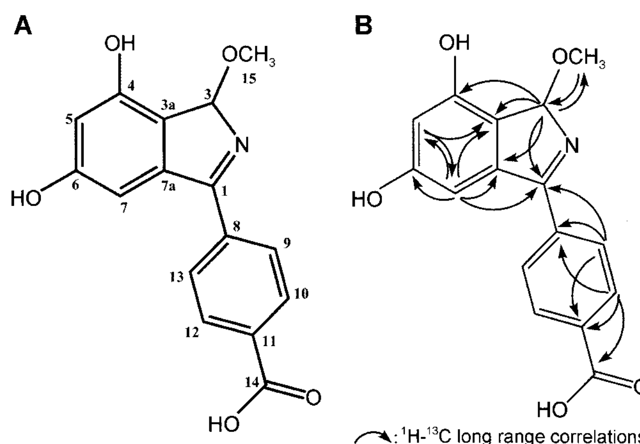

Fig. 1. Structure (**A**) and HMBC correlations (**B**) of clitocybin D.

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from the mycelia by filtration [5]. Next, the mycelia were extracted with 80% aqueous acetone. The culture broth and the evaporated mycelium extract were then combined and the extract was concentrated *in vacuo* to provide an aqueous solution, which was then extracted three times with equal volumes of EtOAc. The concentrated EtOAc extract (710 mg) was then applied to a column of silica gel and eluted with methanol–chloroform [1:50–1:1 (v/v)]. Next, the active fractions were combined and concentrated *in vacuo* to give an oily residue. The residue dissolved in MeOH was then further purified by Sephadex LH-20 column chromatography and eluted with methanol. An active fraction was then rechromatographed on a Sephadex LH-20 column with methanol:water [7:3 (v/v)], followed by preparative HPLC using a YMC-pack ODS-A column (10 mm i.d.×150 mm) eluted with 60% aqueous methanol with a retention time of 8.0 min to give **1** (4 mg, 0.56 % of EtOAc extract).

Compound **1** was obtained as a yellowish powder; $[\alpha]_D^{20}$ −1.80 (*c* 0.5, MeOH); UV ($CHCl_3$) λ_{max} nm (log ε): 210 (1.6), 256 (0.4), 298 (0.9); IR (KBr) ν_{max} (cm^{-1}): 3,398, 2,925, 1,699, 1,516, 1,453, 1,256, 1,211, 950; ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) are summarized in Table 1; HR–EI–MS (*m/z*): 299.0786 $[M]^+$ (calculated for $C_{16}H_{13}NO_5$, 299.0794). The molecular formula of **1** was established as $C_{16}H_{13}NO_5$ from electron ionization (EI) mass and NMR spectral data. The positive EI mass of **1** provided ion peaks at *m/z* 299.0786 $[M]^+$, suggesting a molecular weight of 299. The UV spectrum of **1** showed an absorption maximum at 210, 256, and 298 nm. IR absorptions at 3,398 and 1,699 cm^{-1} implied the presence of hydroxyl groups and a carboxylic moiety, respectively. A heteronuclear multiple-quantum coherency

Table 1. ^1H and ^{13}C NMR data of clitocybin D in methanol-d_4.

Position	Clitocybin D	
	δ_C	δ_H
1	162.8	
3	100.5	6.04 (s)
3a	116.1	
4	155.0	
5	109.8	6.56 (d, *J*=1.8 Hz)
6	161.1	
7	107.2	6.98 (d, *J*=1.8 Hz)
7a	128.3	
8	144.4	
9, 13	122.6	7.88 (d, *J*=9.0 Hz)
10, 12	132.1	8.06 (d, *J*=9.0 Hz)
11	133.3	
14	170.2	
15	58.3	3.53 (s)

Chemical shift in ppm from TMS as internal standard. ^1H and ^{13}C NMR were measured at 600 and 150 MHz, respectively.

(HMQC) experiment established all one-bonded ^1H–^{13}C connectivity, as shown in Table 1. The ^1H NMR spectrum showed the presence of one methoxy proton, one methylene proton, and six aromatic protons. The six aromatic protons displayed a pair of *meta*-coupled signals at δ 6.56 (1H, d, *J*=1.8 Hz) and 6.98 (1H, d, *J*=1.8 Hz), which were assigned as H-5 and H-7, respectively. In addition, an A_2B_2 coupling system with signals at δ 7.88 (2H, d, *J*= 9.0 Hz, H-10, 12) and 8.06 (2H, d, *J*=9.0 Hz, H-9, 13) was also observed. The ^{13}C NMR spectrum of **1** revealed the presence of 16 carbons composed of one methoxy, one sp^3 methylene, six sp^2 methines, and five quaternary carbons including a carbonyl and two oxygenated sp^2 carbons. The HMBC spectrum of **1** revealed two partial structures, an isoindolyl moiety and a 1,4-disubstituted benzene moiety, as shown in Fig. 1. In the isoindol moiety, a methine proton at δ 6.04 showed long-range correlations with C-1 (δ_C 162.8), C-3a (δ_C 116.1), C-4 (δ_C 155.0), C-7a (δ_C 128.3), and C-15 (δ_C 58.3), and from a methoxy proton δ 3.53 to C-3 (δ_C 100.5). In addition, long-range correlations from δ 6.56 to C-3a (δ_C 116.1), C-4 (δ_C 155.0), C-6 (δ_C 161.1), and C-7 (δ_C 107.2), and from δ 6.98 to C-1 (δ_C 162.8), C-3a (δ_C 116.1), C-5 (δ_C 109.8), C-6 (δ_C 161.1), and C-7a (δ_C 128.3), were observed. The long-range correlations from the methine proton at δ 7.88 to C-1 (δ_C 162.8), C-8 (δ_C 144.4), C-11 (δ_C 133.3), and C-13 (δ_C 122.6), and from the methine proton at δ 8.06 to C-8 (δ_C 144.4), C-11 (δ_C 133.3), C-12 (δ_C 132.1), and C-14 (δ_C 170.2), revealed the presence of a 1,4-disubstituted benzene moiety. Based on the above HMBC

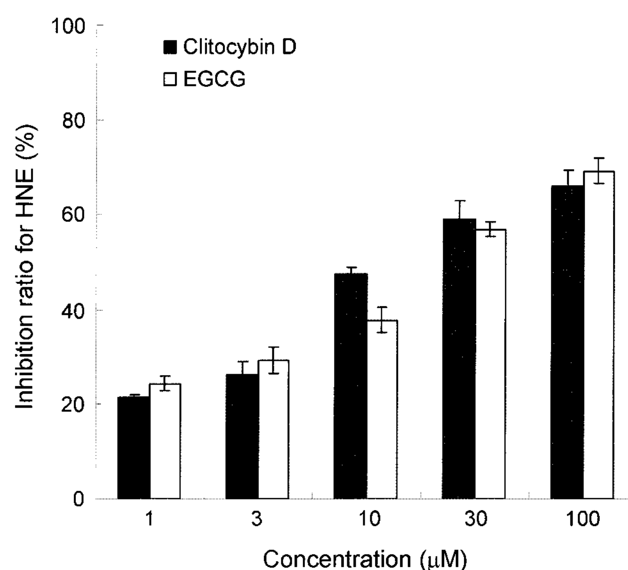

Fig. 2. HNE inhibitory activity of clitocybin D (**1**).
Human neutrophil elastase was reacted with 1.4 mM MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide at 37°C for 1 h in the dark. After the reaction was stopped by the addition of 100 μl of soybean trypsin inhibitor at a concentration of 0.2 mg/ml, the absorbance was immediately measured at 405 nm using an ELISA reader. The values represent the means±SD obtained from three independent experiments performed in triplicate.

experiment (Fig. 1), **1** was assigned as a new compound of isoindolyl class, and the stereochemistry at C-3 of **1** was assigned as *S* configuration, which also showed a negative optical rotation. Thus, the structure of **1** was established to be (*S*)-4-(4,6-dihydroxy-3-methoxy-3H-isoindol-1-yl)-benzoic acid.

The HNE inhibitory activity of **1** was evaluated using a previously described procedure [8]. Briefly, each well of a 96-well plate containing 100 μl of 10 mM Tris-HCl buffer (pH 7.5), 1.4 mM MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide, 0.18U HNE, and the sample at various concentrations was incubated for 1 h at 37°C in the dark. After the reaction was stopped by the addition of 100 μl of soybean trypsin inhibitor at a concentration of 0.2 mg/ml, the absorbance was immediately measured at 405 nm using an ELISA reader. Epigallocatechin gallate (EGCG) was used as a positive control. As a result, compound **1** dose-dependently inhibited HNE activity with an IC_{50} value of 17.8 μM (Fig. 2), which was comparable to the positive control, EGCG (12.9 μM).

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