NOTE

Inhibition of human neutrophil elastase by ergosterol derivatives from the mycelium of *Phellinus linteus*

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The extracellular matrix (ECM) is the largest component of the dermal skin layer, and has a fundamental role in tissue development, differentiation, homeostasis and disease progression.¹ It is continually remodeled by cells in response to environmental factors, such as physical force, hypoxia, trauma and infection.² During the aging process, the balance of synthesis and degradation of ECM proteins necessary for skin integrity and tissue regeneration becomes impaired.³ Elastin, an important structural protein of ECM, is the main component of the elastic fibers that impart resilience and elasticity to elastic tissues, such as skin, lungs, ligaments and arterial walls.⁴ Human neutrophil elastase (HNE), a serine protease primarily found in the azurophilic granules of neutrophils, has broad substrate specificity and can cleave not only elastin but also other ECM proteins, such as collagen, fibronectin, laminin and proteoglycan.⁵ As the pharmacological inhibition of HNE might prevent the loss of skin elasticity, thus preventing skin sagging during aging,⁶ efforts to discover potent HNE inhibitors have increased considerably in the last several years.

Mushrooms have been utilized in folk medicine throughout the world since ancient times. They produce various classes of secondary metabolites, many of which possess interesting biological activities and have the potential to be developed as therapeutic agents.^{7–9} Thus, much attention has been paid to the search for specific pharmacologically significant compounds from mushrooms. *Phellinus linteus*, commonly referred to as Sangwhang in Korea, is a well-known fungus of the genus *Phellinus* in the family Hymenochaetaceae, which is indigenous mainly to the tropical Americas, Africa and East Asia. This mushroom has long been used as a traditional oriental medicine in Korea, China, Japan and other Asian countries for the treatment of various ailments, including stomachaches, inflammation, oral ulcer, gastroenteric disorders, lymphatic disorders, arthritis of the knee and various cancers.^{10–12} Numerous bioactive substances have been isolated from the fruiting bodies of *P. linteus*, such as polysaccharides,

sterols, proteoglycans, cyclophellitol, furan derivatives, hispidin and hispolon, and their biological activities have been verified *in vitro* and *in vivo*.^{13–16} However, there is little information on compounds of the mycelium of *P. linteus*, with the exception of polysaccharides.

In searching for novel HNE-inhibitors from higher fungi, we found that the 70% EtOH extract of the mycelium of *P. linteus* had considerable HNE-inhibitory activity ($IC_{50} = 9.02 \mu\text{g ml}^{-1}$). To search for active compounds from the mycelium of *P. linteus*, its extract was systematically divided into two solvent fractions (CHCl_3 - and *n*-BuOH soluble), and their HNE-inhibition was evaluated. The CHCl_3 -soluble fraction exhibited significant HNE-inhibition, with an IC_{50} of $5.18 \mu\text{g ml}^{-1}$. Further phytochemical studies of this fraction resulted in the isolation of a new ergosterol derivative, (22*E*,24*R*)-ergosta-7,22-dien-2 α ,3 α ,9 α -triol (**1**) together with 14 known compounds (**2**–**15**) (Figure 1). This report describes the isolation and structural elucidation of these ergosterol derivatives, as well as the characterization of their inhibitory effects on HNE and some interesting structural requirements for their activity.

Freeze-dried mycelial culture of *P. linteus* (5 kg, supplied by HanKook Sin Yak Pharm., Nonsan, Korea) was extracted with 70% EtOH (3×101) at room temperature for 7 days, filtered and concentrated to yield a 70% EtOH extract (420 g). This extract was suspended in H_2O (4 l) and then partitioned successively with CHCl_3 (3×41) and *n*-BuOH (3×41) to afford CHCl_3 - and *n*-BuOH-soluble fractions (200 and 85 g, respectively). The CHCl_3 -soluble fraction, which significantly inhibited HNE, was subjected to chromatography on a silica gel column ($80 \times 12 \text{ cm}^2$). Elution with a gradient solvent system consisting of CHCl_3 –MeOH (100 : 1 $\rightarrow$ 1 : 1) yielded five fractions (A–E). Fraction A (7 g) was applied to a silica gel column ($60 \times 6.5 \text{ cm}^2$), and eluted with a hexane–acetone gradient (50 : 1 $\rightarrow$ 20 : 1), yielding **2** (1050 mg). Fraction C (15 g) was applied to the same silica gel column and eluted using a hexane–acetone gradient (5 : 1 $\rightarrow$ 0 : 1), yielding five subfractions (C1–C5). Subfractions C2 and

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Figure 1 Chemical structures of **1–15** isolated from the mycelium of *P. linteus*.

C5 (4 and 3 g, respectively) were then separately chromatographed on a YMC RP-18 column (50 × 3.5 cm², YMC, Tokyo, Japan). Elution of C2 with a MeOH–H₂O gradient (5 : 1 → 7 : 1) yielded **3** (200 mg), **4** (20 mg), **5** (7 mg), **6** (5 mg) and **7** (10 mg). Elution of C5 with a MeOH–H₂O gradient (4 : 1 → 6 : 1) yielded **8** (26 mg), **9** (15 mg) and **10** (17 mg). Chromatography of fraction D (17 g) on the silica gel column (60 × 6.5 cm²) with a gradient solvent system of hexane–acetone (9 : 1 → 7 : 3) yielded six subfractions (D1–D6). Subfraction D3 (1.5 g) was further purified on a YMC RP-18 column (50 × 3.5 cm²) and eluted with a MeOH–H₂O gradient (5 : 1 → 6 : 1), yielding **11** (3 mg), **12** (4 mg) and **13** (7 mg). Compound **14** (12 mg) was isolated from subfraction D5 (0.5 g), using a YMC RP-18 column (50 × 3.5 cm²) eluted with a MeOH–H₂O gradient (4 : 1 → 5 : 1). Fraction E (7 g) was applied to a silica gel column (60 × 6.5 cm²) eluted with a hexane–acetone gradient (7 : 1 → 1 : 1), yielding three subfractions (E1–E3). Subfraction E2 (0.5 g) was subjected to further

chromatography on a YMC RP-18 column and eluted with a MeOH–H₂O gradient (4 : 1 → 5 : 1), yielding **15** (20 mg) and **1** (13 mg).

Compound **1** was obtained as white powder with the following spectral characteristics: $[\alpha]_D^{25}$ –20.0 (c 0.1, CDCl₃); IR (KBr) $V_{\max}$ cm^{–1}: 3420, 2960, 1650, 1460, 1395; HR-EI-MS m/z 430.3445 [M]⁺ (calculated for C₂₈H₄₆O₃, 430.3447); ¹H NMR (300 MHz, CDCl₃): δ_H 5.25 (1 H, dd, J = 15.4, 6.6 Hz, H-23), 5.15 (1 H, dd, J = 15.4, 6.9 Hz, H-22), 5.02 (1 H, dd, J = 5.5, 2.6 Hz, H-7), 4.05 (1 H, m, H-3), 4.00 (1 H, m, H-2), 1.03 (1 H, d, J = 6.6 Hz, H-21), 0.98 (3 H, s, H-19), 0.93 (3 H, d, J = 6.9 Hz, H-28), 0.85 (3 H, d, J = 6.6 Hz, H-27), 0.83 (3 H, d, J = 6.6 Hz, H-26), 0.57 (3 H, s, H-18) (see Supplementary Information); ¹³C NMR (75 MHz, CDCl₃): δ_C 38.8 (C-1), 70.6 (C-2), 67.7 (C-3), 31.9 (C-4), 43.6 (C-5), 30.9 (C-6), 119.8 (C-7), 142.3 (C-8), 76.3 (C-9), 40.0 (C-10), 22.9 (C-11), 39.5 (C-12), 44.0 (C-13), 55.0 (C-14), 21.6 (C-15), 28.3 (C-16), 56.2 (C-17), 12.4 (C-18), 18.0 (C-19), 40.6 (C-20), 21.3 (C-21), 135.6 (C-22), 132.4 (C-23), 43.0

(C-24), 33.3 (C-25), 19.9 (C-26), 20.2 (C-27), 17.8 (C-28) (see Supplementary Information).

High-resolution EI-MS analysis of **1** yielded a molecular ion peak at m/z 430.3445 $[M]^+$, in accordance with the molecular formula $C_{28}H_{46}O_3$. The IR spectrum exhibited absorption bands for hydroxy (3420 cm^{-1}) and olefinic (1650 cm^{-1}) groups. The ^1H NMR spectrum showed two tertiary methyl signals [δ_{H} 0.98 and 0.57 (each s)], four secondary methyl signals [δ_{H} 1.03 (d, $J=6.6\text{ Hz}$), 0.93 (d, $J=6.9\text{ Hz}$), 0.85 (d, $J=6.6\text{ Hz}$) and 0.83 (d, $J=6.6\text{ Hz}$)], two oxymethine signals [δ_{H} 4.05 (m) and 4.00 (m)], and three olefinic proton signals [δ_{H} 5.25 (dd, $J=15.4, 6.6\text{ Hz}$), 5.15 (dd, $J=15.4, 6.9\text{ Hz}$) and 5.02 (dd, $J=5.5, 2.6\text{ Hz}$)], suggesting an ergostane skeleton including a nine-carbon side chain.¹⁷ A typical high-field signal at δ_{H} 0.57 (H_3 -18) suggested **1** to be a Δ^7 sterol.¹⁸ The ^{13}C NMR and DEPT spectra also supported this sterol skeleton in **1**, showing 28 carbon signals, consisting of six methyls, seven methylenes, eight methines (including two oxymethine groups at δ_{C} 70.6 and 67.7), three quaternary carbons (including an oxygenated carbon at δ_{C} 76.3) and four sp^2 carbons (at δ_{C} 142.3, 135.6, 132.4 and 119.8). In addition, the ^1H and ^{13}C NMR data of **1** were similar to those of **15**, indicating that the three hydroxy groups of **1** are located at rings A and B, which was supported by analysis of the 2D NMR spectra. The ^1H - ^1H COSY correlations between $\text{H}_2/\text{H}_3/\text{H}_4$ as well as the long range ^1H - ^{13}C coupling (HMBC) observed between H-2/C-1 and C-3, H-3/C-2, C-4 and C-5, and H-7/C-6, C-8 and C-9 confirmed the positions of three hydroxy groups to be at C-2, C-3 and C-9, respectively (Figure 2a). On the other hand, a careful inspection of the ^1H and ^{13}C NMR spectra of **1** revealed a slight difference in the chemical shift of the C-2 position (δ_{H} 4.00 m; δ_{C} 70.6) compared with that of **15** (δ_{H} 3.60 m; δ_{C} 73.0), indicating that **1** may differ from **15** in the relative configuration of C-2. The strong NOE correlation between δ_{H} 4.00 (H-2) and 0.98 (Me-19) verified the α -orientation for the C-2 hydroxy group rather than the β -orientation found in **15** (Figure 2b). The stereochemistry of the side chain was determined by comparison of the ^1H and ^{13}C NMR data of **1** with those of a (22*E*,24*R*)-methyl- Δ^2 -sterol side chain.¹⁹ On the basis of the above data, the structure of **1** was established as (22*E*,24*R*)-ergosta-7,22-dien-2 α ,3 α ,9 α -triol.

In addition, 14 known compounds were identified as ergosterol (**2**),²⁰ ergosterol peroxide (**3**),²⁰ ergosterol peroxide glycoside (**4**),²¹ 5 α ,6 α -epoxy-ergosta-8(14),22-dien-3 β ,7 α -diol (**5**),²⁰ 5 α ,6 α -epoxy-ergosta-8(9),22-dien-7-on-3 β -ol (**6**),²² 5 α ,6 α ;8 α ,9 α -diepoxy-ergost-22-en-3 β ,7 α -diol (**7**),²³ 3 β -hydroxy-ergosta-7,22-dien-6-one (**8**),²⁴ 3 β ,5 α -dihydroxy-ergosta-7,22-dien-6-one (**9**),²⁴ 3 β ,5 α ,9 α -trihydroxy-ergosta-7,22-dien-6-one (**10**),²¹ 14 α -hydroxy-ergosta-4,7,9(11),22-tetraen-3,6-dione (**11**),²⁴ ergosta-4,7,22-trien-3,6-dione (**12**),²⁵ 3 β ,5 α -dihydroxy-6 β -methoxyergosta-7,22-diene (**13**),²⁶ ergosta-7,22-

dien-3 β ,5 α ,6 β -triol (**14**)²⁶ and ergosta-7,22-dien-2 β ,3 α ,9 α -triol (**15**)¹⁸ by comparing their physicochemical and spectral data to those in the literature.

The inhibitory activity of the isolated compounds **1**–**15** on HNE *in vitro* was evaluated according to a previously described procedure.²⁷ 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 $^\circ\text{C}$ in the dark. Each reaction was stopped by the addition of 100 μl soybean trypsin inhibitor (0.2 mg ml^{-1}), and the absorbance at 405 nm was immediately measured using a microplate reader. Epigallocatechin gallate (EGCG) was used as a positive control, and the results are presented in Table 1. Of the compounds tested, 3 β ,5 α -dihydroxy-6 β -methoxyergosta-7,22-diene (**13**) exhibited the strongest HNE-inhibitory activity with an IC_{50} value of $14.6 \pm 0.8\text{ }\mu\text{M}$, comparable to that of EGCG ($\text{IC}_{50} = 12.5 \pm 0.5\text{ }\mu\text{M}$). Ergosta-7,22-dien-3 β ,5 α ,6 β -triol (**14**), which differs from **13** only in the substituent on C-6, also showed HNE inhibitory activity, albeit five times less potent than that of **13**. In addition, the sterols (**5**, **6**, and **7**) bearing a 5 α ,6 α -epoxy group

Table 1 HNE-inhibitory activity of **1**–**15** isolated from the mycelium of *P. linteus*^a

Compounds	IC_{50}^b (μM)
(22 <i>E</i> ,24 <i>R</i>)-Ergosta-7,22-dien-2 α ,3 α ,9 α -triol (1)	> 100
Ergosterol (2)	64.5 ± 2.5
Ergosterol peroxide (3)	> 100
Ergosterol peroxide glycoside (4)	> 100
5 α ,6 α -Epoxy-ergosta-8(14),22-diene-3 β ,7 α -diol (5)	28.2 ± 1.8
5 α ,6 α -Epoxy-ergosta-8(9),22-dien-7-on-3 β -ol (6)	75.1 ± 3.2
5 α ,6 α ;8 α ,9 α -Diepoxy-ergost-22-en-3 β ,7 α -diol (7)	35.2 ± 1.5
3 β -Hydroxy-ergosta-7,22-dien-6-one (8)	> 100
3 β ,5 α -Dihydroxy-ergosta-7,22-dien-6-one (9)	> 100
3 β ,5 α ,9 α -Trihydroxy-ergosta-7,22-dien-6-one (10)	> 100
14 α -Hydroxy-ergosta-4,7,9(11),22-tetraen-3,6-dione (11)	20.5 ± 1.7
Ergosta-4,7,22-trien-3,6-dione (12)	55.2 ± 2.1
3 β ,5 α -Dihydroxy-6 β -methoxyergosta-7,22-diene (13)	14.6 ± 0.8
Ergosta-7,22-dien-3 β ,5 α ,6 β ,9 α -tetraol (14)	77.5 ± 2.3
Ergosta-7,22-dien-2 β ,3 α ,9 α -triol (15)	> 100
EGCG ^c	12.5 ± 0.5

Abbreviation: HNE, human neutrophil elastase.

^aResults are expressed as means $\pm$ s.d. ($n=3$).

^b IC_{50} indicates the concentration (μM) at which the inhibition percentage of the enzyme activity was 50%, and the values were determined by regression analysis.

^cPositive control.

Figure 2 Key ^1H - ^1H COSY and HMBC (a) and NOE (b) correlations of compound **1**.

Figure 3 Lineweaver–Burk plot for inhibition of HNE by compound **13**.

exhibited considerable HNE inhibition, with IC_{50} values of 28.2 ± 1.8 , 75.1 ± 3.2 and $35.2 \pm 1.5 \mu\text{M}$, respectively. In contrast, the sterols (**9** and **10**) that contain 5-OH and 6-oxo groups exhibited no activity ($IC_{50} > 100 \mu\text{M}$). Those sterols (**11** and **12**) that contain a 3-oxo group, exhibited considerable HNE-inhibitory activity with IC_{50} values of 20.5 ± 1.7 and $55.2 \pm 2.1 \mu\text{M}$, respectively; whereas **8**, **9** and **10**, with a 6-oxo group, displayed weak activity even at the highest concentration tested. These results suggest that the presence of a dihydroxy or epoxy group between C-5 and C-6 in this type of sterol appears to be necessary for effective HNE inhibition; substitution of the 5-OH and/or 6-OH group by a methyl seems to enhance activity. The position of an oxo-group in this sterol nucleus also affects the activity; the presence of an oxo-group at C-3 is considered more effective than at the C-6 position.

On the basis of the above result, **13** was chosen for further investigation to examine the HNE-inhibitory behavior. A kinetic study was carried out in the same reaction medium in the presence of 0, 25 or $50 \mu\text{M}$ of **13** at substrate concentrations ranging from 0.25 to 1 mM. Reactions were started by adding diluted substrate and were recorded over 10 min. The V_{max} and K_{m} were estimated according to Eisenthal and Cornish–Bowden.²⁸ Under the experimental conditions used in this study, the oxidation of HNE by **13** followed Michaelis–Menten kinetics. As shown by the Lineweaver–Burk plot in Figure 3, the values of V_{max} remained the same and the values of K_{m} increased with increasing the concentration of **13**, which indicated that **13** inhibited HNE in a competitive manner.

Ergostane-type sterols have been reported to exhibit a number of biological activities including anti-HIV,²⁹ anti-inflammatory³⁰ and anticancer properties.²⁰ However, to the best of our knowledge, their HNE-inhibitory properties and some interesting structural requirements for this activity are reported here for the first time. Our results suggest that the mycelium of *P. linteus* and its components may represent effective agents for preventing skin aging.

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