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Phellinstatin, a new inhibitor of enoyl-ACP reductase produced by the medicinal fungus *Phellinus linteus*

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ABSTRACT

A new trimeric hispidin derivative, phellinstatin, was isolated from a culture broth of the medicinal fungus *Phellinus linteus* and its structure was established by various spectral analysis. Phellinstatin strongly inhibited *Staphylococcus aureus* enoyl-ACP reductase with an IC_{50} of 6 μM and also showed antibacterial activity against *S. aureus* and MRSA.

The emergence of antibiotic-resistant bacteria has steadily increased to become a serious threat to the human. This emphasizes the need to discover and develop novel antibacterial drugs with new modes of action.[1] Bacterial genomics has revealed a plethora of previously unknown targets of potential use in the discovery of novel antibacterial drugs.[2] Among novel antibacterial targets, bacterial fatty acid synthesis (FAS) is an attractive antibacterial target since FAS is organized differently in bacteria and mammals.[3,4] Bacterial enoyl-ACP reductase that catalyzes the final and rate-limiting step in the bacterial fatty acid synthesis has been validated as a novel target for antibacterial drug development.[5] There are several isoforms, FabI, FabK, and FabL, in enoyl-ACP reductase. Most of bacteria including *Staphylococcus aureus* contain FabI, but *Streptococcus pneumonia* has FabK. *E. faecalis* and *P. aeruginosa* were known to contain both FabI and FabK, while *Bacillus subtilis* contains both FabI and FabL. Indeed, the antibacterial target of triclosan,[6] the broad spectrum biocide in a wide range of consumer goods, and isoniazid,[7] used in the treatment of tuberculosis for 50 years, have been determined to be the FabI and InhA, respectively. Therefore, inhibitors of enoyl-ACP reductase may be interesting lead compounds for developing effective antibacterial drugs.

Mushrooms belonging to the genera *Phellinus* have been used as traditional medicines for the treatment of gastrointestinal cancer, liver or heart diseases, and stomach ailments.[8] Recent studies on the chemical constituents of the fruiting body or cultured broth of the fungi led to isolation of various hispidin class compounds such as hypholomines A and B,[9] phelligridimer A,[10] inoscavins A–C,[11] phelligridins A–G,[12] and interfungins A–C.[13] Hypholomines A and B, and phelligridimer A are dimeric and tetrameric hispidins, respectively. The trimeric hispidin derivative, however, has not been reported before.

In the course of our ongoing screening for new enoyl-ACP reductase inhibitors from microbial resources, we have isolated a new trimeric hispidin derivative, named phellinstatin (**1**),[14] along with the known dimeric hispidin, hypholomine B (**2**),[9] from the culture broth of the medicinal fungus *Phellinus linteus* 08090-29 (Fig. 1). In this paper, we present the isolation, structure determination, and antibacterial activity of **1**.

The producing strain, *P. linteus* 08090-29, was provided from the staff of mushroom taxonomy laboratory at the National Institute of Agricultural Science and Technology. Fermentation was carried out in 1-liter Erlenmeyer flasks containing glucose 2%, polypeptone 0.5%, yeast extract 0.2%, KH_2PO_4 0.1%, and $MgSO_4 \cdot 7H_2O$ 0.05% (adjusted to pH 5.6–5.8 before sterilization). A piece of agar from the mature plate culture of the producing strain was inoculated into a 500 mL Erlenmeyer flask containing 80 mL of sterile seed liquid medium with the above composition and cultured on a rotary shaker (150 rpm) at 28 °C for 3 days. For the production of **1**, 5 mL of the seed culture was transferred into one-liter Erlenmeyer flasks containing 100 ml of the above medium, and cultivated for 15 days using the same conditions.

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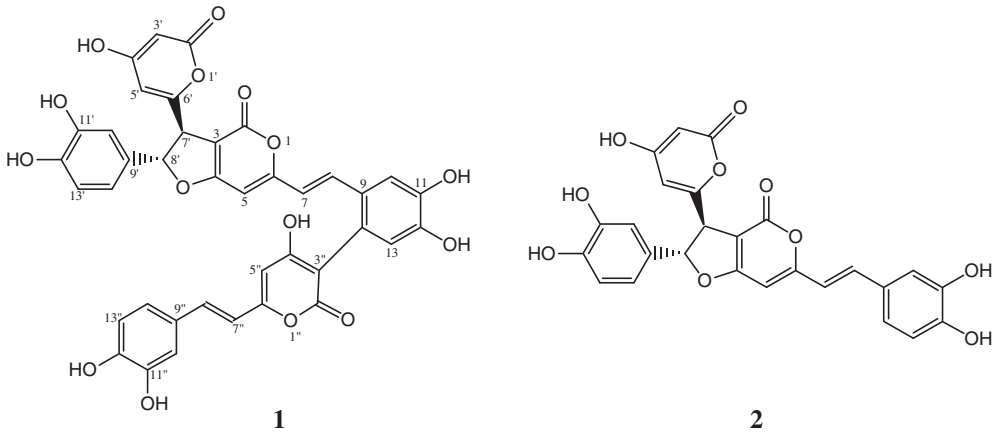

Fig. 1. Chemical structures of phellinstatin (**1**) and hypholomine B (**2**).

The culture supernatant obtained from the culture broth (3 l) was extracted with an equal volume of EtOAc three times and the EtOAc layer was concentrated in vacuo. The resultant residue (4 g) was subjected to ODS (YMC s-150 μm) column chromatography followed by elution with MeOH–H_2O (20:80–100:0). The active fractions eluted with MeOH–H_2O (40:60–50:50) were pooled and concentrated in vacuo to give a yellow residue (412 mg). The residue was applied to thin layer chromatography (TLC) on silica gel 60 RP-18 F_{254} plates (Merck No 1.15389.0001: Darmstadt, Germany) with methanol-water (60:40) to yield two bands, B1 (165 mg) and B2 (43 mg), with R_f values of 0.5 and 0.6, respectively. The B1 band was further purified by TLC on RP-18 plates with ACN-water (30:70) containing 0.1% TFA to yield compound **2** (56 mg) with an R_f value of 0.1. Also the B2 band was further purified by TLC on RP-18 plates with ACN-water (30:70) containing 0.1% TFA to yield compound **1** (27.1 mg) with an R_f value of 0.1 as a yellow powder.

The ^1H and ^{13}C NMR spectroscopic data of **2** together with its molecular weight suggested that this compound is a member of the dimeric hispidin class. Interpretation of the ^1H and ^{13}C NMR data led to the identification of **2** as hypholomine B.[9] The [α] value (+225, *c* 0.1, MeOH) of **2** was almost identical with the literature value for this compound (+220, *c* 0.1, MeOH). In addition, the CD[15] of **2** together with coupling constants and chemicals shifts was in good agreement with that of hypholomine B[16], indicating that **2** has the same absolute configuration as that of hypholomine B.

The molecular formula of **1** was determined to be $C_{39}H_{26}O_{15}$ on the basis of high resolution ESI-MS [$(M-H)^-$, 733.1196 *m/z* (−0.3 mmu error)] in combination with ^1H and ^{13}C NMR data. The IR data suggested the presence of a carbonyl (1635 cm^{-1}) and a hydroxyl (3432 cm^{-1}) moiety. The UV absorption maxima at 284 and 372 nm suggested that **1** was a hispidin derivative. The ^1H NMR data (Table 1) with the COSY spectrum suggested the presence of six aromatic protons attributable to two 1,2,4-trisubstituted benzene rings [δ 6.76 (d, J = 2.0), δ 6.72 (d, J = 8.5, 2.0), and δ 6.79 (d, J = 8.5); δ 7.04 (d, J = 1.5), δ 6.96 (dd, J = 8.5, 1.5), and δ 6.77 (d, J = 8.5)], four olefinic protons of two *trans*-1,2-disubstituted double bonds [δ 6.68 (d, J = 16.0) and δ 7.32 (d, J = 16.0); δ 6.63 (d, J = 16.0) and δ 7.35 (d, J = 16.0)], five olefinic singlets (δ 6.04, δ 6.25, δ 6.41, δ 6.70, and δ 7.26), and two adjacent sp^3 methine doublets [δ 4.28 (d, J = 6.5) and δ 5.76 (d, J = 6.5)]. In the ^{13}C NMR spectrum together with the HMQC spectrum, 15 olefinic carbons, 18 sp^2 quaternary carbons, three carbonyl lactone carbons, and two sp^3 methine carbons were observed. The proton and carbon peaks of 3′ were not detected in CD_3OD due to

Table 1
^1H and ^{13}C NMR spectral data of **1**

Position	δ_H (J, Hz)	δ_C	HMBC	Position	δ_H (J, Hz)	δ_C	HMBC
2		162.6 C		9′	131.4 C		
3		99.9 C		10′	6.76 (1H, d, 2.0)	113.6 CH	C-8′, C-12′, C-14′
4		174.1 C		11′	146.5 C		
5	6.41 (1H, s)	96.3 CH	C-2, C-4, C-6, C-7	12′	147.2 C		
6		165.6 C		13′	6.79 (1H, d, 8.5)	116.5 CH	C-9′, C-11′
7	6.68 (1H, d, 16.0)	117.6 CH	C-6, C-9	14′	6.72 (1H, d, 8.5, 2.0)	119.2 CH	C-10′, C-12′
8	7.32 (1H, d, 16.0)	136.7 CH	C-6, C-9, C-10, C-14	2″	166.9 C		
9		128.0 C		3″	103.2 C		
10	7.26 (1H, s)	112.8 CH	C-8, C-11, C-14	4″	168.8 C		
11		147.0 C		5″	6.25 (1H, s)	101.4 CH	C-3″, C-4″, C-6″
12		148.7 C		6″	160.6 C		
13	6.70 (1H, s)	119.2 CH	C-9, C-11, C-3″	7″	6.63 (1H, d, 16.0)	116.8 CH	C-5″, C-6″, C-9″
14		126.5 C		8″	7.35 (1H, d, 16.0)	137.4 CH	C-6″, C-9″, C-10″, C-14″
2′		167.8 C		9″	128.8 C		
3′	Quenched	90.4 CH		10′	7.04 (1H, d, 1.5)	114.4 CH	C-8″, C-11″, C-12″, C-14″
4′		172.8 C		11″	147.0 CH		
5′	6.04 (1H, s)	103.0 CH	C-3′, C-4′, C-6′, C-7′	12″	148.6 C		
6′		163.7 C		13″	6.77 (1H, d, 8.5)	116.5 C	C-9″, C-11″
7′	4.28 (1H, d, 6.5)	53.3 CH	C-3, C-4, C-5′, C-6′, C-8′, C-9′	14″	6.96 (1H, dd, 8.5, 1.5)	121.9 CH	C-8″, C-10″, 12″
8′	5.76 (1H, d, 6.5)	92.7 CH	C-4, C-6′, C-7′, C-9′, C-10′, C-14′				

^1H and ^{13}C NMR spectra were measured at 500 MHz and 125 MHz, respectively, in CD_3OD.
The assignments were aided by ^1H–^1H COSY, HMQC, and HMBC.

keto-enol tautomerism like **2**. These spectral data with the molecular formula suggested that **1** was composed of three hispidins unlike 2, a dimer of hispidin. The connectivity of three hispidin moieties was determined by the HMBC spectrum (Table 1). The long-range couplings were observed from one sp^3 methine proton at δ 4.28 (H-7′) to an olefinic carbon at δ 103.0 (C-5′) and four sp^2 quaternary carbons at δ 99.9 (C-3), δ 131.4 (C-9′), δ 163.7 (C-6′), and δ 174.1 (C-4). The other adjacent sp^3 methine proton at δ 5.76 (H-8′) was long-range coupled to two aromatic methine carbons at δ 113.6 (C-10′) and δ 119.2 (C-14′) and three sp^2 quaternary carbons at δ 131.4 (C-9′), C-6′, and C-4. In addition, HMBC correlations from an olefinic singlet proton at δ 6.41 (H-5) to the carbon at δ 117.6 (C-7) of one *trans*-1,2-disubstituted double bond and three sp^2 quaternary carbons at C-3, C-4, and C-6 were observed. These spectral data indicated the presence of hypholomine B moiety. The proton at δ 7.32 (H-8) of the *trans*-1,2-disubstituted double bond unit, however, showed the long-range correlations with the aromatic singlet methine carbon at δ 112.8 (C-10) and three sp^2 quaternary carbons at C-6, δ 128.0 (C-9), and δ 126.5 (C-14). Furthermore, the long-range correlations from H-10 to C-8 and two sp^2 quaternary carbons at δ 148.7 (C-12) and C-14 were observed. These HMBC data suggested that the remaining hispidin moiety could be attached to C-14 of hypholomine B moiety. This was confirmed by an HMBC correlation between the aromatic singlet methine proton at δ 6.70 (H-13) of the hypholomine B moiety and three sp^2 quaternary carbons at δ 103.2 (C-3″) of the remaining hispidin moiety. The carbon of C-3″ was in turn long-range coupled with the olefinic singlet proton at δ 6.25 (H-5″). These spectral data clearly indicated the direct linkage of C-3″ of the hispidin moiety with C-14 of hypholomine B moiety. The remaining structure was also corroborated by the HMBC spectrum. Thus, the planar structure of **1** was determined to be a new trimeric hispidin as shown in Fig. 1. The relative configuration of H-7′ and H-8′ was determined by their vicinal coupling constant and NOESY spectrum. The vicinal coupling constant (J = 6.5 Hz) between H-7′ and H-8′ suggested an *anti* configuration of the two protons, which was further confirmed by NOEs between H-7′ and both H-10′ and H-14′, and between H-8′ and H-5′, like the case of phelligridimer A,[10] a tetrameric hispidin. Additionally, considering that the coisolated dimeric hispidin **2** has the same absolute configuration as that of hypholomine B, **1** could have the same absolute configuration at C-7′ and C-8′ as shown in Fig. 1.

Compound **1** is a new class of hispidin in which one more hispidin moiety was linked to C-14 of hypholomine B. Several class of hispidin-derived compounds were reported. Inoscavins A–C,[11] phelligridins A–G,[12] and interfungins A–C[13] isolated from the fruiting bodies of *Inonotus* and *Phellinus* spp. are derived from hispidin and hydroxylbenzaldehyde or cinnamyl derivative. Hypholomines A and B are dimeric hispidins isolated from the fungi *Flammula* spp., *Hypholoma* spp., *Pholiota* spp., *Inonotus* spp., and *Phellinus* spp.[16] Phelligridimer A is a tetrameric hispidin isolated from *Phellinus igniarius*.[10] The trimeric class of hispidin, compound **1**, is reported for the first time in this study.

The inhibitory activity of **1** and **2** against *S. aureus* FabI was evaluated according to our previously reported method[17] as follows; assays contained 50 mM sodium acetate, pH 6.5, 200 μM *t*-*o*-NAC thioester, 200 μM NADPH, and 150 nM *S. aureus* FabI in half-area, 96-well microtitre plates. Compound dissolved in dimethyl sulfoxide was added to each well. The rate of increase in the amount of NADPH in each reaction well was measured at 340 nm at 30 °C by a microtiter ELISA reader using SOFTmax PRO software (Molecular Devices, California, USA). The inhibitory activity was calculated by the following formula: % of inhibition = 100 × [1 − (rate in the presence of compound/rate in the untreated control)].

Compound **1** inhibited *S. aureus* FabI in a dose-dependent fashion with an IC_{50} of 6.0 μM which is two and half times higher activity than that (15.3 μM) of compound **2**. In order to determine whether compounds **1** and **2** inhibit cell growth, their antibacterial activity against *S. aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) were evaluated. Consistent with its FabI-inhibitory activity, compound **1** also exhibited antibacterial activity against *S. aureus* RN4220 as well as methicilline-resistant *S. aureus* (MRSA) CCARM 3167 with MICs of 128 μg/mL. Compound **2**, however, did not show antibacterial activity against *S. aureus* and MRSA at 128 μg/mL.

In summary, phellinstatin is a new trimeric hispidin derivative isolated from the culture broth of the medicinal fungus *Phellinus linteus*. Phellinstatin strongly inhibited *S. aureus* FabI and also showed antibacterial activity against *S. aureus* and MRSA. Phellinstatin may serve as a new class of FabI inhibitor for development of antibacterials.

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14. *Compound* ***1***. a yellow powder; λ_{max} nm (log ε) in MeOH: 202 (4.37), 284 (3.62), 372 (3.73); IR (KBr): 3432, 2924, 1635, 1500, 1251 cm^{-1}; $[\alpha]_D$ = 5.7° (*c* 0.14, MeOH); ESI-MS: *m/z* 733.1196 $(M-H)^-$, $C_{39}H_{26}O_{15}$ requires 733.1199.
15. λ_{ext} nm ($\Delta\varepsilon$): 381 (+5248.6), 294 (−12054), 271 (−1601), 225 (−10052), 221 (−9251), 214 (−11609), 206 (+1966), 196 (−667).
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