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IDENTIFICATION AND ANTIBACTERIAL ACTIVITY OF TWO STEROIDS SECRETED BY THE FUNGUS BEETLE *XYLOGRAPHUS BOSTRICHOIDES* (DUFOUR, 1843)

FENG ZHU*, JIESEN LI, WUCHENG XIE, CHUNYAN WANG AND YILU LIU

Department of Chemical Engineering, Foshan University, Foshan 528000, China

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Abstract

To explore the metabolites secreted by fungus beetle *Xylographus bostrichoides*, the chemical constituents in its secretions were studied. Two steroids were isolated from the secretions and identified as stellasterol and ergosterol peroxide by spectroscopic method comparing the previous data. In the antibacterial assays, both of them showed moderate inhibitory activity *in vitro* against *Staphylococcus aureus* and *Escherichia coli* with minimum inhibitory concentration (MIC) values of 2000 and 1000 μg/ml, respectively. These two secondary metabolites might be the insect pheromones or defensins secreted by the fungus beetle. They might also be indigestible residues derived from the mushroom *Ganoderma applanatum* (Pers.) Pat.

Introduction

Insect secretions and ground-up bodies have commonly been used in forklore medicine not only in China and Bahia, but also in India, Asia, Africa, and American (Pemberton *et al*. 1999, Dossy 2010, Ratcliffe *et al.* 2011). Recently, many important bioactive substances have been isolated from insects (Wang *et al*. 2014, Ratcliffe *et al.* 2014). For examples, ladybug lactones are chemical defensins secreted by the pupae of ladybugs (Daloze *et al*. 1994). Cantharidin is a secretion of *Mylabris phalerata* Pallas and *Mylabris cichorii* L. (Galvis *et al*. 2013). Lucifensin was purified in an extract of the gut of *Lucilia sericata* Meigen larvae (Cerovsky *et al.* 2014). Seven components were obtained from the *n*-butanol extracts of *Coccinella septempunctata* L. adults (Sun *et al*. 2013).

In 2014, the authors collected a wild mushroom *Ganoderma applanatum* (Pers.) Pat. (Fig. 1a) from the Leigang Mountain in Foshan city, Guangdong province, China. But in the spring of 2016, it was found that the mushroom was worm-eaten by a kind of fungus beetle which was identified as *Xylographus bostrichoides* (Fig. 1b) later. The fungus beetle *X. bostrichoides* is a kind of pest belonging to the family Ciidae, often accumulated in the dead wood and food mushrooms (Zhang *et al.* 2004). However, there are no reports on its biochemistry and its secondary metabolites. Herein, we reported the isolation, identification and antibacterial activity of two steroids (Fig. 2) secreted by the fungus beetle *X. bostrichoides*.

Materials and Methods

The fungus beetle was collected from a worm-eaten medicinal mushroom *Ganoderma applanatum* from the Leigang Mountain in Foshan city, Guangdong province, China, and identified as *Xylographus bostrichoides* by Weihong Lu in the School of Life Science and Engineering, Foshan University. Its secretions (Fig. 1c) were collected by feeding them with medicinal mushroom *G. applanatum* for six months.

*Author for correspondence: <zhufeng@fosu.edu.cn>.

The tested bacterial strains *Staphylococcus aureus* and *Escherichia coli* were provided by the School of Life Science and Engineering, Foshan University.

The secretions of *X. bostrichoides* (175 g) were extracted thoroughly with ethyl acetate (3 times × 7 days) in room temperature (25°C). The ethyl acetate in the extracts was removed in a rotary evaporator in vacuum under 50°C, and the residue was subjected to silica gel column chromatography, eluted with petroleum/ethyl acetate (1 : 0 - 0 : 1 gradient system, v/v). The fraction eluted with petroleum/ethyl acetate (4 : 1, v/v) was subjected to silical gel column chromatography repeatedly to give compounds **1** (24 mg) and **2** (12 mg). The structures of compounds **1** and **2** were further identified by silica gel thin layer chromatography (TLC), melting point and nuclear magnetic resornance (NMR) techniques.

The antibacterial activity against *S. aureus* and *E. coli* was tested by following microdilution method (Smania *et al*. 2005, Peng *et al*. 2013), and veterinary penicillin and streptomycin as reference agents. All the compounds were dissolved in dimethylsulfoxide (DMSO) and diluted in a Mueller-Hinton broth. An amount of 100 µl from each dilution (4000 ~ 15.625 µg/ml), as well as, 100 µl of the vehicle (Mueller-Hinton broth plus DMSO), was poured in one of the 96 wells of a sterilized microplate. Each well was inoculated with 5 µl of bacterial inoculum (10^6 CFU/ml). The procedure was performed in duplicate and the microdilution trays were incubated at 36ºC for 18 hrs. The optical density was read in an ELISA apparatus while the microbial growth was confirmed with *p*-iodonitrotetrazolium violet. The minimal inhibitory concentration (MIC) is defined as the lowest concentration for each substance that inhibited bacterial growth.

(a) (b) (c)

Fig. 1. (a) *Ganoderma applanatum* (Pers.) Pat., (b) *Xylographus bostrichoides* and (c) the secretions of *X. bostrichoides*.

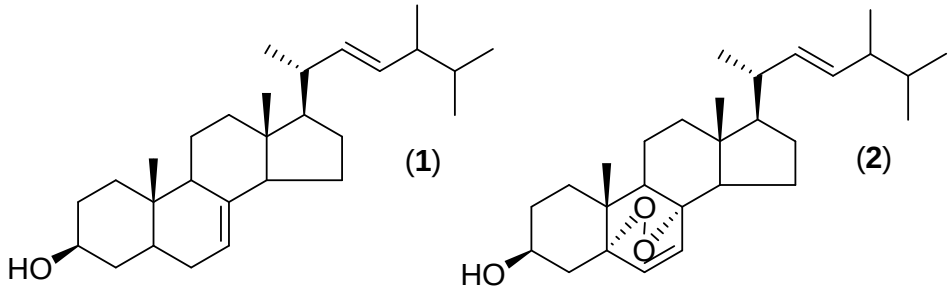

Fig. 2. Structures of steroids stellasterol (**1**) and ergosterol peroxide (**2**) secreted by the fungus beetle *X. bostrichoides*.

Results and Discussion

Two secondary metabolites were obtained from the secretions of the fungus beetle *X. bostrichoides*; their physicochemical properties and ^{1}H NMR data were listed as follows.

Compound **1** (stellasterol): colorless needle; m.p.: 166-168 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.54 (3H, s, H-18), 0.80 (3H, s, H-19), 0.82 (3H, d, J = 6.4 Hz, H-26), 0.84 (3H, d, J = 6.4 Hz, H-27), 0.91 (3H, d, J = 6.8 Hz, H-28), 1.01 (3H, d, J = 6.8 Hz, H-21), 3.60 (1H, m, H-3), 5.19 (3H, m, H-7, 22, 23); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 139.8 (C-8), 135.9 (C-22), 132.1 (C-23), 117.7(C-7), 71.3 (C-3), 56.2 (C-17), 55.3 (C-14), 49.7 (C-9), 43.5 (C-13), 43.0 (C-24), 40.7 (C-20), 40.5 (C-5), 39.7 (C-12), 38.2 (C-4), 37.4 (C-1), 34.4 (C-10), 33.3 (C-25), 31.7 (C-6), 29.9 (C-16), 28.3 (C-2), 23.1 (C-15), 21.8 (C-11), 21.3 (C-21), 20.2 (C-26), 19.9 (C-27), 17.8 (C-28), 13.2 (C-19), 12.3 (C-18).

Compound **2** (ergosterol peroxide): colorless needle; m.p.: 184-186 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.81 (3H, d, J = 6.4 Hz, H-27), 0.82 (3H, s, H-18), 0.83 (3H, d, J = 6.8 Hz, H-26), 0.88 (3H, s, H-19), 0.91 (3H, d, J = 6.8 Hz, H-28), 1.00 (3H, d, J = 6.4 Hz, H-21), 3.97 (1H, m, H-3), 5.14 (1H, dd, J = 8.0, 15.6 Hz, H-22), 5.23 (1H, dd, J = 7.6, 15.2 Hz, H-23), 6.24 (1H, d, J = 8.4 Hz, H-6), 6.50 (1H, d, J = 8.4 Hz, H-7); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 135.6 (C-6), 135.4 (C-22), 132.5 (C-23), 131.0 (C-7), 82.4 (C-8), 79.6 (C-5), 66.7 (C-3), 56.4 (C-17), 51.9 (C-14), 51.3 (C-9), 44.8 (C-13), 43.0 (C-24), 40.0 (C-20), 39.5 (C-12), 37.2 (C-10), 37.1 (C-4), 34.9 (C-1), 33.3 (C-25), 30.3 (C-2), 28.9 (C-16), 23.6 (C-15), 21.1 (C-21), 20.9 (C-11), 20.2 (C-26), 19.8 (C-27), 18.4 (C-19), 17.8 (C-28), 13.1 (C-18).

Compounds **1** and **2** were developed in a silica gel plate with petroleum ether - ethyl acetate (7 : 3, v/v) as developing solvent, and iodine as chromogenic agent after the solvent evaporated. Both of them showed bluish violet in the thin layer chromatogram (Fig. 3). The results suggested that the compounds **1** and **2** are sterols.

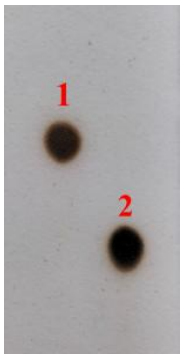

Fig. 3. Thin layer chromatogram of compounds **1** and **2**.
Developing solvent: Petroleum ether-ethyl acetate (7 : 3, v/v); chromogenic agent: iodine.

The ^{1}H and ^{13}C NMR spectrum of compound **1** also proved that it is a steroid. There are six methyl at δ 0.54 (H-18) and 12.3 (C-18), 0.80 (H-19) and 13.2 (C-19), 0.82 (H-26) and 20.2 (C-26), 0.84 (H-27) and 19.9 (C-27), 0.91 (H-28) and 17.8 (C-28), and 1.01 (H-21) and 21.3 (C-21), three olefinic protons at δ_H 5.19 (H-7, H-22, and H-23) and δ_C 117.7(C-7), 135.9 (C-22) and 132.1 (C-23) , and a typical saturated methine at δ 3.60 (H-3) and 71.3 (C-3). All these data were in agreement with that of stellasterol (Lu *et al*. 1985, Seo *et al*. 2009), and its melting point is 166-168 ^{o}C, which was consistence with that of stellasterol, 167-170.5 ^{o}C (Lu *et al*. 1985). Thus the compound **1** was elucidated as stellasterol (Fig. 2).

The ^{1}H and ^{13}C NMR spectrum of compound **2** proved that it is also a steroid. There are six methyl at δ 0.81 (H-27) and 19.8 (C-27), 0.82 (H-18) and 13.1 (C-18), 0.83 (H-26) and 20.2 (C-26), 0.88 (H-19) and 18.4 (C-19), 0.91 (H-28) and 17.8 (C-28), and 1.00 (H-21) and 21.1 (C-21), four olefinic protons at δ 5.14 (H-22) and 135.4 (C-22), 5.23 (H-23) and 132.5 (C-23), 6.24 (H-6) and 135.6 (C-6), and 6.50 (H-7) and 131.0 (C-7), and a typical saturated methine at δ 3.97 (H-3) and 66.7 (C-3). All these data were in agreement with that of ergosterol peroxide (Lu *et al.* 1985, Seo *et al*. 2009), and its melting point is 184-186^{o}C, which was consistence with that of ergosterol peroxide, 182-184^{o}C (Seo *et al*. 2009). Therefore, compound **2** was determined as ergosterol peroxide (Fig. 2).

The antibacterial activity of compounds **1** and **2** against *S. aureus* and *E. coli* was measured *in vitro* by following 96-well plate microdilution method, and veterinary penicillin and veterinary streptomycin as reference agents. The results are listed in Table 1.

Table 1. Antibacterial activity of compounds 1 and 2 against *S. aureus* and *E. coli*. (veterinary penicillin and veterinary streptomycin have been used as reference agents).

Strains	MIC (μg/ml)			
	1	**2**	Penicillin	Streptomycin
S. aureus	2000	1000	640	320
E. coli	2000	1000	320	320

The results showed that the MIC values of compound **1** against both *S. aureus* and *E. coli* were 2000 μg/ml and that of compound **2** against both *S. aureus* and *E. coli* were 1000 μg/ml. Both compounds **1** and **2** exhibited moderate antibacterial activity against *S. aureus* and *E. coli*, but the inhibitory activity of compound **2** is more stronger than compound **1**. Their inhibitory activities against *S. aureus* and *E. coli* are more weaker than the reference agents veterinary penicillin and veterinary streptomycin.

G. applanatum was proved to be a promising mushroom for antitumor and immunomodulating activity (Jeong *et al.* 2008), and lots of triterpenoids and steroids were isolated from this mushroom (Baby *et al.* 2015). Among them, stellasterol and ergosterol peroxide were the main constituents in the fruiting bodies of *G. applanatum.* (Gan *et al*. 1998, Lee *et al*. 2006). It is interesting that stellasterol and ergosterol peroxide were also found in the secretions of the fungus beetle *X. bostrichoides* in this work. It is suggested that these two compounds in the mushroom maybe attractants for this fungus beetle and this also explains why this fungus beetle likes to eat the mushrooms which contain these two steroids. But they may also be indigestible residues derived from the mushroom *G. applanatum*.

The biological actitivies of stellasterol and ergosterol peroxide, such as anti-complement activity (Seo *et al*. 2009), platelet aggregation potentiator activity (Lu *et al.* 1985), antimicrobial and antifungal activity (Smania *et al*. 2003, Smania *et al*. 2005), had been described previously. In present work, both compounds **1** and **2** were found to have moderate antibacterial activity against *S. aureus* and *E. coli*, and the inhibitory activity of compound **2** is more stronger than compound **1**. It is suggested that this fungus beetle may also use compounds **1** and **2** as its chemical weaponry to prevent itself from pathogens.

Two steroids were isolated from the secretions of the fungus beetle *X. bostrichoides* and identified as stellasterol (**1**) and ergosterol peroxide (**2**) by spectroscopic analyses as well as by comparison of their spectroscopic data which are already reported. Both compounds **1** and **2**

showed antibacterial activity *in vitro* against *S. aureus* and *E. coli*. They might be the insect pheromones or defensins secreted by this fungus beetle. They might also be indigestible residues derived from the mushroom *G. applanatum.*

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