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Four novel antibacterial sesquiterpene-α-amino acid quaternary ammonium hybrids from the mycelium of mushroom *Stereum hirsutum*

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ABSTRACT

The mushroom *Stereum hirsutum* is parasitized by *Tremella aurantia* to form a heterogeneous basidiocarp Jin'er, which has been used as food and folk medicine in Chinese society. In present work, the *S. hirsutum* was fermented in YMG broth, and four novel mixed terpenes, stereumamides A-D (**1**–**4**), which are sesquiterpenes combined with α-amino acids to form quaternary ammonium hybrids, were isolated from the *Stereum hirsutum* FP-91666 and their structures were elucidated by spectroscopic data analysis. Stereumamides A and D showed antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium*, with the minimum inhibitory concentration (MIC) values of 12.5–25.0 μg/mL. The stereumamides A-D should be apparently the first example of naturally occurring a quaternary ammonium compound (QAC) conjugated by sesquiterpene with an α-amino acid. QAC is a common antibacterial agent in food industry, which is found in the mycelium of *Stereum hirsutum* would suggest that the complex basidiocarp is a functional food and veritable folk medicine.

1. Introduction

The *Stereum* fungus belongs to the Stereaceae family (basidiomycetes) and is widely distributed throughout the world, which can produce diversiform secondary metabolites. The mushroom *Stereum hirsutum* is very widely distributed and lives on dead wood of limbs and trunks of both hardwoods and conifers, and plays an important role in the wood deterioration process. However, it is parasitized by the fungus *Tremella aurantia* to form a heterogeneous complex basidiocarp Jin'er (golden jelly fungus) [1,2], which has been traditionally used as food and crude medicine in Chinese society.

The fermented mycelia of *S. hirsutum* have been reported to produce abundantly active secondary metabolites, including phytotoxic active acetylenic compounds [3], multiple active sesquiterpenoids [4–6], antibacterial epidioxysterols [7], and benzoate derivatives [8–10]. In the process of studying the biosynthesis of bioactive vibralactone [11], we found that *S. hirsutum* could produce vibaralactone type compounds [12], and based on genome data information *S. hirsutum* would produce more secondary metabolites. To further explore its potential in the production of biologically active metabolites, the OSMAC (one strain-many compounds) strategy was applied to maximize the chemical diversity of this fungus [13]. In the research on the secondary metabolites of *S. hirsutum* FP-91666, we have isolated four novel skeletal mixed terpenoids, stereumamides A-D, which are combination of sesquiterpenes and α-amino acids to form quaternary ammonium hybrids (Fig. 1). Herein, we describe the isolation, structural determination and antibacterial activity of these compounds.

2. Experimental

2.1. General methods

Column chromatography was carried out on silica gel (200–300 mesh, silica gel H and GF254) (Qingdao Marine Chemical 132 Factory, Qingdao, China) and Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden). Precoated silica gel GF254 plates (Qingdao Marine Chemical Factory, 134 Qingdao, China) were used for thin layer chromatography (TLC). Some fractions were purified by the LC3000 Semi-preparation Gradient HPLC (Beijing Chuangxintongheng Science & Technology Co., Ltd., Beijing, China) on RP C_{18} column (250 mm × 10 mm, 5 μ, Thermo Scientific) at ambient temperature. Column chromatography was performed on silica gel G, silica gel 254, silica gel 200–300 mesh (Qingdao Marine Chemical Factory, Qingdao, China), silica gel H (Merck) and Sephadex LH-20 (Amersham

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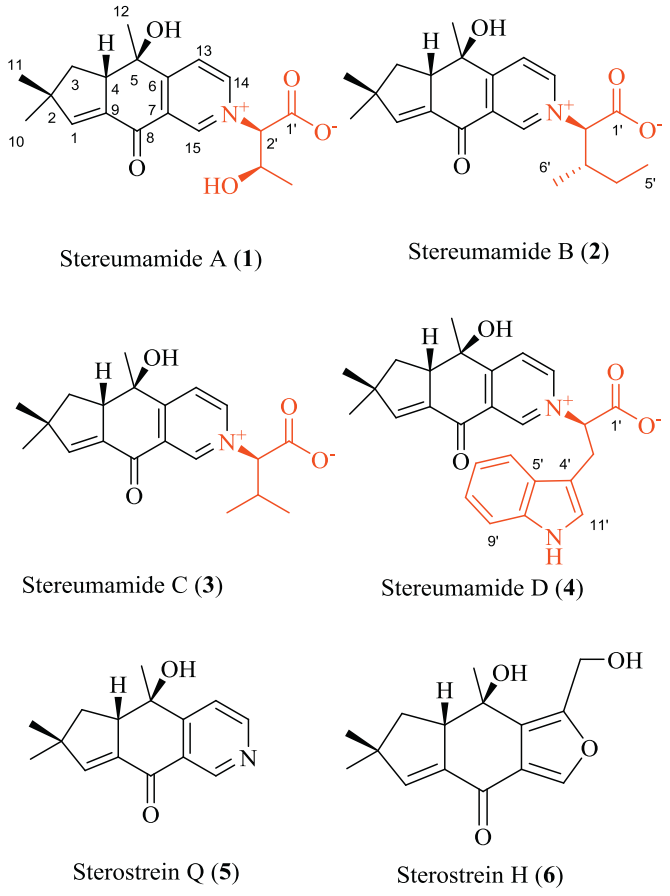

Fig. 1. Structures of compounds 1–6.

Pharmacia) column chromatography (CC). TLC was conducted on silica gel GF254 (Qingdao Marine Chemistry Ltd.). UV spectra were recorded on a Shimadzu UV-2401PC spectrophotometer (Shimadzu, Tokyo, Japan), and λ_{max} (log e) values were reported in nm. NMR experiments were carried out on Bruker AV600M HZ NMR (Bruker) spectrometers with tetramethylsilane (TMS) as an internal standard. ESI-MS and HR-ESI-MS were recorded on a Finnigan LCQ-Advantage mass spectrometer (Thermo, San Jose, CA, USA) and a VG Auto-Spec-3000 mass spectrometer (VG, Manchester, England). Optical rotations were measured on a Jasco DIP-370 digital polarimeter (JASCO, Tokyo, Japan).

2.2. Material

The strain *S. hirsutum* FP-91666 was obtained as a gift from culture collection manager Ms. Rita Rentmeester of Center for Forest Mycology Research, Northern Research Station, Madison, Wisconsin, USA. It was stored in glycerol at −80 °C in State Key Laboratory for Conservation and Utilization of Bio-Resources in Yunnan, Yunnan University, and grown on potato dextrose agar medium for 7 days at 28 °C. It was used to inoculate a 1000 mL Erlenmeyer flask containing 500 mL of the seed medium consisting of yeast extract 0.4%, malt extract 1.0% and glucose 0.4% (pH 7.3 before sterilization). The flasks containing seed culture (15 L) of *S. hirsutum* were shaken on a rotary shaker for 20 days at 28 °C with rotation of 150 rpm.

2.3. Cultivation, extraction and isolation

The fermentation broth (15 L) was filtered through multi-layer and extracted exhaustively with *n*-butanol. The *n*-butanol extract (10.51 g) was subjected to a silica gel G column (200–300 mesh) using a $CHCl_3$–MeOH (100:0–0:100) gradient solvent system to produce nine fractions (Fr.1–Fr.9).

The fraction Fr.2 (0.86 g) was separated on a column of silica gel (200–300 mesh) and eluted with petroleum ether-EtOAc (100:4 → 0:100) gradient solvent system to yield fractions of Fr.2.1-Fr.2.6. The fraction Fr.2.3 was chromatographed on a column of silica gel (GF254) using solvent system petroleum ether-acetone (100:0 → 0:100) gradient solvent system to obtain fractions of Fr.2.3.1-Fr.2.3.3. The fraction Fr.2.3.3 was purified to Sephadex LH-20 (MeOH) and a column of silica gel (H) using $CHCl_3$-MeOH (20: 1) isocratic solvent system to produce compound **5** (12.0 mg). The fraction Fr.4 (1.14 g) was subjected on a column of silica gel (200–300 mesh) using solvent system petroleum ether-EtOAc (100:8 → 0:100) gradient solvent system to give fractions of Fr.4.1-Fr.4.6. Subsequently Fr.4.3 was purified by Sephadex LH-20 $CHCl_3$-MeOH (1:1) and Sephadex LH-20 (MeOH), which gave compound **6** (2.7 mg). The fraction Fr.5 (0.90 g) was separated on a column of silica gel (200–300 mesh) and eluted with petroleum ether-EtOAc (100:0 → 0:100) gradient solvent system to yield fractions of Fr.5.1-Fr.5.3. The fraction Fr.5.2 was isolated with Sephadex LH-20 $CHCl_3$-MeOH (1:1) and Semi-preparation Gradient HPLC to yield fractions of Fr.5.2.1-Fr.5.2.3 (Detection wavelength at 254 nm and a mobile phase of methanol/water (the water reduces from 70% to 20%) at a flow rate of 3 mL/min). These fractions were respectively purified with Sephadex LH-20 (MeOH) to produce compounds **3** (2.0 mg) and **2** (2.1 mg). Fr.6 (1.07 g) was applied to a column of silica gel (200–300 mesh) and eluted with petroleum ether-EtOAc (100:0 → 0:100) gradient solvent system to yield fractions of Fr.6.1-Fr.6.5. The fraction Fr.6.3 was subjected to Sephadex LH-20 ($CHCl_3$–MeOH; 1:1) and then purified by semipreparative HPLC to yield two fractions (A mobile phase of methanol/water (The water reduces from 80% to 20%) at a flow rate of 3 mL/min). Further these two ones were purified with Sephadex LH-20 (MeOH) to produce compound **4** (7.5 mg). Fr.6.5 was separated by Semi-preparation Gradient HPLC (A mobile phase of methanol/water (the water reduces from 60% to 25%) at a flow rate of 3 mL/min) and purified on Sephadex LH-20 (MeOH) to give compound **1** (19 mg).

Stereumamide A ***(1)***: pale brown amorphism; $[\alpha]_D^{20}$ −42.4 (*c* 1.8, MeOH); UV (MeOH) λ_{max} (log ε): 206.0 (4.14), 237.5 (4.01), 261 (3.96), 320.5 (3.15); ^1H and ^{13}C NMR (CD_3OD) data see Table 1; ESI-MS: *m/z* 346 $[M + H]^+$, HR-ESI-MS: (*m/z* 346.1656 $[M + H]^+$, calc. 346.1654).

Stereumamide B ***(2)***: pale brown amorphism; $[\alpha]_D^{20}$ −37.5 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε): 204.5 (4.20), 242.5 (3.95), 268 (3.89), 320.5 (3.23); ^1H and ^{13}C NMR (CD_3OD) data see Table 1; ESI-MS: *m/z* 358 $[M + H]^+$, HR-ESI-MS: (*m/z* 380.1841 $[M + Na]^+$, calc. 380.1838).

Stereumamide C ***(3)***: pale brown amorphism; $[\alpha]_D^{20}$ −27.1 (*c* 0.8, MeOH); UV (MeOH) λ_{max} (log ε): 204.5 (4.02), 261 (3.72), 320.5 (3.11); ^1H and ^{13}C NMR (CD_3OD) data, see Table 1; ESI-MS: *m/z* 344 $[M + H]^+$, HR-ESI-MS: (*m/z* 344.1862 $[M + H]^+$, calc. 344.1862).

Stereumamide D ***(4)***: pale brown amorphism; $[\alpha]_D^{20}$ −67.9 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ε): 203.0 (4.34), 215.0 (4.29), 264.0 (3.80), 320.5 (3.17); ^1H and ^{13}C NMR (CD_3OD) data, see Table 1; ESI-MS *m/z* 431$[M + H]^+$, HR-ESI-MS: (*m/z* 431.1971$[M + H]^+$, calc. 431.1971).

2.4. Antibacterial activities

The antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium* was assayed. The MICs for antibacterial activity were determined by a method modified from the standardized microdilution method [14,15]. Three bacteria were incubated in Nutrient Agar at 25 °C for 18 h overnight. A few colonies of bacteria were collected aseptically with a sterile loop and introduced into 10 mL of sterile 0.9% saline solution. The concentration of the suspension was then standardized by adjusting the optical density to 0.10 at 630 nm, corresponding to bacterial cell suspension of 10^8 colony-forming units/mL (CFU/mL). This cell suspension was diluted