A novel antibacterial tyroscherin derivative with a natural unprecedented morpholine-2, 3-dione structural unit from the fungus *Pseudallescheria boydii*

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**ABSTRACT**

A novel tyroscherin derivative named pseudallecin A (1) with a natural unprecedented morpholine-2, 3-dione structural unit, and a new biogenic synthesis related organic acid named pseudallecin B (2) were purified from a symbiotic fungus *Pseudallescheria boydii* derived from *Pomacea canaliculata*. Their structures were elucidated via spectroscopic analyses and ECD calculation. Pseudallecin A exhibited strong inhibitory activities against both Gram-positive *Escherichia coli* and Gram-negative *Staphylococcus aureus*.

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1. Introduction

Natural products and their derivatives are extremely important molecules for drug discovery due to their diverse therapeutic uses and minimal side effects (Butler 2008). Searching for new antibiotics from microorganisms has always been an important topic (Hou et al. 2019).

*Pomacea canaliculata* is a mollusk of the Pomacea in the family Ampullariidae native to South America (Castillo and Naranjo 2017). Because of the edibility and high nutritional value, it has been introduced to many countries, such as China, Japan,
Thailand, America, New Zealand, and Spain. However, it was abandoned from breeding and spread quickly to various freshwater environment because consumers do not like its taste. Due to its strong adaptability and wide range of feeding habits, *P. canaliculata* greatly endangers the ecosystem balance and agricultural production, and therefore is listed as one of the 100 invasive species in the world (Carlsson et al. 2004; Rawlings et al. 2007; Ricciardi 2007). As an omnivorous snail, *P. canaliculata* carries a large number of parasites, bacteria and fungi (Carlsson et al. 2004). Some of its symbiotic microorganisms might have the ability to produce antibiotics to protect the host or themselves. However, there are few reports about its symbiotic microbial metabolites.

*Pseudallescheria boydii* belonging to the Phylum Ascomycota has all kinds of hosts. It is known as an opportunistic human pathogenic fungus that results in various infections in immunosuppressed and immunocompetent persons (Walts 2001). Its metabolites have been described to have antimicrobial and cytotoxic activities (Lan et al. 2014; Wu et al. 2014; Yan et al. 2015; Lan et al. 2016; Sorres et al. 2017).

During our work to discovery antimicrobial agents from microorganisms (Hu et al. 2019; 2020; Zhu et al. 2021), the metabolites of a fungus *P. boydii* XFQ03 purified from *P. canaliculata* was investigated because of the potent antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* of its ethyl acetate extracts. Our work led to the purification and identification of a novel tyroscherin derivative named pseudallecin A (1) which possessed a natural exceptional morpholine-2, 3-dione structural unit, and a new organic acid named pseudallecin B (2) which has a biogenic synthetic relationship with pseudallecin A (Figure 1). Their antibacterial effects toward *E. coli* and *S. aureus* were assessed.

**2. Results and discussion**

**2.1. Structure elucidation**

Pseudallecin A (1) had a molecular formula C$_{23}$H$_{33}$NO$_4$ (eight double-bond equivalents) deduced by HRESIMS at m/z 388.2488 ([M + H]$^+$, calcd. 388.2486). A set of ortho-positioned aromatic protons at $\delta_{\text{H}}$ 7.09 (d, 8.4 Hz, 2H, H-2/6) and 6.80 (2H, d, 8.4 Hz, H-3/5) in the $^1$H-NMR spectrum of 1, revealed a 1, 4-disubstituted benzene unit in 1. This along with the $^{13}$C-NMR and HSQC spectra revealed 23 carbons composed of four methyls, five sp$^3$ hybridized methenes, six sp$^2$ hybridized methines, and four sp$^2$ hybridized quaternary carbons. The NMR properties were greatly similar to those of tyroscherin, which had ever been isolated from two fungi also belonging to the genus *P. canaliculata*.
Pseudallescheria (Hayakawa et al. 2004; Nirma et al. 2013). However, the carbon chemical shifts of 2 shifted downfield by about 11.0 ppm at C-3 ($\delta_c$ 79.9), and upfield by 3.6 ppm at C-2 ($\delta_c$ 62.9), compared with those of tyroscherin. Moreover, two more sp$^2$ hybridized quaternary carbons at $\delta_c$ 157.4 (C-15) and 153.2 (C-16) were observed. HMBC correlations (Figure S19) from H-2 ($\delta_h$ 3.87) and H-17 ($\delta_h$ 2.58) to C-16 indicated that C-16 was connected to the nitrogen atom attached on C-2. HMBC correlations from H-3 to C-15 suggested that C-15 was linked to the oxygen atom located at C-3. Excluding the benzene ring (C-10/C24 C-60 C-6), the remaining double-bond equivalents indicated the existence of a six-membered ring formed by the linkage between C-15 and C-16. Therefore, the C-15 and C-16 had to be amide carbonyl and ester carbonyl carbons, respectively, according to the total molecular weight.

The $E$ configuration of $\Delta^6$ ($^7$) was deduced by the large coupling constant ($J = 15.3$ Hz). The cis H-2/H-3 was established by their small coupling constant (2.6 Hz) and NOE correlations (Figure S19) between H-2/H-3 and H-17, along with invisible NOE correlations between H-3 and H-1. According to the literature (Stahl et al. 1996; Schmidt et al. 2003), the carbon chemical shift values of C-13 and C-14 methyl groups in the same side-chain (from C-6 to C-14) as 1 and their shift differences (small for syn-configuration, large for anti-configuration) can be used to determine the relative configuration. Of compound 1, the shift difference between C-13 and C-14 was very large as 2.9 ppm. Moreover, this difference (2.9 ppm) and the shifts ($\delta_c$ 19.2/22.1, C-13/C-14) were almost identical to those of tyroscherin and its synthetic stereomers with syn-configuration (C-13/C-14, $\delta_c$ about 19.3/22.2) (Tae et al. 2011) and obviously diverged from those with anti-configuration (C-13/C-14, $\delta_c$ about 19.8/21.2), revealing the syn-configuration of C13/C-14 in 1. Then the experimental ECD was compared with the calculated ones of 2$^S$, 3$^R$, 8$^R$, 10$^R$-1, 2$^R$, 3$^S$, 8$^S$, 10S-1, 2$^S$, 3$^R$, 8$^S$, 10S-1 and 2$^R$, 3$^S$, 8$^R$, 10R-1 (Figure S17), which showed that the curve of 2$^S$, 3$^R$, 8$^R$, 10R-1 had the highest matching with the experimental one. Therefore, the absolute configuration of 1 was assigned. To our best knowledge, the morpholine-2, 3-dione moiety had only been reported as a moiety of some synthetic products (Tam 1986). Pseudallecin A is the first example with such a moiety belonging to natural products.

The molecular formula of pseudallecin B (2) was elucidated as C$_9$H$_{14}$O$_4$ by HRESIMS at $m/z$ 187.0971 ([$\text{M} + \text{H}]^+$, calcd. 187.0970). The $^1$H NMR spectrum contained signals of two methyls ($\delta_H$ 1.17, H-7 and 1.83, H-6), one methoxys ($\delta_H$ 3.74, H-9), one methene ($\delta_H$ 2.72 and 2.45, H-3$^\alpha$ and H-3$^\beta$), one sp$^3$ hybrid methine ($\delta_H$ 2.73, H-2) and one sp$^2$ hybrid methine ($\delta_H$ 7.00, H-5). The HSQC spectrum revealed the carbons directly connected to these protons. In addition, the $^{13}$C NMR spectrum also displayed one quaternary olefinic ($\delta_c$ 130.1, C-4) and two carbonyl ($\delta_c$ 181.5, C-1 and 167.5, C-8) carbons. $^1$H-$^1$H COSY correlations (Figure S19) between H-5 and H-6 indicated the connection between C-5 and C-6. Those between H-2 and H-3/H-7 suggested that C-2 was adjacent to both C-3 and C-7. HMBC correlations (Figure S19) from H-9 and H-5 to C-8 revealed the existence of a $\text{--COOCH}_3$ group which was located on C-4. HMBC correlations between H-5 and C-3 suggested the connection between C-3 and C-4. Thus, the remaining group must be $\text{--COOH}$ (C-1) based on analysis of the molecular formula. HMBC correlations from H-2, H-3 and H-7 to C-1 indicated the $\text{--COOH}$ was substituted
on C-2. The $E$ configuration of the double bond $\Delta^4$ (5) was determined by the NOESY correlations (Figure S19) between H-3 and H-6. The calculated ECD profile of 2 $R\cdot 2$ had a good matching with the experimental one (Figure S18), revealed the $2R$ configuration of 2. According to the structural similarity, pseudallecin B has a biogenic relationship with pseudallecin A. It might be the product that the carbons at positions 7 and 13 of pseudallecin A were oxidized to carboxyl groups, and the carbon at position 10 was oxidized to connect a hydroxyl group and then eliminated to form a double bond.

2.2. Antibacterial activity

Based on the antibacterial bioassay, pseudallecin A (1) showed high inhibitory activities toward both Gram-positive $S. aureus$ and Gram-negative $E. coli$ with MIC values (6.25 $\mu g/mL$) in the same order of magnitude as those of cephradine (1.56 $\mu g/mL$ for $S. aureus$; 3.13 $\mu g/mL$ for $E. coli$), revealing the good potential to be developed as a new broad-spectrum antibiotic. Pseudallecin B (2) only showed weak activity against $E. coli$ (MIC 200 $\mu g/mL$) and was inactive to $S. aureus$ (MIC $> 200 \mu g/mL$).

3. Experimental

3.1. General procedures

Optical rotations were detected by a JASCO P1020 polarimeter. UV and CD spectra were acquired with a Chirascan apparatus (Applied Photophysics, London, UK). HRESIMS was detected via a LCMS-IT-TOF (Shimadzu, Japan) mass spectrometer. NMR spectra were obtained on a Bruker AV (400 MHz) NMR spectrometer (Bruker BioSpin GmbH company, Rheinstetten, Germany). HPLC purification used an Elite system (Elite company, Dalian, China) with a P230 pump and UV230 $\pm$ wavelength detector using a C18 column (250 $\times$ 10 mm, 5 $\mu m$, Welch Technology Co., Ltd). Silica gel (Qingdao Haiyang Co., Ltd., Qingdao, China) for purification and TLC were 50 – 80 $\mu m$ and GF254, respectively. Sephadex LH-20 (GE Healthcare, Sweden). Methanol was chromatographic pure and other solvents were analytical grade.

3.2. Microbial material

$P. boydii$ XFQ03 was isoalted from the body of $P. canaliculata$. $E. coli$, $S. aureus$ and it were provided by and stored at the College of Materials and Energy, South China Agricultural University. This fungus XFQ03 was identified based on analysis of the ITS sequence (No. OL840859 in GenBank). The strain was fermented at 28°C for 25 d in 78 $\times$ 1 L Erlenmeyer flasks without rotation, each containing autoclaved medium (100 mL $H_2O$, 70 g rice).

3.3. Extraction, separation and spectroscopic data

The fermentation was soaked with ethanol for 5 d. The obtained extract was concentrated and suspended with water, and then was extracted twice using ethyl acetate
(EtOAC) to provide a raw extract (31.8 g). The extract was partitioned into five fractions (Fr. a to Fr. e) on the column chromatograph (CC) (40 x 5 cm) with silica gel by gradient elution with the mixed solvent of petroleum ether (PE)/EtOAc (v/v, 91:9, 83:17, 55:45, 17:83, 9:91). Fr. c was eluted with (PE)/EtOAc (v/v, 55:45) and partitioned into five fractions (Fr. c.1 to Fr. c.5) by CC (45 x 2.5 cm). Fr. c.3 eluted with MeOH on Sephadex LH-20 CC (120 x 2.0 cm), generated three subfractions (Fr. c.3.1-c.3.3) according to TLC properties. Fr. c.3.2 was transferred to HPLC with MeOH/H2O (v/v, 20:80 to 100:0, 2.8 mL/min, 130 min) to yield pseudallecin A (1) (3.6 mg, tR = 120 min) and pseudallecin B (2) (3.1 mg, tR = 90 min).

Pseudallecin A (1): colorless gum, C23H33NO4, HRESIMS m/z 388.2488 ([M + H]+, calcd. 388.2486). [α] = −85.4 (c = 0.10, MeOH). 1H NMR (400 MHz, acetone-d6) δ 3.14 (dd, 14.0, 4.8 Hz, 1H, H-1a), 2.76 (dd, 14.0, 9.5 Hz, 1H, H-1b), 3.87 (ddd, 9.5, 4.8, 2.6 Hz, 1H, H-2), 4.94 (ddd, 8.1, 5.0, 2.6 Hz, 1H, H-3), 1.84 (m, 1H, H-4a), 1.92 (m, 1H, H-4b), 2.24 (m, 1H, H-5a), 2.16 (m, 1H, H-5b), 5.45 (dt, 15.3, 7.0 Hz, 1H, H-6), 5.34 (ddd, 15.3, 8.1 Hz, 1H, H-7), 2.19 (m, 1H, H-8), 1.27 (ddd, 13.9, 8.9, 4.2 Hz, 1H, H-9β), 1.02 (ddd, 13.9, 8.9, 5.2 Hz, 1H, H-9α), 1.37 (m, 1H, H-10), 1.13 (m, 1H, H-11α), 1.30 (m, 1H, H-11β), 0.84 (t, 7.2 Hz, 3H, H-12), 0.83 (d, 6.7 Hz, 3H, H-13), 0.94 (d, 6.7 Hz, 3H, H-14), 2.58 (s, 3H, H-17), 7.09 (d, 8.4 Hz, 1H, H-2′/6′), 6.80 (d, 8.4 Hz, 1H, H-3′/5′); 13C NMR (101 MHz, acetone-d6) δ 34.1 (C-1), 62.9 (C-2), 79.9 (C-3), 31.6 (C-4), 28.9 (C-5), 127.6 (C-6), 138.5 (C-7), 35.2 (C-8), 45 (C-9), 32.7 (C-10), 30.7 (C-11), 115 (C-12), 19.2 (C-13), 22.1 (C-14), 157.4 (C-15), 153.2 (C-16), 35.3 (C-17), 127.7 (C-1′), 131.5 (C-2′/6′), 116.4 (C-3′/5′), 157.4 (C-4′).

Pseudallecin B (2): colorless gum, C9H14O4, HRESIMS m/z 187.0971 ([M + H]+, calcd. 187.0970), [α] = 4.6 (c = 0.10, MeOH). 1H NMR (400 MHz, CDCl3) δ 2.73 (m, 1H, H-2), 2.72, (dd, 10.0,16.0 Hz, 1H, H-3α), 2.45, (dd, 10.0,16.0 Hz, 1H, H-3β), 7.00, (q, 7.2 Hz, 1H, H-5), 1.83, (d, 7.2 Hz, 2H, H-6), 1.17, (d, 6.5 Hz, 3H, H-7), 3.74 (s, 3H, H-9); 13C NMR (101 MHz, CDCl3) δ 181.5 (C-1), 62.9 (C-2), 79.9 (C-3), 31.6 (C-4), 28.9 (C-5), 127.6 (C-6), 138.5 (C-7), 35.2 (C-8), 45 (C-9), 32.7 (C-10), 30.7 (C-11), 115 (C-12), 19.2 (C-13), 22.1 (C-14), 157.4 (C-15), 153.2 (C-16), 35.3 (C-17), 127.7 (C-1′), 131.5 (C-2′/6′), 116.4 (C-3′/5′), 157.4 (C-4′).

3.4. Antibacterial assay

Antibacterial evaluation against two types of pathogenic bacteria, E. coli and S. aureus, was performed by the dilution method, identical to previously described (Wu et al. 2018). Cephradine (Aladdin Shanghai Bio-Chem Technology corporation, China) was the positive control and the solvent (5% DMSO/H2O: LB = 1:1, v:v) was the negative one.

3.5. ECD calculations

Geometries of 1 and 2 were generated via OpenBabel 3.1.1 (O’Boyle et al. 2011) and then optimized by xtb at GFN2-xTB level. The conformers with Boltzmann distribution ≥ 2.0% were further optimized at B3LYP/6-31G (d) level and the single point energy were calculated at M062X/def2TZVPP level by Gaussian09 (Gaussian Inc., Wallingford, CT). The excited states (nstates = 30) for the generated stable conformers were calculated via Gaussian09 at B3LYP/6-311+G (d, p) level. The IEFPCM solvent model
(methanol) was adopted for all the Gaussian calculations. ECD curves were plotted through SpecDis 1.71 (University of Wurzburg, Wurzburg, Germany) with a half-bandwidth of 0.2 eV and UV corrections of 0 and +7 nm for 1 and 2, respectively.

4. Conclusion
A novel tyroscherin derivative pseudallecin A (1) with a naturally exceptional morpholine-2, 3-dione structural unit, and a new organic acid named pseudallecin B (2) were isolated and characterized from a symbiotic fungus P. boydii derived from P. canaliculata. Pseudallecin A exhibited strong inhibitory activities against both Gram-positive E. coli and Gram-negative S. aureus, revealing its great potential to be developed as an antibacterial agent towards corresponding bacteria.

Disclosure statement
No potential conflict of interest was reported by the authors.

Supplementary material
Supplementary material relating to this article is available online, alongside HRESIMS, NMR (1D and 2D), UV, calculated and experimental ECD spectra and key 2D NMR correlations of pseudallecins A and B (1 and 2) (Figures S1–S19).

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References
Butler MS. 2008. Natural products to drugs: natural product-derived compounds in clinical trials. Nat Prod Rep. 25(3):475–516.
Carlsson NOL, Brönmark C, Hansson L. 2004. Invading herbivory: the golden apple snail alters ecosystem functioning in Asian wetlands. Ecology (Durham). 85(6):1575–1580.
Castillo G, Naranjo E. 2017. First inventory of the introduced and invasive mollusks in Mexico. Nautilus Greenville Then Sanibel. 131:107–126.
Hayakawa Y, Yamashita T, Mori T, Nagai K, Shin-Ya K, Watanabe H. 2004. Structure of tyroscherin, an antitumor antibiotic against IGF-1-dependent cells from Pseudallescheria sp. J Antibiot. 57(10):634–638.
Hou XM, Liang TM, Guo ZY, Wang CY, Shao CL. 2019. Discovery, absolute assignments, and total synthesis of aspersiversiamides A–C and their potent activity against Mycobacterium marinum. Chem Commun (Camb). 55(8):1104–1107.
Hu Z, Tao Y, Tao X, Su Q, Cai J, Qin C, Ding W, Li C. 2019. Sesquiterpenes with phytopathogenic fungi inhibitory activities from fungus Trichoderma virens from Litchi chinensis Sonn. J Agric Food Chem. 67(38):10646–10652.
Hu Z, Wu Z, Su Q, Li M, Wu S, Meng R, Ding W, Li C. 2020. Metabolites with phytopathogenic fungi inhibitory activities from the mangrove endophytic fungus *Botryosphaeria ramose*. Bioorg Chem. 104:104300.

Lan W, Liu W, Liang W, Xu Z, Le X, Xu J, Lam C, Yang D, Li H, Wang L. 2014. Pseudaboydins A and B: novel isobenzofuranone derivatives from marine fungus *Pseudallescheria boydii* associated with starfish *Acanthaster planci*. Mar Drugs. 12(7):4188–4199.

Lan W, Wang K, Xu M, Zhang J, Lam C, Zhong G, Xu J, Yang D, Li H, Wang L. 2016. Secondary metabolites with chemical diversity from the marine-derived fungus *Pseudallescheria boydii* F19-1 and their cytotoxic activity. RSC Adv. 6(80):76206–76213.

Nirma C, Eparvier V, Stien D. 2013. Antifungal agents from *Pseudallescheria boydii* SNB-CN73 isolated from a *Nasutitermes* sp. termite. J Nat Prod. 76(5):988–991.

O’Boyle NM, Banck M, James CA, Morley C, Vandermeersch T, Hutchison GR. 2011. Open Babel: an open chemical toolbox. J Cheminform. 3:33.

Rawlings TA, Hayes KA, Cowie RH, Collins TM. 2007. The identity, distribution, and impacts of non-native apple snails in the continental United States. BMC Evol Biol. 7(1):97.

Ricciardi A. 2007. Are modern biological invasions an unprecedented form of global change? Conserv Biol. 21(2):320–336.

Schmidt K, Riese U, Z Z L, Hamburger M. 2003. Novel tetramic acids and pyridine alkaloids, miltamarinones B, C, and D, from the insect pathogenic fungus *Paecilomyces Capitolarius*. J Nat Prod. 66(3):378–383.

Sorres J, Nirma C, Barthélemy M, Eparvier V, Stien D. 2017. Tyroscherin and tyroscherin analogs from *Pseudallescheria boydii* SNB-CN85 isolated from termite Termes cf. hispaniolae. Phytochem Lett. 22:142–144.

Stahl M, Schopfer U, Frenking G, Hoffmann RW. 1996. Assignment of relative configuration to acyclic compounds based on (13)C NMR shifts. A density functional and molecular mechanics study. J Org Chem. 61(23):8083–8088.

Tae HS, Hines J, Schneekloth AR, Crews CM. 2011. Unexpected stereochemical tolerance for the biological activity of tyroscherin. Bioorg Med Chem. 19(5):1708–1713.

Tam W. 1986. Carbonylation of β-aminoethanols, diols, and diol amines. J Org Chem. 51(15):2977–2981.

Walts AE. 2001. Pseudallescheria: an underdiagnosed fungus? Diagn Cytopathol. 25(3):153–157.

Wu Q, Jiang N, Bo Han W, Ning Mei Y, Ming Ge H, Kai Guo Z, Seik Weng N, Xiang Tan R. 2014. Antibacterial epipolythiodioxopiperazine and unprecedented sesquiterpene from *Pseudallescheria boydii*, a beetle (coleoptera)-associated fungus. Org Biomol Chem. 12(46):9405–9412.

Wu Z, Xie Z, Wu M, Li X, Li W, Ding W, She Z, Li C. 2018. New antimicrobial cyclopentenones from *Nigrospora sphaeria* ZMT05, a fungus derived from *Oxya chinensis* Thunber. J Agric Food Chem. 66(21):5368–5372.

Yan DF, Lan WJ, Wang KT, Huang L, Jiang CW, Li HJ. 2015. Two chlorinated benzo[5]furan derivatives from the marine fungus *Pseudallescheria boydii*. Nat Prod Commun. 10(4):621–622.

Zhu J, Li Z, Lu H, Liu S, Ding W, Li J, Xiong Y, Li C. 2021. New diphenyl ethers from a fungus *Epicoccum sorghinum* L28 and their antifungal activity against phytopathogens. Bioorg Chem. 115:105232.