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Secondary metabolites from Antarctic marine-derived fungus *Penicillium crustosum* HDN153086

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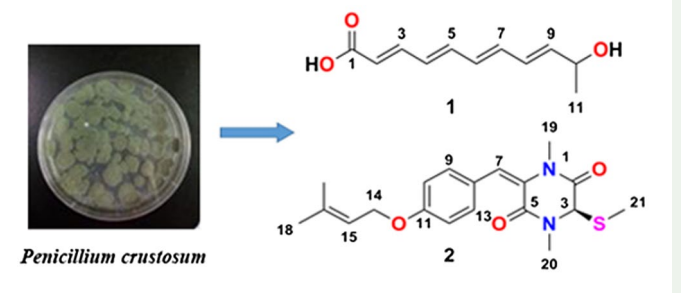
ABSTRACT

A new polyene compound (**1**) and a new diketopiperazine (**2**), as well as three known compounds (**3**–**5**), were isolated from the Antarctic marine-derived fungus *Penicillium crustosum* HDN153086. The structures of **1**–**5** were deduced based on MS, NMR and TD-DFT calculations of specific ECD spectra. These compounds were evaluated for their cytotoxic activities against K562 cell line and only compound **2** exhibited cytotoxicity against K562 cell, with IC_{50} value of 12.7 μM.



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1. Introduction

Antarctic-derived microorganisms has become an important resource for investigation of structurally novel and biologically active compounds (Zoe and Margaret 2009; Ghiglione et al. 2012). In our on-going search for bioactive secondary metabolites from Antarctic-derived fungi (Li et al. 2012; Zhou et al. 2015, 2016), a *Penicillium crustosum* fungal strain HDN153086, isolated from a soil sample collected from Prydz Bay, was selected for its series of UV absorption peaks and cytotoxic activity (inhibitory rate was 27.69% against K562 cell

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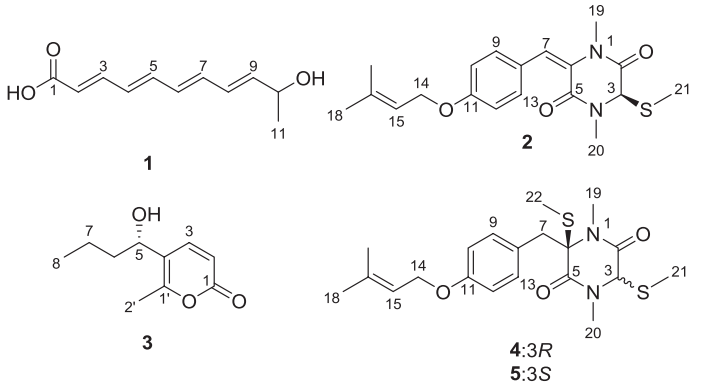

Figure 1. Structures of compounds **1**–**5**.

line at the concentration of 100 μg/mL of the EtOAc extract). Chemical investigation of the organic extract led to the isolation of two new compounds (**1** and **2**), together with three known related compounds (**3**–**5**) (Figure 1). The cytotoxicity of new compounds was assayed and only **2** showed moderate activities against K562 cells, with IC_{50} value of 12.7 μM. In addition, we determined the absolute configuration of compound **3** for the first time. Herein, we reported the details of the isolation, structure elucidation and biological activities of these new compounds.

2. Results and discussion

Compound **1** (a pale yellow amorphous powder) was obtained as an inseparable mixture with the ratio 2:1 deduced from HPLC spectrum (Figure S2). The molecular formula was determined as $C_{11}H_{14}O_3$ based on the HRESIMS de-protonated peaked at *m/z* 193.0874 $[M - H]^-$ (Calcd for $C_{11}H_{13}O_3$, 193.0870), indicating five degrees of unsaturation. The 1D NMR data (Table S1) of **1** showed the presence of one methyl (δ_C 22.0, δ_H 1.24), eight sp^2 methines (δ_C 120.2, δ_H 5.85, δ_C 145.0, δ_H 7.31, δ_C 129.6, δ_H 6.42, δ_C 140.7, δ_H 6.67, δ_C 136.6, δ_H 6.47, δ_C 128.6, δ_H 6.30, δ_C 131.4, δ_H 6.33 and δ_C 140.2, δ_H 5.88) and one oxygenated sp^3 methine (δ_C 67.4, δ_H 4.31). The planar structure of **1** was determined by interpretation of 1D and 2D NMR spectroscopic data. The sequential COSY correlations of H-2/H-3/H-4/H-5/H-6/H-7/H-8/H-9/H-10/H_3-11 established the spin system from C-2 to C-11. The spin system was also confirmed and further extended to C-1 (δ_C 169.1) by HMBC correlations from H-2 to C-1, and from H-3 to C-1 (Figure S1). Then, the planer structure of **1** was constructed. The stereochemistry of the double bonds was determined as *E*, *E*, *E* and *E* stereochemistry for Δ^2, Δ^4, Δ^6 and Δ^8 by the coupling constants ($^3J_{H\text{-}2,\ H\text{-}3}$ = 15.0 Hz, $^3J_{H\text{-}4,\ H\text{-}5}$ = 15.0 Hz, $^3J_{H\text{-}6,\ H\text{-}7}$ = 15.0 Hz, $^3J_{H\text{-}8,\ H\text{-}9}$ = 15.0 Hz). In order to establish the absolute configuration of C-10, Mosher's method was performed. However, we were surprised to find a pair of products, either (*R*)-MPA or (*S*)-MPA ester, which indicated that compound **1** existed as a mixture of enantiomers, and the conclusion was further confirmed by the HPLC analysis on a chiral phase HPLC column (Figure S2). Due to lack of samples, the two enantiomers were not further separated by chiral HPLC column. Therefore, compound **1** was assigned as (2*E*, 4*E*, 6*E*, 8*E*)-10-hydroxyundeca-2,4,6,8-tetraenoic acid.

Fusaperazine F (**2**) was obtained as a white powder. The molecular formula was determined as $C_{19}H_{24}N_2O_3S$ on the basis of HRESIMS protonated peak at *m/z* 361.1581 $[M + H]^+$ ion (Calcd for $C_{19}H_{25}N_2O_3S$, 361.1580), indicating nine degrees of unsaturation. The 1D NMR data (Table S2) of **2** suggested the presence of five methyls, one methylenes, seven methines, and six non-protonated carbons with two carbonyls (δ_C 163.1 and δ_C 164.6). The ^1H and ^{13}C NMR spectra of **2** are close to those of the known compound fusaperazine E (Guimarães et al. 2010). The major differences were the chemical shifts of C-5 (δ_C 163.1 vs. δ_C 159.2), C-19 (δ_C 35.1 vs. δ_C 32.5) and C-20 (δ_C 32.5 vs. δ_C 29.7), indicating the absolute configuration of C-3 or the geometry of double bond $\Delta^{6,7}$ was different. The absolute configuration of C-3 was determined the same as fusaperazine E based on the similar optical rotation between **3** (+159.0) and fusaperazine E (+10.2) (Guimarães et al. 2010). In order to establish the geometry of double bond $\Delta^{6,7}$, NOEs were performed. The NOE_S correlation between H-7 and H-19 clearly suggests an *E* stereochemistry for $\Delta^{6,7}$ which was different with fusaperazine E, resulting in the different chemical shifts of C-5, C-19 and C-20. Therefore, **2** was assigned as (*R*, *E*)-1, 4-dimethyl-3-(4-((3-methylbut-2-en-1-yl)oxy)benzylidene)-6-(methylthio)piperazine-2,5-dione (Figure S1).

Compound **3** was obtained as a white powder. The positive ESI-MS protonated peak at *m/z* 183.08 $[M + H]^+$ and corresponding to the molecular composition of $C_{10}H_{14}O_3$. Further interpretation of 1D NMR demonstrated that the planar structure of **3** was same as the known compound xylariolide D (Hu et al. 2010). However, the absolute configuration of C-5 was not reported previously. To figure out the absolute configuration of the C-5 in **3**, the computational ECD calculations were attempted using a truncated model compound **3a**. A molecular mechanics conformational analysis of **3** was performed using Spartan software, followed by reoptimization using DFT at the B3LYP/6 – 31 + G(d) level with Gaussian 09 software. According to the good agreement of the Boltzmann-weighted CD curve of the truncated model (5*S*)-**3a** with the experimental one (Figure S16), the absolute configuration of **3** was determined as 5*S*. Thus, the structure of **3** was established to be (*S*)-5-(1-hydroxybutyl)-6-methyl-2H-pyran-2-one.

Compounds **1**–**5** were evaluated for their antitumor activities *in vitro* against the K562 cell line by the MTT method. Compound **2** showed cytotoxicity with the IC_{50} value of 12.7 μM.

3. Experimental

3.1. General experimental procedures

Optical rotations were obtained on a JASCOP-1020 digital polarimeter. UV spectra were recorded on Waters 2487, while the ECD spectrum was measured on JASCO J-815 spectropolarimeter. ^1H NMR, ^{13}C NMR, DEPT and 2D NMR spectra were recorded on an Agilent 500 MHz DD2 spectrometer. HRESIMS and ESIMS data were obtained using a Thermo Scientific LTQ Orbitrap XL mass spectrometer. Column chromatography (CC) was performed with Sephadex LH-20 (Amersham Biosciences). MPLC was performed using a C_{18} column (Agela Technologies, YMC-Pack ODS-A, 3 × 40 cm, 5 μm, 20 mL/min). Preparative HPLC collection used a C_{18} column (Waters, YMC-Pack ODS-A, 10 × 250 mm, 5 μm, 3 mL/min). Chiral-phase HPLC used a Daicel Chiralpack IC column (4.6 × 250 mm, 5 μm, 1 mL/min).

3.2. Biological material

The fungal strain was isolated from the Antarctic sediment from Prydz Bay (E 77.57°, S 68.34°, collected in February, 2015) and identified as *Penicillium crustosum* based on sequence of ITS region (GenBank No. MG728115) with 100% similarity. The strain was deposited at the Key Laboratory of Marine Drugs, the Ministry of Education of China, School of Medicine and Pharmacy, Ocean University of China, Qingdao, People's Republic of China.

3.3. Fermentation, extraction and isolation

Erlenmeyer flasks (500 mL) containing 150 mL fermentation media were directly inoculated with spores. The media contained maltose (20 g), mannitol (20 g), glucose (10 g), sodium glutamate (10 g), yeast extract (3 g), corn syrup (1 g), KH_2PO_4 (0.5 g), and $MgSO_4 \cdot 7H_2O$ (0.3 g) dissolved in 1 L of naturally collected seawater (Huiquan Bay, Yellow Sea). The flasks were cultured with shaking at 180 rpm and 28 °C for 9 days.

The whole fermentation broth (120 L) was filtered through cheese cloth to separate the supernatant from the mycelia. The supernatant was extracted with EtOAc (3 × 120 L) and the extract was evaporated under reduced pressure to give a crude gum (40 g). The crude extract was subjected to a silica gel (300–400 mesh) CC and was separated into six fractions (Fr.1–Fr.6) using a step gradient elution of CH_2Cl_2/CH_3OH. The fraction Fr.1 (2.6 g), eluted with CH_2Cl_2, was fractionated on a Sephadex LH-20 CC using CH_2Cl_2/CH_3OH (7:3) as the eluting solvent to afford three fractions, Fr.1.1–Fr.1.3 (243.5 mg, 980.0 mg and 867.3 mg, respectively). Fr.1.2 was separated MPLC (C-18 ODS) using a stepped gradient elution of MeOH/H_2O (20:80 to 100:0) to yield seven subfractions (Fr.1.2.1 to Fr.1.2.7), Fr.1.2.6 (386.7 mg) was then separated by semi-preparative HPLC eluted with MeOH/H_2O (70:30) to obtain compounds **2** (3.0 mg, t_R = 33.5 min), **4** (60.0 mg, t_R = 25.0 min), and **5** (110.0 mg, t_R = 29.0 min). Fr.1.3 was separated by MPLC (C-18 ODS) using a stepped gradient elution of MeOH/H_2O (5:95 to 100:0) to yield seven subfractions (Fr.1.3.1 to Fr.1.3.7), Fr.1.3.4 (681.5 mg) was separated by semi-preparative HPLC eluted with MeOH/H_2O (46:54) to yield three subfractions (Fr.1.3.4.1 to Fr.1.3.4.3) and Fr.1.3.4.2 (119.3 mg) was then separated by semi-preparative HPLC CH_3CN/H_2O (27:73) to obtain compound **3** (10.0 mg, t_R = 22.5 min). The fraction Fr.2 (1.5 g) was separated on Sephadex LH-20 CC using CH_2Cl_2/CH_3OH (7:3) as the eluting solvent to afford five fractions (Fr.2.1–Fr.2.5), Fr.2.3 (132.8 mg) was applied on Sephadex LH-20 CC using CH_3OH to provide three subfractions (Fr.2.3.1–Fr.2.3.3). Fr.2.3.2 (18.7 mg) was further purified by semi-preparative HPLC CH_3OH/H_2O (57:43) to obtain compound **1** (3.0 mg, t_R = 16.0 min).

3.3.1. Compound 1

Pale yellow amorphous powder; $[\alpha]_D^{25}$-7.5 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (logε): 229 (1.26), 334 (4.19); ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz): see Table S1; HRESIMS *m/z* 193.0874 $[M - H]^-$ (Calcd for $C_{11}H_{13}O_3$, 193.0870).

3.3.2. Compound 2

White powder; $[\alpha]_D^{25}$ + 159.0 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (logε): 225 (4.25), 316 (4.47); ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz): see Table S2; HRESIMS *m/z* 361.1581 $[M + H]^+$ (Calcd for $C_{19}H_{25}N_2O_3S$, 361.1580).

3.4. Cytotoxic assay

Inhibition of K562 cell proliferation of compounds **1**–**5** were measured using the MTT assay (Mosmann 1983). Ninety microliters of those cell suspensions were plated in 96-cell plates at a density of 390,000 cell mL^{-1}. Then 1 µL of the test compound solutions (in DMSO) at different concentrations was added to each well and further incubated for 72 h. Following drug exposure, the cells were fixed with 20 µL MTT (5 mg/mL), and then add 100 µL triplex solution to the cell layer after 4 h. The absorbance of MTT solution was measured at 570 nm. Dose-response curves were generated, and the IC_{50} values, the concentration of compound required to inhibit cell proliferation by 50%, were calculated from the linear portion of log-dose-response curve.

3.5. Computation section

Conformational searches were run by employing the 'systematic' procedure implemented in Spartan'14 using MMFF (Merck molecular force field). All MMFF minima were reoptimized with DFT calculations at the B3LYP/6 − 31 + G(d) level using the Gaussian09 program (Frisch et al. 2010). The geometry was optimized starting from various initial conformations, with vibrational frequency calculations confirming the presence of minima. Time-dependent DFT calculations were performed on two lowest-energy conformations for (5*S*)-**3a** (>5% population) using 30 excited states, and using a polarizable continuum model (PCM) for MeOH. ECD spectra were generated using the program SpecDis (Bruhn et al. 2011) by applying a Gaussian band shape with 0.4 eV width for **3**, from dipolelength rotational strengths. The dipole velocity forms yielded negligible differences. The spectra of the conformers were combined using Boltzmann weighting, with the lowest-energy conformations accounting for about 100% of the weights. The calculated spectra were shifted by 5 nm to facilitate comparison to the experimental data.

4. Conclusion

Two new compounds (**1** and **2**), together with three known related compounds (**3–5**) were isolated from the Antarctic derived fungus *Penicillium crustosum* HDN153086, which including one polyene compound (**1**), three diketopiperazines (**2**, **4** and **5**), and xylariolide D (**3**). Compound **1** showed conjugated tetraene which was unusual in natural products (Komori and Itoh 1985; Fleury et al. 2009). Compound **2** showed cytotoxicity against K562 cells, while fusaperazine E (Guimarães et al. 2010), which was an isomer differed in the geometry of double bond $\Delta^{6,7}$, was inactive. The compounds **4** and **5** were discovered from various specific environment, such as terrestrial plant (Yang et al. 2017), inland soil (Rahbæk et al. 1998; Wang et al. 2015; Li et al. 2018) and marine organism (Capon et al. 2007; Wang et al. 2012) of Pacific coastal area. As far as we known, compounds **4** and **5** were isolated from Antarctic derived fungus for the first time.

Supplementary material

Supplementary material relating to this article is available online, including HRESIMS and NMR spectra for compounds **1** and **2**, ECD spectra of compound **3**.

Disclosure statement

No potential conflict of interest was reported by the authors.

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