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Antimicrobial secondary metabolites from a marine-derived fungus *Penicillium* sp. OM07

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ABSTRACT

Eight compounds, diketopiperazine dimer WIN 64821 (**1**), ergosterol peroxide (**2**), ergosterol (**3**), 3β,5*α*,9*α*-trihydroxyergosta-7,22-dien-6-one (**4**), 3,4-dihydroxy-6,7-dimethyl-quinolin-2-carboxylic (**5**), norhaman (**6**), dihydrocitrinin (**7**), and phenol A acid (**8**) were isolated and characterized from the culture broth of the marine-derived *Penicillium* sp. OM07 strain was isolated from sediment collecting at Son Cha, Hue, Vietnam. Their structures were determined by analyses of MS and NMR data. All compounds were evaluated for their antimicrobial activity against a panel of clinically significant microorganisms. Most showed high antifungal activity against *Candida albicans* ATCC10231 strain with MIC values ranging from 8 µg/mL to 256 µg/mL. All compounds had inhibitory activity against from one to three Gram-positive tested strains with MIC values from 64–256 µg/mL.

Keywords: *Penicillium*, diketopiperazine dimer, antimicrobial activity, ergosterol, marine sediment.

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INTRODUCTION

Marine-derived fungi from the *Penicillium* genus have received remarkable interest as a valuable source of novel natural products with potential applications in industry, agriculture, and medicine [1]. Marine *Penicillium* fungi have been found in sediments, mangroves, sponges, and algae and have been shown to have high novelty for more than 390 new metabolites in the last decade, including alkaloids, polyketides, terpenes, steroids, and macrolides [2] that possess important biological activities such as antimicrobial, anticancer, anti-inflammatory and larvicidal [3, 4]. In Vietnam, few publications have been on the chemical constituents of the marine fungi *Penicillium* [5, 6].

During our screening program, the extract of the *Penicillium* sp. OM07 strain exhibited antimicrobial activity against three Gram-positive bacteria strains: *Staphylococus aureus* ATCC25923, *Enterococcus faecalis* ATCC29212, and *Bacillus cereus* ATCC14579 with MIC values of 256, 128 and 64 µg/mL, respectively. Further investigation on the chemical constituents resulted in the identification of eight compounds (**1-8**) from the fungus *Penicillium* sp. OM07 strain isolated from sediment samples collected at Son Cha (Hue, Vietnam).

MATERIALS AND METHODS

Collection of marine sediment sample

The marine sediment sample (Name of sample: 243 A) was collected in the Son Cha - Hue sea area, Vietnam, in May 2021. It was collected at 34.2 m depth, with a geographic coordinate of 16.4°45’28”–108.0°88’13” and a water temperature of 28°C. The sample was collected into a 50 mL sterile Falcon tube, preserved on ice, and processed within 24 hours.

Isolation and identification of the fungus OM07

The sediment sample was homogenized and treated using a wet-heat technique at 60°C for 6 min. A ten-fold dilution series diluted the suspension to 10^{-3}. Then, aliquots of 50 µL were spread on Petri dishes PDA solid medium (30 g/L potato extract, 20 g/L dextrose, 5 g/L soluble starch, 30 g/L instant ocean, 15 g/L agar). The plates were incubated at 28°C for 7 days. The colony of fungus OM07 was transferred onto a new Petri dish of medium PDA for purification (Figure 1).

Figure 1. Morphological appearance of OM07 strain’s colonies

The taxonomy of the strain OM07 was identified by using 18S rRNA gene sequence analysis and compared with fungal 18S rRNA sequences in the GenBank database by the NCBI Blast program. The results showed that strain M893 belonged to the genus *Penicillium*.

Fermentation fungus OM07

The strain OM07 was activated and inoculated into 1 L of PDB broth medium pH 7.0 (comprising 30 g/L potato extract, 20 g/L dextrose, 5 g/L soluble starch, 30 g/L instant ocean). After 7 days of incubation at 28°C with shaking of 100 rpm, the culture broth was spread on the medium surface of 50 flasks (each 3L flask containing 1 L of PDA medium, pH 7.0). The flasks were incubated at 28°C and harvested for twenty-five days.

General Experiment procedures

Melting points were recorded on a Buchi B-545 instrument. Optical rotations were recorded on an Atago Polax-2L polarimeter, using a sodium (589, D line) lamp. HR-ESI-MS spectra were obtained using an AGILENT 6550 iFunnel Q-TOF LC/MS system. ESI-MS was performed on an Agilent 6120 instrument, and CD spectra were recorded on a Chirascan CD spectrometer. Using TMS as the internal standard, 1D and 2D NMR spectra were recorded on Bruker Avance 500 MHz and 600 MHz spectrometers. Column chromatography (CC) was performed with silica gel (230–400 mesh, Merck). Thin layer chromatography monitored the fractions; spots were visualized by UV light (254 and 365 nm) and by heating silica gel plates sprayed with Cerium (IV)sulfate reagent. All column chromatography solvents were distilled before being used.

Extraction and isolation

The fermentation products of strain *Penicillium* sp. OM07 were cut into small pieces and sonicated in ethyl acetate (three times × 2 hours each) at 40^{o}C. The combined ethyl acetate extracts were concentrated under reduced pressure to give 50.0 g of ethyl acetate (EtOAc) extract. The EtOAc extract was separated on a silica gel column chromatography (CC) and eluted with a solvent gradient CH_2Cl_2/MeOH (5% to 100% MeOH) to yield 9 fractions F1-F9. Fraction 3 (0.8 g) was separated on a Sephadex LH-20 CC and eluted with MeOH to obtain five fractions F3.1–F3.5. Fraction 3.3 (0.18 g) was separated on a silica gel CC eluted with CH_2Cl_2/acetone (95/5) to obtain **3** (5 mg). Fraction 5 (3.2 g) was separated on a Sephadex LH-20 CC eluted with MeOH to obtain five fractions F5.1–F5.5. Fraction F5.3 (0.16 g) was continuously separated by silica gel column chromatography and eluted with CH_2Cl_2/acetone gradient (5% to 100% acetone) to give five fractions F5.3.1–F5.3.5. Fraction 5.3.4 was crystallized with CH_2Cl_2/acetone (9/1, V/V) to yield **5** (4 mg). Fraction F6 (2.8 g) was further purified by gel filtration over Sephadex LH-20 CC with MeOH as eluent to give 5 fractions F6.1–F6.5. Fraction F6.2 (0.2 g) was continuously separated by silica gel CC, eluted with CH_2Cl_2/MeOH gradient to give **2** (5 mg) and **4** (4 mg). Fraction F7 (2.2 g) was separated by silica gel CC, eluted with CH_2Cl_2/MeOH gradient (5% to 100% MeOH) to give 6 fractions F7.1–F7.6, fraction F7.3 (0.3 g) was continuously separated by Sephadex LH-20 CC to give 4 fractions F7.3.1–F7.3.4, fraction 7.3.2 was crystallized to yield **1** (6 mg). Similarly, fraction F7.4 (0.42 g) was separated on a silica gel CC, eluted with CH_2Cl_2/acetone (9/1) to yield **6**. Fraction F8 (3.5 g) was further purified by gel filtration over Sephadex LH-20 CC, eluted with MeOH to give 7 fractions F8.1–F8.7. Fraction 8.3 (0.25 g) was continuously subjected by silica gel CC, eluted with CH_2Cl_2/MeOH gradient (5% to 100% MeOH) to yield **7** (5 mg), and **8** (4 mg).

Diketopiperazine dimer WIN 64821 (1): white solid, HRMS *m/z* 665.2876 $[M+H]^+$ $[\alpha]_D^{25}$ +225.2^o (*c* 1.73, MeOH). ^{1}H-NMR (600 MHz, $CDCl_3$-*d₁*): δ_H (ppm) 2.66 (1H, dd, *J* = 10.2, 14.4 Hz, H-17a), 2.76 (1H, dd, *J* = 8.4, 13.8 Hz, H-10a), 3.16 (1H, dd, *J* = 9.0, 13.8 Hz, H-10b), 3.45 (1H, dd, *J* = 3.6, 14.4 Hz, H-17b), 3.95 (1H, t, *J* = 8.3 Hz, H-11), 4.06 (1H, dd, *J* = 3.6, 10.2 Hz, H-14), 4.94 (1H, s, H-2), 5.61 (1H, s, NH-15), 5.71 (1H, s, NH-1), 6.69 (1H, d, *J* = 7.8 Hz, H-7), 6.81 (dt, *J* = 0.6, 7.8 Hz, H-5), 7.09 (2H, d, *J* = 7.8 Hz, H-19, H-23), 7.19 (1H, dt, *J* = 0.6, 7.8 Hz, H-6), 7.22 (2H, t, *J* = 7.8 Hz, H-20, H-22), 7.23 (1H, m, H-21), 7.34 (1H, d, *J* = 7.8 Hz, H-4). ^{13}C-NMR (150 MHz, $CDCl_3$-d_1): δ_C (ppm) 36.6 (CH_2, C-17), 36.7 (CH_2, C-10), 56.4 (CH, C-14), 57.1 (CH, C-11), 59.8 (C, C-3), 79.8 (CH, C-2), 110.1 (CH, C-7), 119.9 (CH, C-5), 124.7 (CH, C-4), 127.5 (CH, C-21), 128.9 (2CH, C-19, C-23), 129.2 (2CH, C-20, C-22), 129.7 (CH, C-6), 129.8 (C, C-8), 135.4 (C, C-18), 148.7 (C, C-9), 167.4 (C=O, C-13), 168.5 (C=O, C-16).

Ergosterol peroxide (2): colorless amorphous powder, mp: 182–183^{o}C, $[\alpha]_D^{25}$ = -43.1^o (*c* 0.05, $CHCl_3$), ^{1}H-NMR (600 MHz, $CDCl_3$-d_1) δ_H: 0.82 (3H, d, *J* = 6.6 Hz, CH_3-27), 0.83 (3H, s, CH_3-18), 0.84 (3H, d, *J* = 6.6 Hz, CH_3-26), 0.88 (3H, s, CH_3-19), 0.91 (3H, d, *J* = 6.6 Hz, CH_3-28), 1.00 (3H, d, *J* = 6.6 Hz, CH_3-21), 3.97 (1H, m, H-3), 5.14 (1H, dd, *J* = 8.4, 15.6 Hz, H-22), 5.22 (1H, dd, *J* = 7.8, 15.6 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 (150 MHz, $CDCl_3$-d_1) δ_C: 12.9 (CH_3, C-18), 17.6 (CH_3, C-28), 18.2 (CH_3, C-19), 19.7

(CH_3, C-27), 20.0 (CH_3, C-26), 20.7 (CH_3, C-21), 20.9 (CH_2, C-11), 23.4 (CH_2, C-15), 28.7 (CH_2, C-16), 30.1 (CH_2, C-2), 33.1 (CH, C-25), 34.7 (CH_2, C-1), 37.0 (CH_2, C-4), 37.0 (C, C-10), 39.4 (CH_2, C-12), 39.8 (CH, C-20), 42.8 (CH, C-24), 44.6 (CH, C-13), 51.1 (CH, C-9), 51.7 (CH, C-14), 56.2 (CH, C-17), 66.5 (CH, C-3), 79.4 (C, C-5), 82.2 (C, C-8), 130.8 (CH, C-7), 132.3 (CH, C-23), 135.2 (CH, C-6), 135.4 (CH, C-22).

Ergosterol (3): colorless needle, mp 163–164°C, $[\alpha]_D^{25}$ = -101° (*c* 0.05, $CHCl_3$), ^{1}H-NMR (600 MHz, $CDCl_3$-d_1) δ_H: 0.63 (3H, s, CH_3-18), 0.82 (3H, d, *J* = 6.6 Hz, CH_3-26), 0.84 (3H, d, *J* = 6.6 Hz, CH_3-27), 0.91 (3H, d, *J* = 6.6 Hz, CH_3-28), 0.95 (3H, s, CH_3-19), 1.03 (3H, d, *J* = 6.6 Hz, CH_3-21), 3.63 (1H, m, H-3), 5.17 (1H, dd, *J* = 6.6, 15.0 Hz, H-22), 5.23 (1H, dd, *J* = 7.8, 15.0 Hz, H-23), 5.38 (1H, m, H-7), 5.57 (1H, dd, *J* = 2.4, 5.4 Hz, H-6). ^{13}C-NMR (150 MHz, $CDCl_3$-d_1) δ_C (ppm): 12.1 (CH_3, C-18), 16.3 (CH_3, C-19), 17.6 (CH_3, C-28), 19.6 (CH_3, C-26), 19.9 (CH_3, C-27), 21.1 (CH_3, C-21), 21.13 (CH_2, C-11), 23.0 (CH_2, C-15), 28.3 (CH_2, C-16), 32.0 (CH_2, C-2), 33.1 (CH, C-25), 37.0 (CH, C-10), 38.4 (CH_2, C-1), 39.1 (CH_2, C-12), 40.4 (CH, C-20), 40.8 (CH_2, C-4), 42.8 (C, C-13), 42.9 (CH, C-24), 46.3 (CH_2, C-9), 54.6 (CH, C-14), 55.8 (CH, C-17), 70.5 (CH, C-3), 116.3 (CH, C-7), 119.6 (CH, C-6), 132.0 (CH, C-23), 135.6 (CH, C-22), 139.8 (C, C-5), 141.3 (C, C-8).

3β,5*α*,9*α*-Trihydroxyergosta-7,22-dien-6-one (4): white solid, mp 225–228°C, $[\alpha]_D^{25}$ -72° (0.07, MeOH). ^{1}H NMR (600 MHz, $CDCl_3$-d_1): δ_H (ppm) 0.62 (3H, s, CH_3-18), 0.82 (3H, d, *J* = 6.6 Hz, CH_3-26), 0.83 (3H, d, *J* = 6.6 Hz, CH_3-27), 0.92 (3H, d, *J* = 6.6 Hz, CH_3-28), 1.03 (3H, s, CH_3-19), 1.04 (3H, d, *J* = 6.6 Hz, CH_3-21), 4.06 (1H, m, H-3), 5.17 (1H, dd, *J* = 7.8, 15.0 Hz, H-22), 5.25 (1H, dd, *J* = 7.8, 15.0 Hz, H-23), 5.67 (1H, d, *J* = 1.8 Hz, H-7). ^{13}C-NMR (150 MHz, $CDCl_3$-d_1): δ_C (ppm) 12.3 (CH_3, C-18), 17.6 (CH_3, C-28), 19.6 (CH_3, C-27), 19.96 (CH_3, C-26), 20.5 (CH_3, C-19), 21.1 (CH_3, C-21), 22.4 (CH_2, C-15), 25.5 (CH_2, C-1), 27.9 (CH_2, C-16), 28.9 (CH_2, C-11), 30.1 (CH_2, C-2), 33.1 (CH, C-25), 34.9 (CH_2, C-12), 37.2 (CH_2, C-4), 40.3 (CH, C-20), 41.8 (C, C-10), 42.8 (CH, C-24), 45.3 (C, C-13), 51.8 (CH, C-14), 56.0 (CH, C-17), 67.2 (CH, C-3), 74.7 (C, C-9), 79.7 (C, C-5), 119.9 (CH, C-7), 132.5 (CH, C-23), 135.1 (CH, C-22), 164.3 (C, C-8), 197.6 (C, C-6).

3,4-Dihydroxy-6,7-dimethyl-quinolin-2-carboxylic (5): yellow solide, mp. 154–155°C, ^{1}H-NMR (600 MHz, DMSO-d_6): δ_H (ppm): 2.46 (3H, s, CH_3-10), 2.49 (3H, s, CH_3-11), 7.70 (1H, s, H-8), 7.91 (1H, s, H-5), 11.65 (1H, br s, OH). ^{13}C-NMR (150 MHz, DMSO-d_6): δ_C (ppm) 19.5 (CH_3, C-6), 20.2 (CH_3, C-7), 125.8 (CH, C-8), 128.7 (CH, C-5), 130.1 (C, C-4a), 138.4 (C, C-8a), 138.9 (C, C-7), 141.6 (C, C-4), 144.6 (C, C-6), 146.4 (C, C-3), 150.0 (C, C-2), 160.6 (C=O).

Norhaman (6): white solid, mp. 198–200°C, ^{1}H-NMR (500 MHz, DMSO-d_6): δ_H (ppm): 7.23 (1H, td, *J* = 0.5; 8.0 Hz, H-6), 7.55 (1H, td, *J* = 1.0, 8.0 Hz, H-7), 7.60 (1H, d, *J* = 8.0 Hz, H-8), 8.09 (1H, dd, *J* = 0.5, 5.0 Hz, H-3), 8.22 (1H, d, *J* = 8.0 Hz, H-5), 8.31 (1H, d, *J* = 5.0 Hz, H-4), 8.90 (1H, d, *J* = 0.5 Hz, H-1). ^{13}C-NMR (125 MHz, DMSO-d_6): δ_C (ppm): 112.0 (CH, C-8), 114.6 (CH, C-3), 119.2 (CH, C-6), 120.6 (C, C-4b), 121.7 (CH, C-5), 127.4 (C, C4a), 128.1 (CH, C-7), 134.1 (CH, C-1), 136.1 (C, C-8b), 137.98 (CH, C-4), 140.7 (C, C-8a).

Dihydrocitrinin (7): colorless solid, ESI-MS *m/z* 253 $[M+H]^+$, ^{1}H NMR (500 MHz, CD_3OD-d_4) δ_H (ppm): 1.23 (3H, d, *J* = 6.5 Hz, CH_3-9), 1.24 (3H, d, *J* = 6.5 Hz, CH_3-10), 2.05 (3H, s, CH_3-11), 2.68 (1H, dq, *J* = 2.5, 6.5 Hz, H-4), 3.92 (1H, dq, *J* = 2.5, 6.5 Hz, H-3), 4.59 (1H, d, *J* = 15.0 Hz, H-1b), 4.66 (1H, d, *J* = 15.0 Hz, H-1a). ^{13}C NMR (125 MHz, CD_3OD-d_4), δ_C (ppm): 10.0 (CH_3, C-11), 18.2 (CH_3, C-9), 20.6 (CHC-$_3$ 10), 36.8 (CH, C-4), 60.2 (CH_2, C-1), 75.6 (CH, C-3),101.5 (C, C-7), 110.7 (C, C-8a), 112.8 (C, C-5), 142.7 (C, C-4a), 156.2 (C, C-8), 159.2 (C, C-6), 169.8 (C=O, C-12).

Phenol A acid (**8**): white solid, $[\alpha]_D^{25}$ = -45° (*c*. 0.1, MeOH), ^{1}H-NMR (600 MHz, CD_3OD-d_4) δ_H (ppm): 1.16 (3H, d, *J* = 7.2 Hz, CH_3-3'), 1.18 (3H, d, *J* = 6.0 Hz, CH_3-4′), 2.11 (3H, s, CH_3-7), 3.09 (1H, m, H-1′), 3.90 (1H, m, H-2′), 6.26 (1H, s, H-5).^{13}C-NMR (150 MHz, CD_3OD-d_4): δ_C (ppm) 10.6 (CH_3-7), 16.3 (CH_3, C-4′), 19.6 (CH_3, C-3′), 43.4 (CH, C-1'), 71.9 (CH, C-2′), 102.8 (C, C-1), 104.5 (C, C-5), 113.9 (CH, C-3), 149.1 (C, C-4), 160.3 (C, C-6), 160.8 (C, C-2), 173.0 (C, C=O).

Evaluating antimicrobial activity of the OM07 strain

Antimicrobial activity test using the serial dilution method of Andrews (2001) [7] was carried out at the Institute of Marine Biochemistry, Vietnam Academy of Science and Technology. The samples were diluted in DMSO in the decreasing concentration range of 256, 128, 64, 32, 16, 8, 4, and 2 µg/mL. Next, 50 µL of bacteria and yeast solution at a concentration of 2.10^5 CFU/mL were added and the mixture was incubated at 37°C for 24 hours. The MIC value was determined at the sample with the lowest concentration which was able to inhibit the growth of microorganisms after 24 hours completely. Streptomycin and nystatin antibiotics were positive controls for bacteria and yeast, respectively. Seven tested strains used in this study were provided by the American Type Culture Collection (ATCC), including three Gram-negative strains: *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853, *Salmonella enterica* ATCC13076, three Gram-positive strains: *Enterococcus faecalis* ATCC29212, *Staphylococus aureus* ATCC25923, *Bacillus cereus* ATCC 14579 and one yeast strain *Candida albicans* ATCC10231. The independent experiments were performed in triplicate.

RESULTS AND DISCUSSION

From the fermentation broth of the *Penicillium* sp. OM07 strain, eight compounds, diketopiperazine dimer WIN 64821 (**1**), ergosterol peroxide (**2**), ergosterol (**3**), 3β,5*α*,9*α*-trihydroxyergosta-7,22-dien-6-one (**4**), 3,4-dihydroxy-6,7-dimethyl-quinolin-2-carboxylic (**5**), norharman (**6**), dihydrocitrinin (**7**), and phenol A acid (**8**) were isolated. Their structures were determined by spectral data analysis, including MS and 2D-NMR.

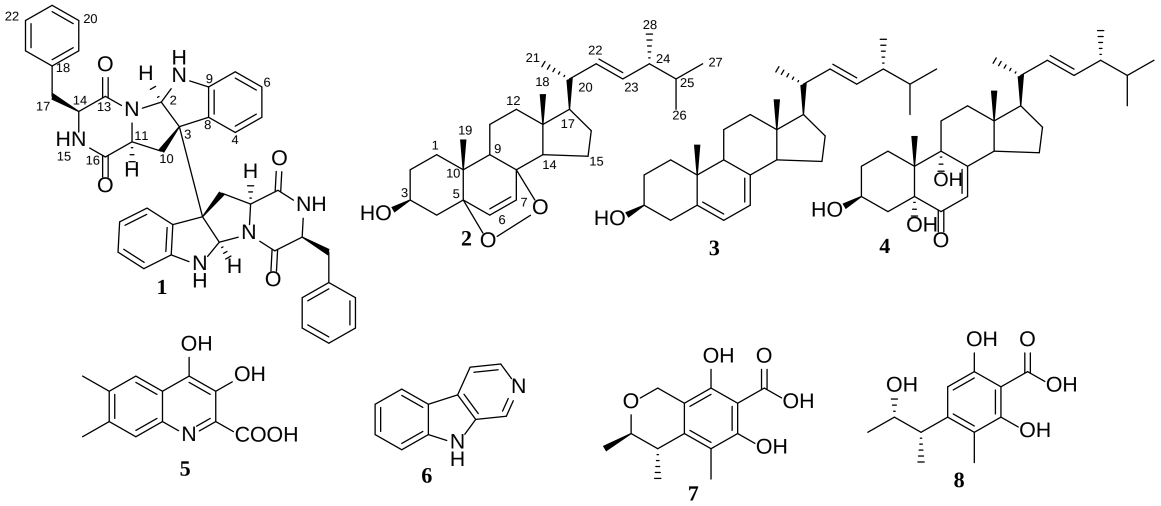

Figure 2. Secondary metabolites **1**-**8** from *Penicillium* sp. OM07

Compound **1** was isolated as an optically active white solid $[\alpha]_D^{25}$ +225.2° (*c* 1.73, MeOH). Its positive HR-ESI MS showed a pseudo-molecular ion $[M+H]^+$ at *m/z* 665.2891 (calcd. 665.2871 for $[C_{40}H_{37}N_6O_4]^+$), leading to the molecular formula of $C_{40}H_{36}N_6O_4$. However, analysis of the ^{13}C-NMR and DEPT with the aid of HSQC spectra revealed the presence of only twenty carbon signals, including two carbonyl groups at δ_C 167.4, 168.5, two sp^3 methylene groups, three sp^3 methine groups, nine sp^2 methine groups and four non-protonated carbons. The ^{1}H-NMR spectrum of **1** showed signals of four aromatic protons at δ_H 6.69 (1H, d, *J* = 7.8 Hz, H-7), 6.81 (1H, dt, *J* = 0.6, 7.8 Hz, H-5), 7.19 (1H, dt, *J* = 0.6, 7.8 Hz, H-6), 7.34 (1H, d, *J* = 7.8 Hz, H-4), characteristic of a 1,2-disubstituted benzene ring, and five aromatic protons at δ_H 7.09 (2H, d, *J* = 7.8 Hz, H-19, H-23), 7.22 (2H, t, *J* = 7.8 Hz, H-20, H-22), 7.23 (1H, m, H-21), characteristic of a monosubstituted benzene ring. Signals of seven protons in the aliphatic region were also noted. This observation suggested a dimeric structure for compound **1**. The chemical shifts of CH-14/CH-14′ (δ_C 56.4, δ_H 4.06), CH-11/CH-11′ (δ_C 57.1, δ_H 3.95), and CH-2/CH-2′ (δ_C 79.8, δ_H 4.94) suggested their linkage to nitrogen. Phenylalanine and tryptophan-derived subunits were readily identified after analysis of COSY, HSQC, and HMBC NMR data (Fig 3). In the ^{1}H-^{1}H COSY spectrum of **1**, four spin-spin interaction systems of the proton: H-4 (δ_H 7.34)/H-5 (δ_H 6.81)/H-6 (δ_H 7.19)/H-7 (δ_H 6.69); H-10 (δ_H 2.76, 3.16)/H-11 (δ_H 3 .95); H-14 (δ_H 4.06)/H-17 (δ_H 2.66, 3.45), and H-19 (δ_H 7.09)/H-20 (δ_H 7.22)/H-21 (δ_H 7.23)/H-22 (δ_H 7.22)/H-23 (δ_H 7.09) were indicated by the presence of four fragments shown in Figure 3. In the HMBC spectrum of **1**, correlations between the proton of NH-15 (δ_H 5.61) with C-13 (δ_C 167.4), C-16 (δ_C 168.5), C-14 (δ_C 56.4), C-11 (δ_C 57.1) and C-17 (δ_C 36.6) indicated the presence of a diketopiperazine ring system. In the NOESY spectrum, the NOE cross-peaks between H-14 and H-2, H-11, and H-2 suggested that H-2, H-11, and H-14 were oriented on the same side. Analyses of the ^{1}H and ^{13}C NMR, HSQC, HMBC, COSY, and NOESY spectra of **1** indicated a diketoperazine dimer skeleton similar to that found in *Aspergillus flavus* [8]. The planar structure of **1** was established, as shown in Figure 2. The relative configuration of **1** was determined by NOESY spectra and in comparation of ^{13}C-NMR chemical shifts, coupling constant in the ^{1}H-NMR spectrum and optical rotation value with those for several known diketoperazine dimer, including WIN 64821 [$[\alpha]_D^{25}$ +200.0° (*c* 0.15, MeOH), $[\alpha]_D^{26}$ +350.0° (*c* 0.20, MeOH)] [9], *ent*-WIN 64821 $[\alpha]_D^{25}$ -200° (*c* = 0.07, MeOH) [9], and the other asymmetric analogs [$[\alpha]_D^{21}$ -292 (*c* = 0.97, CH_2Cl_2), $[\alpha]_D^{24}$ -330 (*c* = 0.52, CH_2Cl_2)] [10], $[\alpha]_D^{26}$ -321.4° (*c* 0.15, pyridine), $[\alpha]_D^{27}$ -377° (*c* 0.68, MeOH), $[\alpha]_D^{25}$ -114° (*c* 0.24, pyridine) [10]. Compound **1** was thus identified as a diketopiperazine dimer WIN 64821 [9, 11, 12].

Compound **2** was obtained as white amorphous solid and optically active $[\alpha]_D^{25}$ = -43.1° (*c* 0.05, $CHCl_3$). The ESI-MS spectrum indicated the pseudo-molecular ion peak at *m/z* 429.7 $[M+H]^+$. The ^{1}H-NMR spectrum of **2** showed the presence of two singlet methyls at δ_H 0.83 (3H, s, CH_3-18), 0.88 (3H, s, CH_3-19), four doublet methyls at δ_H 0.82 (3H, d, *J* = 6.6 Hz, CH_3-27), 0.84 (3H, d, *J* = 6.6 Hz, CH_3-26), 0.91 (3H, d, *J* = 6.6 Hz, CH_3-28), 1.00 (3H, d, *J* = 6.6 Hz, CH_3-21), four *sp*^{2} methines at δ_H 5.14 (1H, dd, *J* = 8.4, 15.6 Hz, H-22), 5.22 (1H, dd, *J* = 7.8, 15.6 Hz, H-23), 6.24 (1H, d, *J* **=** 8.4 Hz, H-6), 6.50 (1H, d, *J* **=** 8.4 Hz, H-7) and one methine bearing oxygen at δ_H 3.97 (1H, m, H-3). The ^{13}C-NMR and DEPT spectra of **2** exhibited the presence of 28 carbons, including six methyl groups, seven methylene groups, seven *sp*^{3} methine groups, four *sp*^{2} methine groups, one oxymethine at δ_C 66.5 (C-3) and four quaternary *sp*^{3} carbons. The coupling constants of two olefinic protons H-22, H-23 were 15.6 Hz suggested *E* configuration of double bond. The configuration of C-24 was suggested to be *R* based on the ^{13}C-NMR chemical shift of C-28 (δ_C 17.6). It was reported that the ^{13}C-NMR value of C-28 resonates at δ_C 17.6 in the 24*R* epimer of known sterol, and the 24*S* epimer has a relative 0.4 ppm downfield chemical shift [13]. Thus, analysis of the NMR spectra and comparison with reported data, compound **2** was identified as ergosterol peroxide [14].

Compound **3** was isolated as a colorless needle and optically active $[\alpha]_D^{25}$= -101° (*c* 0.05, $CHCl_3$). The 1D-NMR of **3** showed the typical signals of ergosterol as compound **2**, with the presence of 28 carbons, including two *tert*-methyl groups at δ_C 12.1 (C-18), 16.3 (C-19), and four *sec*-methyl groups at δ_C 17.6 (C-28), 19.6 (C-26), 19.9 (C-27), 21.1 (C-21). In addition, the signals of one oxymethine [δ_C 70.5 (C-3)/δ_H 3.63 (1H, m, H-3)], four *sp*^{2} methines of two double bonds [δ_H 5.23 (1H, dd, *J* = 7.8, 15.0 Hz, H-23)/δ_C 132.0 (C-23), 5.17 (1H, dd, *J* = 6.6, 15.0 Hz, H-22)/δ_C 135.6 (C-22), 5.38 (1H, m, H-7)/ δ_C 116.3 (C-7); 5.57 (1H, dd, *J* = 2.4, 5.4 Hz, H-6)/119.6 (C-6) were also observed. The coupling constant of two olefinic protons, H-22 and H-23, was 15.0 Hz suggested *E* configuration of double bond. Thus, through analysis of the NMR spectra and comparison with reported data, compound **3** was identified as ergosterol [14].

Comparison of the 1D-NMR signal of **4** with those of **3** depicted the presence of the carbonyl group at δ_C 197.6 (C-6), two oxygenated quaternary sp^3 carbons and the absence of one double bond (one sp^2 methine group and one quaternary sp^2 carbon) and one sp^3 methine. Analysis of the ^{1}H-NMR spectrum of **4** revealed signals of three olefinic protons at δ_H 5.17 (1H, dd, J = 7.8, 15.0 Hz, H-22), 5.25 (1H, dd, J = 7.8, 15.0 Hz, H-23) and 5.67 (1H, d, J = 1.8 Hz, H-7). The signal of six methyl groups at δ_H 0.62 (3H, s, CH_3-18), 0.82 (3H, d, J = 6.6 Hz, CH_3-26), 0.83 (3H, d, J = 6.6 Hz, CH_3-27), 0.92 (3H, d, J = 6.6 Hz, CH_3-28), 1.03 (3H, s, CH_3-19), 1.04 (3H, d, J = 6.6 Hz, CH_3-21), one oxygenated methine proton at δ_H 4.06 (1H, m, H-3) and the protons of the aliphatic region at δ_H 1.32-2.75 also observed on the ^{1}H-NMR spectrum. The ^{13}C-NMR and DEPT spectrum of **4** showed the presence of twenty-eight carbons, including one carbonyl group (δ_C 197.6), 6 methyl groups (δ_C 12.3, 17.6, 19.6, 19.96, 20.5 and 21.1), seven methylene groups, nine methine groups (three sp^2 methines at δ_C 119.9 (C-7), 132.5 (C-23), 135.1 (C-22), one oxymethine group at δ_C 67.23 (C-3)) and five non-protonated carbons. Analysis of 1D-NMR spectral data of compound **4** shows that this compound is an ergosterol. Comparing the 1D-NMR with reference data determined the structure **4** as 3β,5α,9α-trihydroxyergosta-7,22-dien-6-one. The substance was reported to inhibit the Hela cell line at a minimum concentration of 8 µg/mL [15].

Compound **5** was isolated as a microcrystalline yellow solid. Its positive HR-ESI-MS showed the pseudo-molecular ion $[M+H]^+$ at m/z 234.0761 (calcd 234.0766 for $[C_{12}H_{12}NO_4]^+$), leading to the molecular formula of $C_{12}H_{11}NO_4$. In the ^{1}H NMR spectrum, signals of two singlet aromatic protons at δ_H 7.70 (H-8) and 7.91 (H-5), two singlet methyls at δ_H 2.46 (CH_3-10) and 2.49 (CH_3-11), and broad signal of hydroxyl proton at δ_H 11.65 were observed. The ^{13}C-NMR and DEPT spectra of **5** revealed the presence of a carboxylic carbon at δ_C 160.6, nine aromatic carbons, and two methyl carbons at δ_C 19.5 and 20.2. In the HMBC spectrum of **5**, the presence of the A-ring was established by cross-peaks of the proton of the CH_3-10 group with C-5/C-6/C-7; CH_3-11 with C-6/C-7/C-8, and H-8 with C-8a (Fig. 3). Additionally, the HMBC correlation between H-5 with C-4/C-4a/C-8a demonstrated the connection of C-4 to C-4a. The signals of two sp^2 quaternary carbons at δ_C 146.4 and 150.0 and a carboxylic carbon at δ_C 160.6 were remaining to be assigned. The carbon chemical shifts of C-2 (δ_C 150.0), C-3 (δ_C 146.4), and C-4 (δ_C 141.6) suggested their linkages to nitrogen or oxygen atoms. However, all three carbons, C-2, C-3, and C-4, were observed downfield, and thus the carboxylic group's position could be assigned to be linked at C-2. Thus, the structure of compound **5** was identified as 3,4-dihydroxy-6,7-dimethyl-quinoline-2-carboxylic [16].

Compound **6** was isolated as a white solid, mp. 198–200°C. The ESI-MS spectrum of **6** showed the protonated adduct $[M+H]^+$ at m/z 169. The ^{1}H-NMR spectrum of **6** showed the signals of an 1,2-disubstituted benzene ring at δ_H 7.23 (1H, td, J = 0.5; 8.0 Hz, H-6), 7.55 (1H, td, J = 1.0, 8.0 Hz, H-7), 7.60 (1H, d, J = 8.0 Hz, H-8), 8.22 (1H, d, J = 8.0 Hz, H-5), and three aromatic protons at δ_H 8.09 (1H, dd, J = 0.5, 5.0 Hz, H-3), 8.31 (1H, d, J = 5.0 Hz, H-4), 8.90 (1H, d, J = 0.5 Hz, H-1). Analyses of the ^{13}C-NMR and DEPT spectra with the HSQC of **6** indicated the presence of eleven carbons, including seven sp^2 methine carbons and four sp^2 quaternary carbons. Analysis of the COSY spectrum revealed the presence of two spin–spin coupling systems: H-5/H-6/H-7/H-8 and H-3/H-4. In the HMBC spectrum, the interactions between H-1 with C-8b, C-4a, C-3; H-3 with C-1, C-4a; H-4 with C-4a determined the positions of C-1, C-4a and C-8b (Fig 3). The positions of C-4b and C-8a were confirmed through the interactions between H-6 with C-4b, H-5 with C-4b, C-8a, H-7 with C-8a, and H-8 with C-4b, C-8a. Thus, detailed analysis of the 1D, 2D-NMR spectra, and MS data allowed for determining structure **6** as 9H-pyrido[3,4-b]indole. Its NMR data were consistent with those reported in the literature [17, 18].

Compound **7** was isolated as a colorless solid. In the ^{1}H-NMR spectrum of **7**, the presence of three methyl signals, including one singlet at δ_H 2.05 (3H, s, CH_3-11), and two doublets at δ_H 1.23 (3H, d, J = 6.5 Hz, CH_3-9), 1.24 (3H, d, J = 6.5 Hz, CH_3-10), one oxymethine proton at δ_H 3.92 (1H, dq, J = 2.5, 6.5 Hz, H-3), one methine proton at δ_H 2.68 (1H, dq, J = 2.5, 6.5 Hz, H-4), one oxymethylene group at δ_H 4.59 (1H, d, J = 15.0 Hz, H-1b), 4.66 (1H, d, J = 15.0 Hz, H-1a) were observed. The ^{13}C-NMR and DEPT spectrum of **7** showed the presence of 13 carbons, including one carbonyl group (δ_C 169.8), 3 methyl groups (δ_C 10.0, 18.2 and 20.6), one methylene group (δ_C 60.1), one methine group (δ_C 36.8), one oxymethine group at δ_C 75.6 and six non-protonated carbons. Comparing the 1D-NMR with reference data determined the structure of **7** as dihydrocitrinin [19].

Compound **8** was isolated as a white solid, $[\alpha]_D^{25}$ -45° (c 0.1, MeOH). The ^{13}C-NMR and DEPT spectra indicated the presence of 12 carbons, including one carbonyl group at δ_C 178.3 (C=O), three methyl groups at δ_C 10.6 (CH_3-7), 16.3 (CH_3-4′), 19.6 (CH_3-3′), one sp^2 methine group at δ_C 104.5 (C-5), one oxymethine group at δ_C 71.9 (C-2′), one sp^3 methine group at δ_C 43.4 (C-1′), and five quaternary sp^2 carbons. The ^{1}H-NMR spectrum of **8** is consistent with the ^{13}C-NMR with the presence of one singlet aromatic proton at δ_H 6.26 (1H, s, H-5), one oxymethine proton at δ_H 3.90 (1H, m, H-2′), one methine proton at δ_H 3.09 (1H, m, H-1′), three methyl signals including one singlet at δ_H 2.11 (3H, s, CH_3-7), and two doublets at δ_H 1.16 (3H, d, J = 7.2 Hz, CH_3-3′), 1.18 (3H, d, J = 6.0 Hz, CH_3-4′) were observed. Comparing the 1D-NMR with reference data determined the structure of **8** as phenol A acid [6, 20].

Antimicrobial assay

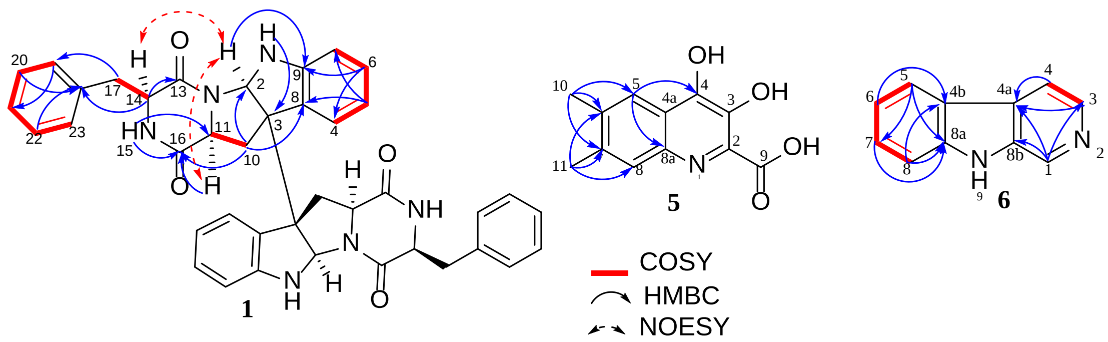

Figure 3. Key HMBC, NOESY and COSY correlations of **1, 5** and **6**

Table 1. Antibacterial and antifungal activities of compounds **1-8** (MIC: µg/mL)

	Gram-positive			Gram-negative			Yeast
Compd.	*E. faecalis*	*S. aureus*	*B. cereus*	*E. coli*	*P. aeruginosa*	*S. enterica*	*C. albicans*
1	64	128	> 256	> 256	> 256	> 256	256
2	128	256	> 256	> 256	> 256	256	128
3	64	256	128	> 256	> 256	> 256	64
4	128	256	> 256	> 256	> 256	> 256	128
5	128	256	256	32	256	64	> 256
6	> 256	32	> 256	> 256	> 256	> 256	> 256
7	64	64	64	> 256	> 256	> 256	16
8	128	128	256	> 256	> 256	> 256	8
Streptomycin	256	256	128	32	256	128	-
Nystatin	-	-	-	-	-	-	8

All the isolates were evaluated for their antibacterial activity against *Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* (ATCC27853), *Salmonella enterica* (ATCC13076), *Enterococcus faecalis* (ATCC299212), *Staphylococcus aureus* (ATCC25923) and *Bacillus cereus* (ATCC14579), and antifungal property against *Candida albicans* (ATCC10231) (Table 1). All the compounds inhibited effect on the growth of one to three Gram-positive strains with MIC values of 32–256 µg/mL. In particular, compound **5** showed antimicrobial activity against all three Gram-positive and three Gram-negative strains with MIC values from 32–256 µg/mL. Compounds **3**, **7**, and **8** inhibited all three Gram-positive strains against antifungal *C. albicans* with MIC values from 8–256 µg/mL. In addition, compounds **1, 2,** and **4** selectively inhibited two of three Gram-positive strains, *E. faecalis, S. aureus*, and *C. albicans* with a MIC value of 64–256 µg/mL. Compound **6** selectively inhibited *S. aureus* strain with a MIC value of 32 µg/mL.

CONCLUSION

Analysis of an antimicrobial extract prepared from culture broth of the marine-derived fungus *Penicillium* sp. OM07 led to the isolation of eight compounds identified as diketopiperazine dimer WIN 64821 (**1**), ergosterol peroxide (**2**), ergosterol (**3**), 3β,*5α,9α*-trihydroxyergosta-7,22-dien-6-one (**4**), 3,4-dihydroxy-6,7-dimethyl-quinolin-2-carboxylic (**5**), norhaman (**6**), dihydrocitrinin (**7**), and phenol A acid (**8**). Most showed high antifungal activity against *Candida albicans* ATCC10231 strain with MIC values ranging from 8 µg/mL to 256 µg/mL. All compounds had inhibitory activity against from one to three Gram-positive tested strains with MIC values in the 32–256 µg/mL range.

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