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Ergostane steroids from *Aspergillus* sp. M904 in Vietnam

Trinh Thi Thanh Van[1], Nguyen Mai Anh[1,2], Vu Van Nam[1], Nguyen Thuy Linh[1], Le Thi Hong Minh[1], Brian T. Murphy[3], Doan Thi Mai Huong[1,2,*], Pham Van Cuong[1,2,]**

[1]*Institute of Marine Biochemistry, VAST, Vietnam*
[2]*Graduate University of Science and Technology, VAST, Vietnam*
[3]*University of Illinois Chicago, Illinois 60612-7231, USA*

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ABSTRACT

Seven ergostane steroids were isolated from the cultures of *Aspergillus* sp. M904, an endophytic fungus isolated from the marine sediment sample collected at a depth of ten meters in Binh Dinh, Vietnam. These compounds include 5α,6α-epoxy-(22*E*,24*R*)-ergosta-8(14),22-diene-3β,7α-diol (**1**), 5α,6α-epoxy-(22*E*,24*R*)-ergosta-8(9),22-diene-3β,7α-diol (**2**), ergosterol (**3**), ergosterol peroxide (**4**), 3β,5α,6α-trihydroxy-ergosta-7,22-diene (**5**), 3β,5α,9α-trihydroxy-ergosta-7,22-diene-6-one (**6**), 3β-hydroxy-5α,9α-epoxy-ergosta-7,22-dien-6-one (**7**). Their structures were identified through MS and NMR data analyses. All compounds were evaluated for their antimicrobial activity against a panel of clinically significant microorganisms. Compounds **1–7** had inhibitory activity against from one to six tested strains with MIC values from 64–256 µg/mL.

Keywords: *Aspergillus*, steroid, ergostane, antimicrobial.

*/** Corresponding author at: Institute of Marine Biochemistry, 18 Hoang Quoc Viet Str., Cau Giay Dist., Hanoi, Vietnam.
E-mail addresses: huongdm@imbc.vast.vn/phamvc@imbc.vast.vn

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INTRODUCTION

Marine fungi have proved promising sources for many structurally interesting and biologically active secondary metabolites [1, 2]. Previous studies have shown that compounds derived from marine fungi, such as alkaloids, terpenes, polyketones, peptides, sterols, and lactones, may show suitable activities, including antimicrobial, antioxidant, antitumor, anticoagulant, and enzyme inhibitory activities [3–6]. Thus, marine fungi comprise an essential source for developing novel drugs with low toxicity and high efficacy [7]. The genus *Aspergillus* (Moniliaceae) is one of the largest and most intensively investigated fungal genera, and it survives in both terrestrial and marine environments. *Aspergillus* has been proven to be an excellent resource for new natural products [8].

In this study, a preliminary experiment demonstrated that the ethyl acetate extract of *Aspergillus* sp. M904 showed antibacterial activity against *Enterococcus faecalis* ATCC299212, *Staphylococcus aureus* ATCC25923, and *Candida albicans* ATCC10231 with MIC values of 256, 64, 128 µg/mL, respectively. Further investigation on the chemical constituents resulted in the identification of seven compounds including 5*α*,6*α*-epoxy-(22*E*,24*R*)-ergosta-8(14),22-diene-3*β*,7*α*-diol (**1**), 5*α*,6*α*-epoxy-(22*E*,24*R*)-ergosta-8(9),22-diene-3*β*,7*α*-diol (**2**), ergosterol (**3**), ergosterol peroxide (**4**), 3*β*,5*α*,6*α*-trihydroxy-ergosta-7,22-diene (**5**), 3*β*,5*α*,9*α*-trihydroxy-ergosta-7,22-diene-6-one (**6**), 3*β*-hydroxy-5*α*,9*α*-epoxy-ergosta-7,22-dien-6-one (**7**).

MATERIALS AND METHODS

General Experiment procedures

Melting points were recorded on a Boetius. The ESI-MS spectra were recorded on an Agilent 1100 LC-MSD Trap spectrometer. The NMR spectra were recorded on a Bruker 600.36 MHz spectrometer operating at 150.98 MHz for ^{13}C NMR, and at 600.36 MHz for ^{1}H-NMR. The solvents for NMR spectra were $CDCl_3$, CD_3OD, and DMSO-d_6, using TMS as internal standard. TLC silica gel Merck 60 F_{254} was used for thin-layer chromatography and preparative TLC. Column chromatography (CC) was carried out using silica gel (230-400 mesh, Merck), silica gel RP-18 (30-50 µm, YMC), or Sephadex LH-20 (Sigma). Fractions were monitored by thin-layer chromatography. Spots were visualized by UV light (254 nm and 365 nm) and by heating silica gel plates sprayed with sulfuric acid 10% reagent. Medium-pressure liquid chromatography (MPLC) was performed on a Biotage-Isolera One system (Sweden).

Marine materials

The fungus strain *Aspergillus* sp. M904 was isolated from a marine sediment sample collected in April 2021 at a depth of ten meters from the coast of Binh Dinh, Vietnam.

Isolation, identification, and fermentation of fungus M904

First, 0.5 g of sediment sample was crushed by glass chopsticks in a falcon tube. 4.5 mL of sterile seawater was added, the mixture was homogenized by vortexing for 1 minute, and the suspension was treated using a wet-heat technique (60°C for 6 minutes). Next, 0.5 mL of this suspension was transferred to another 4.5 mL of sterile seawater and this step was repeated to set up a ten-fold dilution series of 10^{-3}. At the final dilution step, aliquots of 50 µL were spread on Petri dishes of solid medium PDA - potato dextrose agar (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 30°C for 7 days. The colony of the M904 strain was transferred onto a new petri dish of PDA medium for purification.

The taxonomy of the strain M904 was identified using 18S rRNA gene sequence analysis and compared with fungal 18S rRNA sequences in the GeneBank database by the NBCI Blast program. The results showed that the strain M904 belonged to the genus *Aspergillus*.

The strain M904 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, and 30 g/L

instant ocean). After 7 days of incubation at 30°C with shaking at 150 rpm speed, the culture broth was spread on the medium surface of 50 flasks containing 1 L of solid medium PDA. The fermentation was incubated in an incubator at 30°C and harvested after twenty days.

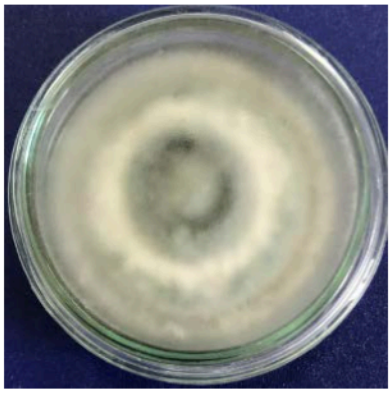

Figure 1. Morphological appearance of M904 strain's colonies

Extraction and isolation

The *Aspergillus sp.* M904 solid medium was cut into small pieces and extracted with ethyl acetate (3 times × 5 liters × 2 h sonication each) at 40°C. The combined ethyl acetate extracts were concentrated under reduced pressure to obtain 53 g of the crude extract. The ethyl acetate extract (53 g) was eliminated from the salt and then separated by *silica gel* column chromatography on Medium-Performance Liquid Chromatography (MPLC) equipment with a gradient of CH_2Cl_2/MeOH (0–100% MeOH) to give 9 fractions F1–F9. Fraction F3 (1.27 g) was separated on a *silica gel* column chromatography with a mixture of CH_2Cl_2/acetone gradient as mobile phase to give 5 fractions F3.1–F3.5. Fraction F3.2 (65 mg) was continuously separated by *silica gel* column chromatography and eluted with *n*-hexane/acetone gradient to obtain 3 subfractions: F3.2.1–F3.2.3. Subfraction F3.2.1 (28 mg) was further purified by Sephadex LH-20 column chromatography with CH_2Cl_2/MeOH (1/9) as eluent to yield **3** (3.0 mg). Fraction F3.4 (95 mg) was separated on a Sephadex LH-20 column chromatography, eluted with CH_2Cl_2/MeOH (1/9) to give 2 subfractions F3.4.1–F3.4.2. The subfraction F3.4.2 was continuously subjected by *silica gel* column chromatography and eluted with CH_2Cl_2/acetone gradient to yield **4** (3.5 mg). Fraction 4 (1.95 g) was subjected by Sephadex LH-20 column chromatography with CH_2Cl_2/MeOH (1/9) as eluent to give 3 fractions F4.1–F4.3. Fraction F4.3 (220 mg) was separated on a *silica gel* column chromatography with a mixture of *n*-hexane/acetone gradient as mobile phase to obtain 5 subfractions F4.3.1-F4.3.5. The subfraction F4.3.5 was further purified by gel filtration over Sephadex LH-20 column chromatography and eluted with CH_2Cl_2/MeOH (1/9) to give **2** (4.0 mg). Similarly, fraction F5 (2.30 g) was separated on a Sephadex LH-20 column chromatography and eluted with MeOH/H_2O (9/1) to give 4 fractions F5.1–F5.4. Two fractions, F5.3 (85 mg) and F5.4 (75 mg) were separated by *silica gel* column chromatography and eluted with CH_2Cl_2/acetone gradient to give **7** (2.0 mg) and **1** (4.1 mg). Fraction F6 (3.7 g) was further purified by gel filtration over Sephadex LH-20 column chromatography with MeOH as eluent to give 3 fractions F6.1–F6.3. Fraction F6.2 (230 mg) was separated on a *silica gel* column chromatography with a mixture of ethyl acetate/acetone gradient as mobile phase to obtain 4 subfractions F6.2.1–F6.2.4. Subfraction F6.2.4 (51 mg) was separated on a *silica gel* column chromatography with a mixture of ethyl acetate/acetone gradient as mobile phase to give **5** (4 mg).

5α,6α-epoxy-(22*E*,24*R*)-ergosta-8(14),22-diene-3β,7α-diol (1): white solid; mp. 123–124°C, ESI-MS: *m/z* 429 $[M+H]^+$, ^{1}H-NMR (600 MHz, $CDCl_3$): δ_H (ppm) 0.87 (6H, s, H-18, H-19), 0.92 (3H, d, *J* = 6.6 Hz, H-28), 0.82 (3H, d, *J* = 6.6 Hz, H-26), 0.84 (3H, d, *J* = 7.2 Hz, H-27), 1.02 (3H, d, *J* = 6.6 Hz, H-21), 1.40 (1H, m, H-11a), 1.48 (1H, m, H-11b), 2.27 (1H, m, H-13a), 2.62 (1H, m, H-13b), 1.38 (1H, m, H-16a), 1.74 (1H, m, H-16b), 1.50 (1H, m, H-2a), 1.92 (1H, m, H-2b), 1.43 (1H, m, H-1a), 1.66 (1H, m, H-1b), 1.44 (1H, m, H-25), 1.17 (1H, m, H-10a), 1.91 (1H, m, H-10b), 2.23 (1H, m, H-9), 2.11 (2H, m, H-4), 1.43 (1H, m, H-20), 1.85 (1H, m, H-24), 1.22 (1H, m, H-17), 3.14 (1H, d, *J* = 3.6 Hz, H-6), 4.42 (1H, d *J* = 3.6 Hz, H-7), 3.92 (1H, m, H-3), 5.22 (1H, dd, *J* = 7.2, 15.0 Hz, H-23), 5.18 (1H, dd, *J* = 7.2, 15.0 Hz,

H-22). ^{13}C-NMR (150 MHz, $CDCl_3$): δ_C 16.5 (CH_3, C-19), 17.6 (CH_3, C-28), 18.1 (CH_3, C-18), 19.0 (CH_2, C-11), 19.7 (CH_3, C-26), 19.9 (CH_3, C-27), 21.2 (CH_3, C-21), 25.0 (CH_2, C-15), 27.2 (CH_2, C-16), 31.1 (CH_2, C-2), 32.2 (CH_2, C-1), 33.1 (CH, C-25), 35.8 (CH_2, C-10), 36.6 (C, C-12), 38.7 (C, C-9), 39.2 (CH, C-4), 39.6 (CH, C-20), 42.9 (CH, C-24), 43.0 (C, C-13), 56.8 (CH, C-17), 61.3 (CH, C-6), 65.1 (CH, C-7), 67.8 (C, C-5), 68.7 (CH, C-3), 125.2 (CH, C-22), 132.3 (CH, C-23), 135.3 (C, C-8), 152.6 (C, C-14).

5*α*,6*α*-epoxy-(22*E*,24*R*)-ergosta-8(9),22-diene-3*β*,7*α*-diol (2): amorphous powder; mp. 169–170°C, ESI-MS: *m/z* 429 $[M+H]^+$, ^{1}H-NMR (600 MHz, $CDCl_3$): δ_H (ppm) 0.59 (3H, s, H-18), 0.82 (3H, d, *J* =7.2 Hz, H-28), 0.84 (3H, d, *J* = 6.6 Hz, H-21), 0.91 (3H, d, *J* = 6.6 Hz, H-27), 1.02 (3H, dd, *J* = 6.6 Hz, H-26), 1.14 (3H, s, H-19), 1.20 (1H, m, H-17), 1.26 (1H, m, H-11b), 1.28 (1H, m, H-15b), 1.29 (1H, m, H-16b), 1.40 (1H, m, H-12b), 1.45 (1H, m, H-4b), 1.47 (1H, m, H-25), 1.60 (1H, m, H-2b), 1.79 (1H, m, H-16a), 1.80 (1H, m, H-2a), 1.84 (1H, m, H-24), 1.95 (1H, m, H-12a), 1.97 (2H, m, H-1), 2.05 (2H, m, H-14, H-15a), 2.08 (1H, m, H-11a), 2.18 (1H, m, H-20), 2.19 (1H, m, H-4a), 3.31 (1H, d, *J* = 2.4 Hz, H-6), 3.95 (lH, m, H-3), 4.22 (1H, br.s, H-7), 5.17 (1H, dd, *J* = 7.2, 15.0 Hz, H-22), 5.22 (lH, dd, *J* = 7.2, 15.0 Hz, H-23). ^{13}C-NMR (150 MHz, $CDCl_3$): δ_C (ppm): 11.3 (CH_3, C-19), 17.7 (CH_3, C-28), 19.7 (CH_3, C-18), 19.9 (CH_3, C-21), 20.9 (CH_3, C-26), 22.8 (CH_3, C-27), 23.4 (CH_2, C-11), 23.9 (CH_2, C-15), 29.0 (CH_2, C-16), 30.2 (CH_2, C-2), 30.8 (CH_2, C-1), 33.1 (CH, C-25), 35.7 (CH_2, C-12), 38.0 (C, C-10), 39.2 (CH_2, C-4), 40.4 (CH, C-14), 42.1 (C, C-13), 42.8 (CH, C-24), 49.6 (CH, C-20), 53.7 (CH, C-17), 62.6 (CH, C-6), 65,7 (C, C-5), 67.1 (CH, C-7), 68.6 (CH, C-3), 126.9 (C, C-8), 132.0 (CH, C-23), 134.5 (C, C-9), 135.6 (CH, C-22).

Ergosterol (3): white powder; mp. 149–150°C, ESI-MS: *m/z* 396 $[M+H]^+$, ^{1}H-NMR (600 MHz, $CDCl_3$): δ_H (ppm) 0.63 (3H, s, H-18), 0.82 (3H, d, *J* = 6.6 Hz, H-27), 0.84 (3H, d, *J* = 6.6 Hz, H-26), 0.92 (3H, d, *J* = 7.2 Hz, H-28), 0.95 (3H, s, H-19), 1.03 (3H, d, *J* = 6.6 Hz, H-21), 3.63 (1H, m, H-3), 5.17 (1H, dd, *J* = 7.2, 15.0 Hz, H-22), 5.22 (1H, dd, *J* = 7.2, 15.0 Hz, H-23), 5.38 (1H, q, J = 2.4 Hz, H-7), 5.57 (1H, dd, J = 2.4, 6.0 Hz, H-6). ^{13}C-NMR (150 MHz, $CDCl_3$): δ_C 12.1 (CH_3, C-18), 16.3 (CH_3, C-19), 17.6 (CH_3, C-28), 19.6 (CH_3, C-21), 19.9 (CH_3, C-26), 21.1 (CH_3, C-27), 21.1 (CH_2, C-15), 23.0 (CH_2, C-11), 28.3 (CH_2, C-12), 32.0 (CH_2, C-2), 33.1 (CH, C-25), 37.0 (C, C-10), 38.4 (CH_2, C-1), 39.1 (CH_2, C-16), 40.4 (CH, C-20), 40.8 (CH_2, C-4), 42.8 (CH, C-24), 42.9 (C, C-13), 46.3 (CH, C-9), 54.6 (CH, C-17), 55.8 (CH, C-14), 70.5 (CH, C-3), 116.3 (CH, C-7), 119.3 (CH, C-6), 132.0 (CH, C-23), 135.6 (CH, C-22), 139.8 (C, C-8), 141.3 (C, C-5).

Ergosterol peroxide (4): colorless amorphorus powder, mp. 183–184°C, ESI-MS: *m/z* 429 $[M+H]^+$, ^{1}H-NMR (600 MHz, $CDCl_3$): δ_H (ppm) 0.81 (3H, s, H-18), 0.82 (3H, d, *J* = 6.6 Hz, H-27), 0.83 (3H, d, *J* = 6.6 Hz, H-26), 0.88 (3H, d, *J* = 7.2 Hz, H-19), 0.91 (3H, s, H-28), 1.00 (3H, d, *J* = 6.6 Hz, H-21), 3.97 (1H, m, H-3), 5.14 (1H, dd, *J* = 7.2, 15.5 Hz, H-22), 5.22 (1H, dd, *J* = 7.2, 15.5 Hz, H-23), 6.23 (1H, d, *J* = 8.4 Hz, H-7), 6.50 (1H, d, *J* = 8.4 Hz, H-6). ^{13}C-NMR (150 MHz, $CDCl_3$): δ_C 12.9 (CH_3, C-18), 17.6 (CH_3, C-28), 18.3 (CH_3, C-19) 19.6 (CH_3, C-26), 19.9 (CH_3, C-27), 20.6 (CH_2, C-15), 20.9 (CH_3, C-21), 23.4 (CH_2, C-11), 28.6 (CH_2, C-16), 30.1 (CH_2, C-2), 33.1 (CH, C-25), 34.7 (CH_2, C-1), 36.9 (CH_2, C-4), 36.9 (C, C-10), 39.4 (CH_2, C-12), 39.7 (CH, C-20), 42.8 (CH, C-24), 44.6 (C, 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-8), 82.2 (C, C-5), 130.8 (CH, C-7), 132.3 (CH, C-23), 135.2 (CH, C-22), 135.4 (C, C-6).

3*β*, 5*α*, 6*α*-trihydroxyergosta-7,22-diene (5): white solid; mp. 246–247°C, ESI-MS: *m/z* 431 $[M+H]^+$, ^{1}H-NMR (600 MHz, $CDCl_3$): δ_H (ppm) 0.60 (3H, s, H-18), 0.82 (3H, d, *J* = 6.6 Hz, H-26), 0.84 (3H, d, *J* = 6.6 Hz, H-27), 0.92 (3H, d, *J* = 7.2 Hz, H-28), 1.03 (3H, d, *J* = 6.6 Hz, H-21), 1.25 (3H, s, H-19), 3.62 (1H, m, H-3), 4.09 (1H, d, *J* = 5.4 Hz, H-6), 5.17 (1H, dd, *J* = 7.2, 15.5 Hz, H-22), 5.24 (1H, dd, *J* = 7.2, 15.5 Hz, H-23), 5.35 (1H, d, *J* = 5.4 Hz, H-7). ^{13}C-NMR (150 MHz, $CDCl_3$): δ_C 12.6 (CH_3, C-18), 17.6 (CH_3, C-28), 18.4 (CH_3, C-19), 19.7 (CH_3, C-27), 19.9 (CH_3, C-26), 21.1 (CH_3, C-21), 22.1 (CH_2, C-11), 22.9 (CH_2, C-15), 27.9 (CH_2, C-16), 30.9 (CH_2, C-1), 33.0 (CH_2, C-2), 33.1 (CH, C-25), 37.2 (C, C-10), 39.3 (C, C-12), 39.5 (CH, C-20), 40.4 (CH_2 , C-4), 42.8 (CH, C-24), 43.5 (CH, C-9), 43.8 (C, C-13), 54.8 (CH, C-14), 56.0 (CH, C-17), 67.8 (CH, C-3), 73.1 (CH, C-6), 76.5 (CH, C-5), 117.6 (CH, C-7), 132.3 (CH, C-23), 135.5 (CH, C-22), 144.0 (C, C-8).

3β, 5α, 9α-trihydroxyergosta-7,22-diene-6-one (6): White solid, ESI-MS: *m/z* 445 $[M+H]^+$, ^{1}H-NMR (600 MHz, $CDCl_3$): δ_H (ppm) 0.62 (3H, s, H-18), 0.82 (3H, d, *J* = 6.6 Hz, H-28), 0.84 (3H, d, *J* = 7.2 Hz, H-21), 0.92 (3H, d, *J* = 6.6 Hz, H-27), 1.02 (3H, s, H-19), 1.03 (3H, d, *J* = 6.6 Hz, H-26), 1.36 (1H, m, H-1a), 1.45 (1H, m, H-15a), 1.46 (1H, m, H-17), 1.48 (1H, m, H-25), 1.50 (1H, m, H-16a), 1.52 (1H, m, H-2a), 1.60 (1H, m, H-15b), 1.73 (1H, m, H-12a), 1.75 (1H, m, H-4a), 1,76 (1H, m, H-11a), 1.78 (1H, m, H-1b), 1.87 (1H, m, H-24), 1.88 (1H, m, H-12b), 1.91 (1H, m, H-11b), 1.97 (1H, m, H-2b), 2.05 (1H, m, H-20), 2.10 (1H, m, H-4b), 2.34 (1H, m, H-16b), 2.75 (1H, m, H-14), 4.06 (1H, m, H-3), 5.17 (1H, dd, *J* = 7.2, 15.0 Hz, H-22), 5.23 (1H, dd, *J* = 7.2, 15.0 Hz, H-23), 5.65 (1H, d, *J* = 1.8 Hz, H-7). ^{13}C-NMR (150 MHz, $CDCl_3$): δ_C 12.3 (CH_3, C-18), 17.6 (CH_3, C-27), 19.6 (CH_3, C-28), 19.9 (CH_3, C-21), 20.5 (CH_3, C-19), 21.1 (CH_3, C-26), 22.4 (CH_2, C-15), 25.5 (CH_2, C-16), 27.9 (CH_2, C-1), 28.8 (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).

3β-hydroxy,5α,9α-epoxyergosta-7,22-dien-6-one (7): white solid, ESI-MS: *m/z* 427 $[M+H]^+$, ^{1}H-NMR (600 MHz, $CDCl_3$): δ_H (ppm) 0.62 (3H, s, H-18), 0.82 (3H, d, *J* = 6.6 Hz, H-26), 0.84 (3H, d, *J* = 7.2 Hz, H-27), 0.92 (3H, d, *J* = 7.2 Hz, H-28), 1.02 (3H, s, H-19), 1.03 (3H, d, *J* = 6.6 Hz, H-21), 4.05 (1H, m, H-3), 5.17 (1H, dd, *J* = 7.2, 15.5 Hz, H-22), 5.24 (1H, dd, *J* = 7.2, 15.5 Hz, H-23), 5.64 (1H, d, *J* = 1.8 Hz, H-7).

Evaluating antimicrobial activity

Antimicrobial activity test using the serial dilution method of Hadecek (2000) [9] 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 from 256, 128, 64, 32, 16, 8, 4 and 2 µg/mL. Next, 50 uL 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 h. The MIC value was determined at the sample with the lowest concentration, which was able to inhibit the growth of microorganisms after 24 h completely. Streptomycin and cycloheximide 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, *Staphylococcus 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 culture of *Aspergillus sp*. M904, seven sterol compounds were isolated (Fig. 2). Their structures were determined by their spectral data analysis, including MS and NMR.

Compound **1** was obtained as a white solid, mp. 123–124°C. Its positive ESI-MS showed the pseudomolecular ion peak $[M+H]^+$ at *m/z* 429. The ^{1}H-NMR spectrum showed two singlet methyls at δ_H 0.87 (6H, s, H-18, H-19), four doublet methyls at δ_H 0.82 (3H, d, *J* = 6.6 Hz, H-26), 0.84 (3H, d, *J* = 7.2 Hz, H-27), 0.92 (3H, d, *J* = 6.6 Hz, H-28), 1.02 (3H, d, *J* = 6.6 Hz, H-21), three oxymethines at δ_H 3.14 (1H, d, *J* = 3.6 Hz, H-6), 4.42 (1H, d *J* = 3.6 Hz, H-7), 3.92 (1H, m, H-3), two olefinic protons at δ_H 5.18 (1H, dd, *J* = 15.0, 7.2 Hz, H-22), 5.22 (1H, dd, *J* = 15.0, 7.2 Hz, H-23) and the remaining protons in the aliphatic region at δ_H 1.31-2.14 ppm. A large coupling constant observed between H-22 and H-23 (*J* = 15.0 Hz) supported a *trans* relationship between H-22 and H-23. The ^{13}C-NMR and HSQC spectra indicated twenty-eight carbon signals containing six methyl groups, seven methylenes, ten methine groups, and five non-protonated carbons. The NMR spectra of compound **1** were similar to an ergostane steroidal skeleton. The chemical shifts of the carbon signal at δ_C 61.3 (CH, C-6), 65.1 (CH, C-7), 67.8 (C, C-5), and 68.7 (CH, C-3) suggested their linkage to oxygen. In the HMBC

spectrum of **1**, the HMBC correlations of H-18 to C-12, C-14, C-17; H-19 to C-1, C-5, C-9, and C-10 demonstrated the locations of Me-18 and Me-19 at C-13 and C-10, respectively. The correlations of H-21 with C-22, C-17, C-20; H-28 with C-23, C-24, C-25; H-26 and H-27 with C-24, C-25 determined the placement of four methyl groups at C-20, C-24 and C-25, respectively. In addition, the HMBC correlations from H-6 (δ_H 3.14) to C-5, C-7, C-8, and from H-7 (δ_H 4.42) to C-8, C-9, C-14, along with the MS data of **1** suggested the formation of an epoxide ring in the 5,6-position (Fig. 3). The proton coupling constants and NOESY spectrum analysis suggested the relative configuration of compound **1**. The NOESY correlations of H-6 (δ_H 3.14, d, J = 3.6 Hz) with H-7 (δ_H 4.42, d, J = 3.6 Hz) suggested the α configuration of the 5,6-epoxide ring and α - orientation of the hydroxyl group at C-7. The configuration of C-24 was considered *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 resonated at δ_C 17.68 in the 24*R* epimer of a known sterol, (22*E*,24*R*)-24-methylcholesta-5,22-dien-3β-ol, with the same chain, and the 24*S* epimer, (22*E*,24*S*)-24-methylcholesta-5,22-dien-3β-ol, had a relative 0.4 ppm downfield chemical shift [10, 11]. Thus, the structure of compound **1** was determined as 5α,6α-epoxy-(22E,24R)-ergosta-8(14),22-diene-3β,7α-diol based on comparing the reported data and NMR analyses [12, 13].

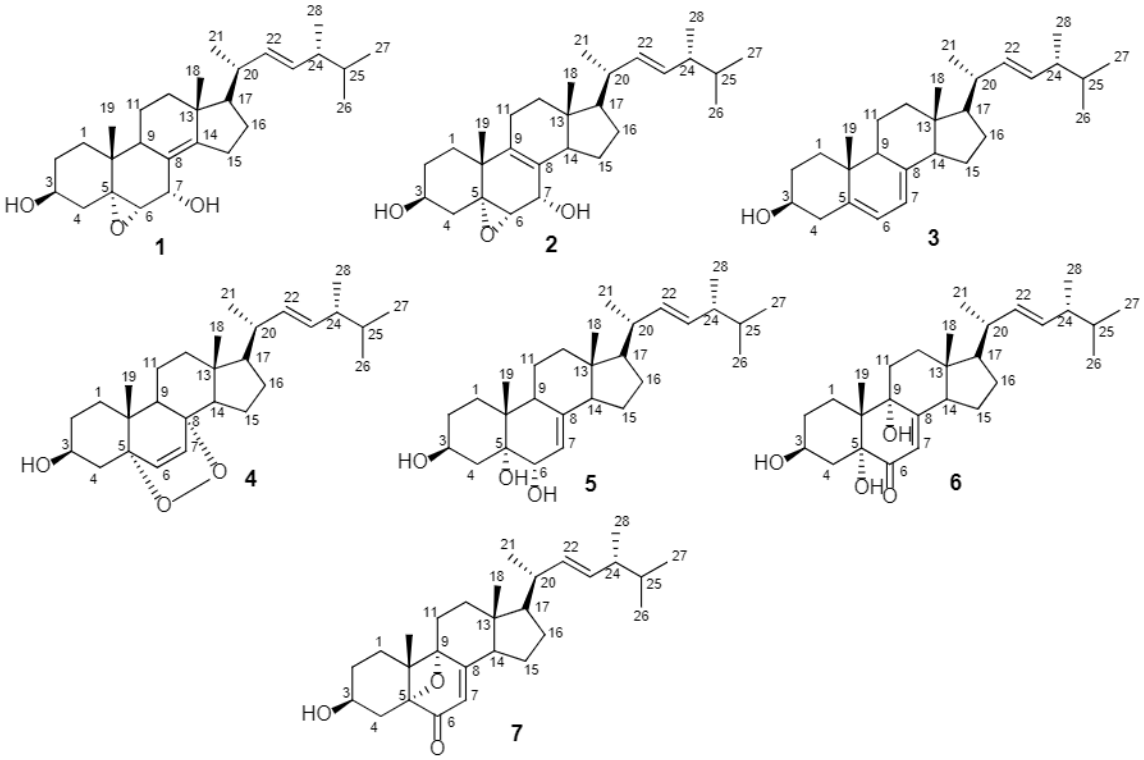

Figure 2. Secondary metabolites **1–7** from *Aspergillus* sp. M904

Compound **2** was obtained as an amorphous powder, mp 169-170°C. Its positive ESI-MS showed the pseudomolecular ion peak $[M+H]^+$ at *m/z* 429. The 1D-NMR and MS spectral data of compound **2** were very similar to those of compound **1**, except the differences in carbon chemical shifts of C-8 (δ_C 126.9), C-9 (δ_C 134.5) and C-14 (δ_C 40.4). The ^{1}H-NMR spectrum of **2** showed two singlet methyls at δ_H 0.59 (3H, s, H-18), 1.14 (3H, s, H-19), four doublet methyl signals at δ_H 0.82 (3H, d, J = 7.2 Hz, H-28), 0.84 (3H, d, J = 6.6 Hz, H-21), 0.91 (3H, d, J = 6.6 Hz, H-27), 1.02 (3H, dd, J = 6.6 Hz, H-26), three oxygenated proton signals at

δ_H 3.31 (1H, d, *J* = 2,4 Hz, H-6), 3.95 (lH, m, H-3), 4.22 (1H, br. s, H-7) and two olefinic proton signals at δ_H 5.17 (1H, dd, *J* = 7.2, 15.0 Hz, H-22), 5.22 (lH, dd, *J* = 7.2, 15.0 Hz, H-23). The ^{13}C-NMR and HSQC spectra of compound **2** indicated twenty-eight carbon signals containing six methyl carbons, seven methylene carbons, five sp^3 methine carbons, three oxymethines, two olefinic methines at δ_C 132.0 (CH, C-23), 135.6 (CH, C-22) and five non-protonated carbons. The signals at δ_C 62.6 (CH, C-6), 65,7 (C, C-5) in the ^{13}C-NMR spectrum of **2** suggested the presence of an epoxy ring. Thus, the NMR and MS spectrum data of **2** led to the structure of 5α,6α-epoxy-(22*E*,24*R*)-ergosta-8(9),22-diene-3β,7α-diol [12, 13].

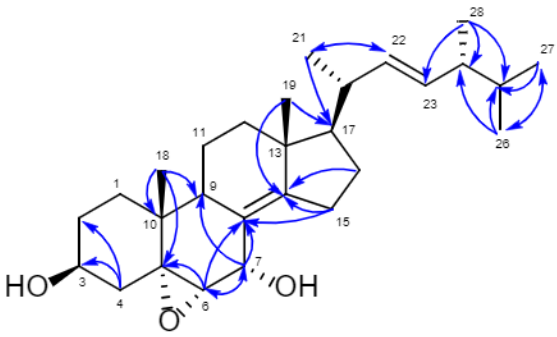

Figure 3. Key HMBC (→) correlation of compound 1

Compound **3** was isolated as a white solid, mp. 149–150°C. Its positive ESI-MS showed the pseudomolecular ion peak $[M+H]^+$ at *m/z* 397. The observation of six methyl signals in the ^{1}H-NMR spectrum at δ_H 0.63 (3H, s, H-18), 0.82 (3H, d, *J* = 6.6 Hz, H-27), 0.84 (3H, d, *J* = 6.6 Hz, H-26), 0.92 (3H, d, *J* = 7.2 Hz, H-28), 0.95 (3H, s, H-19), 1.03 (3H, d, *J* = 6.6 Hz, H-21) indicated that the sterol fragment of compound **3** is an ergosterol derivative. The oxygenated proton signal at δ_H 3.63 (1H, m, H-3) and four olefinic proton signals at δ_H 5.17 (1H, dd, *J* = 7.2, 15.0 Hz, H-22), 5.22 (1H, dd, *J* = 7.2, 15.0 Hz, H-23), 5.38 (1H, q, J = 2.4 Hz, H-7), 5.57 (1H, dd, J = 6.0, 2.4 Hz, H-6) were also observed. The ^{13}C-NMR spectrum of compound **3** showed the signals of twenty-eight carbons, including six methyl groups at δ_C 12.1 (CH_3, C-18), 16.3 (CH_3, C-19), 17.6 (CH_3, C-28), 19.6 (CH_3, C-21), 19.9 (CH_3, C-26), 21.1 (CH_3, C-27), seven methylene groups at δ_C 21.1 (CH_2, C-15), 23.0 (CH_2, C-11), 28.3 (CH_2, C-12), 32.0 (CH_2, C-2), 38.4 (CH_2, C-1), 39.1 (CH_2, C-16), 40.8 (CH_2, C-4), six sp^3 methine groups at δ_C 33.1 (CH, C-25), 40.4 (CH, C-20), 42.8 (CH, C-24), 46.3 (CH, C-9), 54.6 (CH, C-17), 55.8 (CH, C-14), six olefinic carbon signals at δ_C 116.3 (CH, C-7), 119.3 (CH, C-6), 132.0 (CH, C-23), 135.6 (CH, C-22), 139.8 (C, C-8), 141.3 (C, C-5), one oxygenated carbon signal at δ_C 70.5 (CH, C-3) and two quaternary carbons at δ_C 37.0 (C, C-10), 42.9 (C, C-13). Based on the above evidence, structure **3** was determined as ergosterol [12, 14, 15].

Compound **4** was obtained as an amorphous powder, mp. 183–184°C, ESI-MS *m/z* 429 $[M+H]^+$. The proton signals of compound **4** in the ^{1}H-NMR spectrum were characteristic for an ergosta steroidal skeleton with six methyl signals at δ_H 0.81 (3H, s, H-18), 0.82 (3H, d, *J* = 6.6 Hz, H-27), 0.83 (3H, d, *J* = 6.6 Hz, H-26), 0.88 (3H, d, *J* = 7.2 Hz, H-19), 0.91 (3H, s, H-28), 1.00 (3H, d, *J* = 6.6 Hz, H-21), one oxymethine at δ_H 3.97 (1H, m, H-3) and four olefinic protons at δ_H 5.14 (1H, dd, *J* = 7.2, 15.5 Hz, H-22), 5.22 (1H, dd, *J* = 7.2, 15.5 Hz, H-23), 6.23 (1H, d, *J* = 8.4 Hz, H-7), 6.50 (1H, d, *J* = 8.4 Hz, H-6). The ^{13}C-NMR spectrum of compound **4** showed twenty-eight carbon signals, of which there were four olefinic carbon signals at δ_C 130.8 (CH, C-7), 132.3 (CH, C-23), 135.2 (CH, C-22), 135.4 (CH, C-6), two quaternary carbons at δ_C 79.4 (C, C-8), 82.2 (C, C-5) and one oxygenated carbon at δ_C 66.5 (CH, C-3). The chemical shift values of two carbons at δ_C 79.4 (C, C-8) and 82.2 (C, C-5) suggested their linkages to oxygen as peroxide groups. From the above evidence and comparison with the NMR data in the previously reported literature, compound **4** was identified as ergosterol peroxide [12, 13].

Compound **5** was isolated as a white solid, mp. 246–247°C, ESI-MS: *m/z* 431 $[M+H]^+$. Analysis of the ^{1}H-NMR spectrum of **5** exhibited that compound **5** was a polyhydroxylated sterol. The ^{1}H-NMR spectrum of compound **5** showed six up-field shifted methyl signals at δ_H 0.60 (3H, s, H-18), 0.82 (3H, d, *J* = 6.6 Hz, H-26), 0.84 (3H, d, *J* = 6.6 Hz, H-27), 0.92 (3H, d, *J* = 7.2 Hz, H-28), 1.03 (3H, d, *J* = 6.6 Hz, H-21),

1.25 (3H, s, H-19) supported a steroidal skeleton. In addition, the oxygenated proton and olefinic proton signals at δ_H 3.62 (1H, m, H-3), 4.09 (1H, m, H-6), 5.17 (1H, dd, *J* = 7.2, 15.5 Hz, H-22), 5.24 (1H, dd, *J* = 7.2, 15.5 Hz, H-23), 5.35 (1H, d, *J* = 5.4 Hz, H-7) were also observed. The ^{13}C-NMR spectrum of compound **5** showed twenty-eight carbon signals, of which there were four olefinic carbon signals at δ_C 117.6 (CH, C-7), 132.3 (CH, C-23), 135.5 (CH, C-22), 144.0 (C, C-8) and three oxygenated carbon signals at δ_C 67.8 (CH, C-3), 73.1 (CH, C-6), 76.5 (CH, C-5). Based on the above evidence, structure **5** was determined as 3β, 5α, 6α, trihydroxy-ergosta-7,22-diene [16, 17].

Compound **6** was obtained as a white amorphous powder, mp. 225–226°C, ESI-MS: *m/z* 445 $[M+H]^+$. The ^{13}C-NMR and DEPT spectra of compound **6** showed twenty-eight carbon signals containing six methyl groups, seven methylene groups, five methine groups, three olefine methines at δ_C 119.9 (CH, C-7), 132.5 (CH, C-23), 135.1 (CH, C-22), one oxymethine at δ_C 67.2 (CH, C-3), one carbonyl group at δ_C 197.6 (C, C-6) and four non-protonated carbons. The NMR spectra and physical data of **6** led to the structure of 3β, 5α, 9α-trihydroxyergosta-7,22-diene-6-one [13, 18].

Compound **7** was isolated as amorphous powder, ESI-MS: *m/z* 427 $[M+H]^+$. The ^{1}H-NMR data of compound **7** were identical to those of compound **6**, even though compound **6** and compound **7** had very different R_f values when spotted on the same TLC plate. The ^{1}H-NMR of compound **7** showed the proton methyl signals at δ_H 0.62 (3H, s, H-18), 0.82 (3H, d, *J* = 6.6 Hz, H-26), 0.84 (3H, d, *J* = 7.2 Hz, H-27), 0.92 (3H, d, *J* = 7.2 Hz, H-28), 1.02 (3H, s, H-19), 1.03 (3H, d, *J* = 6.6 Hz, H-21), the proton oxygenated and olefinic at δ_H 4.05 (1H, m, H-3), 5.17 (1H, dd, *J* = 7.2, 15.5 Hz, H-22), 5.24 (1H, dd, *J* = 7.2, 15.5 Hz, H-23), 5.64 (1H, d, *J* = 1.8 Hz, H-7). Based on these data, comparison with the reference data, and the MS data of compound **7**, the structure of compound **7** was elucidated as 3β-hydroxy,5α,9α-epoxy ergosta-7,22-dien-6-one [19].

All isolated compounds were tested against a panel of gram-positive bacteria (*Enterococcus faecalis* ATCC299212, *Staphylococcus aureus* ATCC25923, *Bacillus cereus* ATCC14579), gram-negative bacteria *(Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853, *Salmonella enterica* ATCC13076) and yeast (*Candida albicans* ATCC10231). Compounds **1–7** had inhibitory activity against from one to six tested strains with MIC values from 64–256 µg/mL. Compound **6** selectively inhibited *E. faecalis* strain with a MIC value of 64 µg/mL. Compound **1** indicated antibacterial activity against three strains of gram-positive bacteria (*E. faecalis*, *S. aureus*, *B. cereus*), one Gram negative bacteria (*E. coli*) and one fungus (*C. albicans*) with MIC values of 64 -256 µg/mL (Table 1). Compound **2** inhibited *E. faecalis*, *S. aureus*, and *C. albicans* with a MIC value of 64, 256, and 128 µg/mL, respectively. In addition, compounds **3, 5** and **7** indicated antibacterial activity against three strains of gram-positive bacteria (*E. faecalis*, *S. aureus*, *B. cereus*) and fungi (*C. albicans*) with MIC values of 64–256 µg/mL.

Table 1. Antibacterial and antifungal activities of compounds **1–7** (MIC: µg/mL)

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

CONCLUSION

Seven ergostane steroid compounds were isolated from the ethyl acetate extract of *Aspergillus* sp. M904 strain. Their structures were identified by using the MS and NMR spectroscopy and comparing with the reference data, including 5α,6α-epoxy-(22*E*,24*R*)-ergosta-8(14),22-diene-3β,7α-diol (**1**), 5α,6α-epoxy-(22*E*,24*R*)-ergosta-8(9),22-diene-3β,7α-diol (**2**), ergosterol (**3**), ergosterol peroxide (**4**), 3β,5α,6α,trihydroxy-ergosta-7,22-diene (**5**), 3β,5α,9α-trihydroxy ergosta-7,22-diene-6-one (**6**), 3β-hydroxy, 5α,9α-epoxy ergosta-7,22-dien-6-one (**7**). Compounds **1–7** had inhibitory activity against from one to six tested strains with MIC values from 64–256 µg/mL.

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