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Ergosteroid derivatives from an algicolous strain of Aspergillus ustus

Xiang-Hong Liu\textsuperscript{a}, Feng-Ping Miao\textsuperscript{a}, Xiao-Rui Liang\textsuperscript{ab} & Nai-Yun Ji\textsuperscript{a}
\textsuperscript{a} Key Laboratory of Coastal Biology and Bioresource Utilization, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, P.R. China
\textsuperscript{b} Department of Basic Sciences, Naval Aeronautical and Astronautical University, Yantai 264001, P.R. China

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Ergosteroid derivatives from an algicolous strain of *Aspergillus ustus*

Xiang-Hong Liu\(^a\), Feng-Ping Miao\(^a\), Xiao-Rui Liang\(^{ab}\) and Nai-Yun Ji\(^a*\)

\(^a\)Key Laboratory of Coastal Biology and Bioresource Utilization, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, P.R. China; \(^b\)Department of Basic Sciences, Naval Aeronautical and Astronautical University, Yantai 264001, P.R. China

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One new ergosteroid derivative, isocyathisterol (1), and eight known compounds (2–9) were isolated from the culture of an algicolous strain (cf-42) of *Aspergillus ustus* obtained from the fresh tissue of marine green alga *Codium fragile*. The structure and absolute configuration of 1 were unequivocally identified by using NMR and mass spectroscopic methods as well as quantum chemical calculations. Compound 1 exhibited weak antibacterial activity.

Keywords: ergosteroid; isocyathisterol; algicolous fungus; *Aspergillus ustus*

1. Introduction

Marine alga-endophytic fungi have attracted much attention for phytochemistry researches due to the high molecular diversity and intriguing bioactivity of their secondary metabolites (Bugni & Ireland 2004; Kjer et al. 2010; Qiao et al. 2010; Miao et al. 2012; Ji et al. 2013; Liu et al. 2013). Among them, terpenes and steroids derived from the mevalonic acid pathways often occurred. In our ongoing searches for the bioactive products from marine organisms, a fungal strain (cf-42) of *Aspergillus ustus* obtained from the marine green alga *Codium fragile* was chemically examined. As a result, one new steroid, isocyathisterol (1), and eight known compounds, ergosta-4,6,8(14),22-tetraen-3-one (2) (Kwon et al. 2002), ergosterol (3) (Kwon et al. 2002), 3β,5α-dihydroxy-6β-methoxyergosta-7,22-diene (4) (Kwon et al. 2002), ergosterol endoperoxide (5) (Kwon et al. 2002), 5α,8α-epidioxyergosta-6,9(11),22-trien-3β-ol (6) (Greca et al. 1990), 1(10→6)abeo-ergosta-5,7,9,22-tetraen-3α-ol (7) (Koshino et al. 1989), citreoanthrasteroid (8) (Nakada & Yamamura 2000) and 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11β-methoxy-3α-ol (9) (Nakada & Yamamura 2000), were isolated and identified (Figure 1). Details of the isolation, structural elucidation and bioactivity of compound 1 are the main subjects of this article.

2. Results and discussion

Compound 1 was obtained as colourless crystals. A molecular formula of C\(_{28}\)H\(_{42}\)O\(_2\) was assigned by analysis of HR-EI-MS (m/z 410.3184 [M]+, calcd for C\(_{28}\)H\(_{42}\)O\(_2\), 410.3185). The IR absorption bands at 3394 and 1655 cm\(^{-1}\) corresponded to the presence of a hydroxyl group and a conjugated carbonyl group. The \(^1\)H NMR spectrum (Table S1) exhibited two methyl singlets, four methyl doublets, and one singlet and two doublets as well as two double doublets ascribed to five olefinic protons. The \(^{13}\)C NMR spectrum (Table S1) indicated 28 resonances, which were
classified into 6 methyls, 6 methylenes, 11 methines and 5 quaternary carbons on the basis of DEPT and HSQC experiments. A detailed NMR data comparison with those reported for cyathisterol revealed the close similarity of them (Kawahara et al. 1994). The $^1$H–$^1$H COSY and HMBC correlations further confirmed the same planar structure of 1 and cyathisterol (Kawahara et al. 1994).

The relative configuration of 1 was determined by coupling constants and NOE correlations. Me-18 and Me-19 were located on the same face according to their NOE correlations with H-11b, which was axial and opposite to H-9 based on the large coupling constant between them. The axial orientation of H-9, H-14 and H-17 was also established by the analysis of coupling constants, and the same orientation of H-14 and H-17 was further supported by their correlation in the NOESY spectrum. The configurations of side-chain moiety (C-20 to C-25) were deduced to be in agreement with those of 2–9 through the biogenic consideration, which were supported by the identical NMR data of 1 and cyathisterol. In addition, H-7 exhibited NOE correlations with H-15a and H-15b, which established the $\beta$-orientation of OH-8 by analysis of the stereo structure produced via the Dreiding force field in MarvinSketch (2012). Collectively, the above-mentioned data evidenced the structure of 1 to be ergosta-4,6,22-trien-8$\beta$-ol-3-one, trivially named isocyathisterol. It was deduced to be a C-9 and/or C-14 isomer of cyathisterol according to their different NMR data around C-8 (Kawahara et al. 1994). In addition, the specific optical rotation ($\text{[a]}_{25}^\text{D} + 61.3$) of 1 differed from that ($\text{[a]}_{25}^\text{D} + 133$) of cyathisterol (Kawahara et al. 1994), which further supported the different configurations of these two steroids.

In order to establish the absolute configuration of compound 1, its electronic circular dichroism (ECD) spectrum was determined, which displayed two positive Cotton effects at 218 and 340 nm and one negative Cotton effect at 268 nm. Then, the ECD spectrum was computed with the time-dependent density function theory (TD-DFT) in methanol at the gas-phase B3LYP/6-31G(d) level (Frisch et al. 2010). The calculated ECD spectrum was produced by SpecDis software (Bruhn et al. 2011), which agreed with the experimental data (Figure S1). Thus, the absolute configuration of 1 was suggested to be 8$R$, 9$R$, 10$R$, 13$R$, 14$R$, 17$R$, 20$R$, and 24$R$.

Figure 1. Structures of compounds 1–9.
The remaining known compounds, including ergosta-4,6,8(14),22-tetraen-3-one (2) (Kwon et al. 2002), ergosterol (3) (Kwon et al. 2002), 3β,5α-dihydroxy-6β-methoxyergosta-7,22-diene (4) (Kwon et al. 2002), ergosterol endoperoxide (5) (Kwon et al. 2002), 5α,8α-epidioxyergosta-6,9(11),22-trien-3β-ol (6) (Greca et al. 1990), 1(10 → 6)abeo-ergosta-5,7,9,22-tetraen-3α-ol (7) (Koshino et al. 1989), citreanthrasteroid (8) (Nakada & Yamamura 2000) and 1(10 → 6)abeo-ergosta-5,7,9,22-tetraen-11β-methoxy-3α-ol (9) (Nakada & Yamamura 2000), were identified by comparing the NMR data with the literature values.

Compound 1 was tested for biological activities against several target organisms including bacteria, fungi and brine shrimp (Table 1). The results demonstrated that it exhibited weak antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* (inhibitory diameters of 6.7 and 5.7 mm, respectively) at 30 μg/disc, and no antifungal activity against *Colletotrichum lagenarium* and *Fusarium oxysporum* as well as toxicity against *Artemia salina* was detected.

### 3. Experimental

#### 3.1. General

NMR spectra were recorded at 500 and 125 MHz for 1H and 13C, respectively, on a Bruker Avance III 500 NMR spectrometer (Bruker Corp., Billerica, MA, USA) using TMS as internal standard. Low- and high-resolution mass spectra were determined on an Autospec Premier P776 mass spectrometer (Waters Corp., Milford, MA, USA). IR spectrum was obtained on a JASCO FT/IR-4100 Fourier Transform Infrared spectrometer (JASCO, Tokyo, Japan). UV spectrum was measured on a DU 800 Spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA). Optical rotation was determined on a JASCO P-1020 polarimeter (JASCO, Tokyo, Japan). Melting point was measured using an X-4 micro-melting point apparatus (Beijing Tech Instrument Co., Ltd., Beijing, China). ECD spectrum was recorded on a Chirascan CD Spectrometer (Applied Photophysics Ltd., Surrey, UK). Quantum chemical calculations were operated via Gaussian 09 software (IA32W-G09RevC.01). HPLC separation was carried out on an Elite HPLC system (P270 pump, UV230 + detector, Dalian Elite Analytical Instruments Co., Ltd, Dalian, China) using an Eclipse XDB-C18 (5 μm, 9.4 mm × 250 mm) column. Column chromatography (CC) was performed with silica gel (200–300 mesh, Qingdao Haiyang Chemical Co., Qingdao, China) and Sephadex LH-20 (Pharmacia, Uppsala, Sweden). TLC was carried out with pre-coated silica gel plates (GF-254, Qingdao Haiyang Chemical Co.). The solvents were of analytical grade except for the spectral-grade methanol for HPLC.

#### 3.2. Fungal material, fermentation, extraction and isolation

The *A. ustus* cf-42 strain was obtained from the fresh tissue of the marine green alga *C. fragile* collected from Zhoushan Island, which was identified by analysis of the morphology and ITS region of the rDNA. The sequence data have been deposited at GenBank with the accession number JX036023, and the strain has been preserved at the Coastal Natural Product Laboratory of Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, and the China Center for Type Culture Collection (No. CCTCC M 2011420). The fresh mycelia were grown on the potato dextrose agar plates and were then inoculated into 50 Erlenmeyer flasks (1 L), each

<table>
<thead>
<tr>
<th>Compound</th>
<th><em>E. coli</em></th>
<th><em>S. aureus</em></th>
<th><em>C. lagenarium</em></th>
<th><em>F. oxysporum</em></th>
<th><em>A. salina</em> (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.7 mm$^a$</td>
<td>5.7 mm$^a$</td>
<td>0 mm$^a$</td>
<td>0 mm$^a$</td>
<td>5.1$^b$</td>
</tr>
</tbody>
</table>

$^a$ Inhibitory diameter at 30 μg/disc.

$^b$ Lethal rate at 100 μg/mL.
containing 300 mL potato dextrose broth media (50% sea water). The fermentation was performed statically at room temperature (ca. 20°C) for 35 days (Liu et al. 2013).

The extraction and preliminary isolation was shown in a previous literature (Liu et al. 2013). Fr. 5 eluted with PE/EtOAc (10:1) and was further purified by CC on Sephadex LH-20 (CHCl₃/MeOH, 1:1) and silica gel (PE/EtOAc, 10:1) and preparative HPLC (MeOH/H₂O, 85:15) to yield 2 (3.0 mg). Fr. 6 eluted with PE/EtOAc (5:1) and was further purified by CC on Sephadex LH-20 (CHCl₃/MeOH, 1:1) and silica gel (PE/EtOAc, 6:1) to yield two subfractions (Fr 6.1 and 6.2). Fr. 6.1 was further purified by recrystallisation in MeOH to yield 3 (15.2 mg) and a subfraction, which was further purified by preparative HPLC (MeOH/H₂O, 85:15) to afford 1 (3.0 mg). Fr. 6.2 was further purified by preparative HPLC (MeOH/H₂O, 85:15) to yield 2 (1.4 mg), 8 (1.8 mg) and 9 (1.3 mg). Fr. 8 eluted with PE/EtOAc (2:1) and was further purified by CC on Sephadex LH-20 (CHCl₃/MeOH, 1:1) and silica gel (PE/EtOAc, 4:1) to yield three subfractions (Frs 8.1–8.3). Fr. 8.3 was further purified by CC on silica gel (PE/EtOAc, 5:1) and preparative HPLC (MeOH/H₂O, 85:15) to yield 6 (14.1 mg). Fr. 9 eluted with PE/EtOAc (2:1) and was further purified by CC on Sephadex LH-20 (CHCl₃/MeOH, 1:1) and silica gel (PE/EtOAc, 4:1) to yield two subfractions (Frs 9.1 and 9.2). Fr. 9.2 was rechromatographed by CC on silica gel (PE/EtOAc, 3:1) and preparative HPLC (MeOH/H₂O, 85:15) to yield four subfractions (Frs 9.2.1–9.2.4). Fr. 9.2.4 was purified by preparative HPLC (MeOH/H₂O, 85:15) to afford 5 (23.7 mg). Fr. 10 eluted with PE/EtOAc (2:1) and was further purified by CC on Sephadex LH-20 (CHCl₃/MeOH, 1:1) and silica gel (PE/EtOAc, 4:1) to yield three subfractions (Frs 8.1–8.3). Fr. 8.3 was further purified by CC on silica gel (PE/EtOAc, 5:1) and preparative HPLC (MeOH/H₂O, 85:15) to yield 6 (14.1 mg). Fr. 9 eluted with PE/EtOAc (2:1) and was further purified by CC on Sephadex LH-20 (CHCl₃/MeOH, 1:1) and silica gel (PE/EtOAc, 4:1) to yield two subfractions (Frs 9.1 and 9.2). Fr. 9.2 was rechromatographed by CC on silica gel (PE/EtOAc, 3:1) and preparative HPLC (MeOH/H₂O, 85:15) to yield four subfractions (Frs 9.2.1–9.2.4). Fr. 9.2.4 was purified by preparative HPLC (MeOH/H₂O, 85:15) to afford 5 (23.7 mg). Fr. 10 eluted with PE/EtOAc (2:1) and was further purified by CC on Sephadex LH-20 (CHCl₃/MeOH, 1:1) and silica gel (PE/EtOAc, 4:1) to yield three subfractions (Frs 8.1–8.3).

3.3. Computational details

A conformational search for compound 1 was performed via the Dreiding force field in MarvinSketch (2012), and the geometries were further optimised at the B3LYP/6-31G(d) level in methanol without vibrational imaginary frequencies. The predominant conformation was subjected to the theoretical calculations of ECD spectrum at the B3LYP/6-31G(d) level in methanol using the TD-DFT method, which was drawn via SpecDic software with sigma = 0.35 and UV shift = 10 nm (Bruhn et al. 2011). All the above-mentioned calculations were performed with the integral equation formalism-polarisable continuum model as implemented in Gaussian 09 (Frisch et al. 2010).
3.4. **Bioassays**

Antibacterial activity against *E. coli* and *S. aureus*, antifungal activity against phytopathogenic *C. lagenarium* or *F. oxysporum*, and toxicity against brine shrimp (*A. salina*) of compound 1 were tested as described previously (Miao et al. 2012) with chloramphenicol, amphotericin B and K₂Cr₂O₇ as positive controls, respectively. The bioassay results are shown in Table 1.

4. **Conclusion**

Nine ergosteroid derivatives (1–9), including a new one (1), with the weak antibacterial activity, were isolated and identified from an alga-endophytic strain (cf-42) of *A. ustus*, which further added the molecular diversity and evidenced the metabolic talent of this fungus.

**Supplementary material**

Supplementary details relating to this paper are available online, alongside experimental details, 1D/2D NMR, IR, and mass spectra as well as Cartesian coordinates.

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**References**


