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Antiviral Merosesquiterpenoids Produced by the Antarctic Fungus

Aspergillus ochraceopetaliformis SCSIO 05702

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‡Key Laboratory of Plant Resources Conservation and Sustainable Utilization, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, People’s Republic of China
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*Supporting Information

ABSTRACT: Five new highly oxygenated α-pyrene merosesquiterpenoids, ochraceopones A–E (1–5), together with one new double bond isomer of asteltoxin, isoasteltoxin (6), and two known asteltoxin derivatives, asteltoxin (7) and asteltoxin B (8), were isolated from an Antarctic soil-derived fungus, Aspergillus ochraceopetaliformis SCSIO 05702. Their structures were determined through extensive spectroscopic analysis, CD spectra, quantum mechanical calculations, and X-ray single-crystal diffraction. Ochraceopones A–D (1–4) are the first examples of α-pyrene merosesquiterpenoids possessing a linear tetracyclic carbon skeleton, which has not been previously described. All the isolated compounds were tested for their antiviral, cytotoxic, antibacterial, and antitubercular activities. Among these compounds, ochraceopone A (1), isoasteltoxin (6), and asteltoxin (7) exhibited antiviral activities against the H1N1 and H3N2 influenza viruses with IC50 values of >20.0/12.2 ± 4.10, 0.23 ± 0.05/0.66 ± 0.09, and 0.54 ± 0.06/0.84 ± 0.02 μM, respectively. A possible biosynthetic pathway for ochraceopones A–E (1–5) was proposed.

During the last century, there were three major influenza pandemics: the 1918 H1N1 Spanish, the 1957 H2N2 Asian, and the 1968 H3N2 Hong Kong outbreaks. In June 2009, the World Health Organization (WHO) identified a new strain of swine origin, H1N1, raising the level of influenza pandemic alert from phase three to phase six.1,2 Natural products have been and continue to be a rich source of antiviral drugs.3 Meroterpenoids are hybrid natural products of both terpenoid and non-terpenoid origin, and more than 330 naturally occurring meroterpenoids have been isolated from various fungal sources.4 Naturally occurring α-pyrene meroterpenoids comprise a diverse group of fungal polyketide-terpenoid hybrid metabolites that have attracted a great deal of attention for their unusual structure features4,5 and resulting broad spectrum of biological activities including inhibition of acetylcholinesterase6,7 and cholesterol acyltransferase,8 as well as antimicrobial,9 phytotoxic,10 and anti-insect11 activities. The complex polycyclic, highly oxygenated structures and the extraordinary range of biological activities of α-pyrene meroterpenoids have led to many biosynthesis12,13 and total synthesis14−16 programs.

As part of our investigations aimed at exploring structurally novel bioactive secondary metabolites from fungal species inhabiting unique environments,17−19 a subculture of an isolate...
48 of the fungal strain *Aspergillus ochraceopetaliformis* SCSIO 4905702, obtained from a soil sample that was collected near the 50 Great Wall station (Chinese Antarctic station), was grown in a 51 nutrient-deprived culture medium. Its ethyl acetate extract 52 displayed significant *in vitro* antiviral activity against H1N1 53 influenza virus and contained a variety of secondary metabolites 54 with similar UV absorptions at 207, 290, and 340 nm, as shown 55 by HPLC analysis with a photodiode array. Further chemical 56 investigations of the culture extract afforded five new \( \alpha \)-pyrone 57 merosesquiterpenoids, which we have named ochraceopones 58 A–E (1–5), together with one new double-bond isomer of 59 asteltoxin, isoasteltoxin (6), and two known asteltoxin 60 derivatives, asteltoxin (7) \(^{20}\) and asteltoxin B (8) \(^{21,22}\). 61 Ochraceopones A–D (1–4) are the first examples of \( \alpha \)-pyrone 62 merosesquiterpenoids possessing a linear tetracyclic carbon 63 skeleton, which has not been previously described. Compounds 64 1–8 were tested for their antiviral, cytotoxic, antibacterial, and 65 antitubercular activities. Details of the isolation, structure 66 elucidation, and bioactivities of these compounds are reported 67 herein.

### RESULTS AND DISCUSSION

69 Compound 1 was obtained as an amorphous powder and 70 crystallized from methanol to give yellow crystals. The 71 molecular formula of 1 was determined to be \( C_{23}H_{30}O_7 \) from 72 the HRESIMS peak at m/z 473.1791 ([M + Na]\(^+\)). Its IR 73 spectrum exhibited absorptions at 3368 cm\(^{-1}\) (hydroxy) and 74 1684 and 1647 cm\(^{-1}\) (carbonyl). The \(^{13}C\) NMR (Table 1), 75 DEPT, and HMQC spectra revealed the presence of 23 76 carbons, namely, seven methyls, one methylene, four methines, 77 and 11 nonprotonated carbons. The HMBC correlations from 78 H-6 to C-1, C-2, and C-3, H-7 to C-3, C-4, and C-5, and H-12 79 to C-4 and C-5 suggested the presence of an \( \alpha \)-pyrone 80 moiety (A ring). Furthermore, the HMBC spectrum showed 81 the following correlations: H-12 with C-7, C-8, and C-9, H-4 82 with C-7, C-11, and C-13, H-12 with C-8, C-10, and C-16, H-2 83 with C-7, C-11, and C-12, H-21/H-22 with C-13, C-14, and 84 C-15, and H-16 with C-10, C-11, C-12, and C-15. This 85 evidence, as well as one proton spin system derived from 86 \(^1H\)-H COSY correlations, H-7/H-12/H-20, led to the 87 establishment of a highly oxygenated decalin system (C and 88 D rings). The \( \alpha \)-pyrone moiety (A ring) and decalin system (C 89 and D rings) were found to be directly connected via one 90 oxygenated methine (C-6), which was further supported by 91 COSY correlations of H-6/H-7/H-12/H-20 and by the key 92 HMBC correlation from H-6 to C-1 and C-3 (Figure 1). 93 However, the 2D NMR spectra did not provide sufficient 94 information to elucidate the unambiguous connecting pattern 95 between C-3 and C-8. If the carbons C-3 and C-8 were 96 connected via one oxygen bridge, this kind of connecting 97 pattern was consistent with the degrees of unsaturation and 98 molecular formula. The NOESY correlations of H-7/H-12/H-20 99 and H-7/H-13/H-19 indicated a trans-diaxial-like relationship of 100 H-7/H-12/H-13/H-19, which not only indicated 101 the connection of C-3 to C-8 through an ether linkage but also 102 determined the absolute stereochemistry of 1 to be 6R, 7S, 8S, 103 9R, 10R, 11R, and 12R. Compound 2 was obtained as a yellow, amorphous powder.

### Table 1. \(^1H\) and \(^{13}C\) NMR Data for 1–4 (500, 125 MHz, CD3OD, TMS, \( \delta \) ppm)

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<thead>
<tr>
<th>Position</th>
<th>( \delta_C ) (C)</th>
<th>( \delta_H ) (J in Hz)</th>
<th>( \delta_C ) (C)</th>
<th>( \delta_H ) (J in Hz)</th>
<th>( \delta_C ) (C)</th>
<th>( \delta_H ) (J in Hz)</th>
<th>( \delta_C ) (C)</th>
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<td>166.9, C</td>
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<td>109.2, C</td>
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Compound 3 was isolated as a yellow, amorphous solid, and its molecular formula was established as C_{22}H_{26}O_{8} by an HRESIMS peak at m/z 459.1624 [M + Na]^+ (calcld, 459.1631), with 9 degrees of unsaturation. Comparison of their NMR data revealed the difference occurred at the B ring. One methylene signal at δ_{C} 21.4 for C-6 was observed in 3, instead of the oxygenated methine between C-2 and C-7 in 1 and 2. This deduction was further supported by the COSY correlations of H_{2}-6/H-7/H_{12}/H_{17}-20 and by the HMBC correlation of C-6 with C-1, C-3, C-8, and C-12. The NOE correlations of H-7/H_{12}-20 and H_{12}/H_{19}-19 also indicated the trans-diaxial-like relationship of H-7/H_{19}-19. Additional NOE correlations of H-9/H_{19}-19 and H_{19}/H_{12}-12 located H-9, H-12, and H_{19}-19 on the same face, consistent with the NOE correlations of the C ring in 1 and 2 (Figure 3). This assignment was further confirmed by ECD calculations, and the absolute configurations of 3 were determined as 7R, 8S, 9R, 10R, 11R, 12R, respectively (Figure 4).

The molecular formula of compound 4 was determined to be C_{22}H_{28}O_{8} by HRESIMS, indicating 18 amu less than 2 and 10 degrees of unsaturation. Further comparison of the NMR data of 4 with those of 2 revealed a Δ⁶ double bond for 4 as a replacement for the oxygenated methine (C-6) and methine (C-7) in 2, which was supported by HMBC correlations from olefinic methine H-6 (δ_{C} 6.23, 1H, s) to C-1, C-3, C-8, and C-12. The NOE correlations of H-9/H_{19}-19 and H_{19}/H_{12}-12 indicated H-9, H-12, and H_{19}-19 were on the same face (Figure 3). The absolute configurations of 4 were further determined as 8S, 9R, 10R, 11R, 12R, respectively, by comparing the calculated ECD curve with its experimental values (Figure 4), consistent with the corresponding absolute configurations of the C ring in compounds 1–3.

Compound 5 was isolated as a yellow crystal. The molecular formula was established to be C_{22}H_{28}O_{8} (eight degrees of unsaturation) by HRESIMS with analysis of the °H and ^{13}C NMR spectroscopic data. Comparison of UV−vis and °H and ^{13}C NMR data with those of compound 3 (Tables 1 and S1) revealed a high degree of similarity, indicating the same α-pyrene moiety (A ring). The main differences between 5 and 3 were the connecting pattern of the decalin system (C and D rings). Detailed analysis of the 1D- and 2D-NMR spectral data revealed that the decalin ring of 5 is quite similar to those of arisugacin C\textsuperscript{+} which was further supported by the °H−°H COSY, HMBC, and NOEY experiments. This deduction was unambiguously confirmed by the X-ray crystallographic analysis (Figure 2), which determined the absolute configuration of 5.

Figure 1. Key °H−°H COSY (bold), HMBC (arrows), and NOEY (dashed arrows) correlations of 1.

Figure 2. ORTEP drawing of compounds 1 and 5 (Cu Kα).

Figure 3. Key NOESY correlations of compounds 2–6.
Stereochemistry of 5 to be 7R, 8S, 9R, 11S, and 12R. α-Pyrene merosesquiterpenoids possessing an angular tetracyclic carbon skeleton are frequently isolated from the genera *Penicillium* and *Aspergillus* as anti-cholinesterase active constituents. Compound 6 was obtained as a yellow oil with the molecular formula C_{23}H_{30}O_{7} as determined by HRESIMS, indicating 9 degrees of unsaturation. Its 1H NMR and 13C NMR (DEPT) spectra include signals for six nonprotonated carbons, seven unsaturated methine carbons, four oxygenated methine carbons, one methylene carbon, and five methyl carbons, including one methoxyl group. The connectivity of the protons and C atoms was established by the 1H, 13C, and HMQC spectra (Table S2). The general features of its NMR spectroscopic data (Table S2) closely resembled those of asteltoxin (7). Detailed comparison of NMR data of these two compounds suggested that compound 6 had the same 2,8-dioxabicyclo[3.3.0]octane and α-pyrone units. Significant differences in NMR spectra were observed in the signals for the polyene chain. These differences suggested that compound 6 and asteltoxin (7) differed only in the geometry of the double bond in the polyene chain. The double bond between C-11 and C-12 possessed the Z-geometry, which was further deduced by the key ROESY correlation between H-10 (δH 7.04) and H-13 (δH 7.66) (Figure 3). The ROESY correlations of H-6/H-21 indicated a cis-diaxial-like relationship of the 2,8-dioxabicyclo[3.3.0]octane ring in 6. Additional NOESY correlations of H-4-H-21, H-6/H-8, H-6/H-21, and H-7/H-12 in 6 located H-2, H-6, H-7, and H-21 on the same face, which positioned H-3 and H-20 on the opposite face (Figure 3). The key NOESY correlations for the junction of the 2,8-dioxabicyclo[3.3.0]octane ring in 6 were in good agreement with the data for asteltoxin (7). Furthermore, the absolute configurations of asteltoxin (7) were revealed as 3R, 4R, 5R, 6S, 7R, 8R, 9E, 11E, 13E by total synthesis in 2003. Thus, the structure of 6 was suggested as the Δ^{11} double-bond isomerism of asteltoxin (7) and named isoasteltoxin.

The five α-pyrene merosesquiterpenoids 1−5 are probably biosynthesized via polyketide and mevalonate hybrid biogenetic pathways (Scheme 1). In the proposed biogenesis of 1−5, an all-trans farnesyl pyrophosphate is produced via the mevalonate pathway, which condenses with acetyl-CoA and malonyl-CoA to form an intermediate a, which further produces the angular tetracyclic carbon skeleton.

Figure 4. Comparison between calculated (CAM-B3LYP/TZVP) and experimental ECD spectra of 2−4 in MeOH.

Scheme 1. Postulated Biogenetic Pathway for Ochraceopones A–E (1−5)
Table 2. Antiviral Activity of 1, 6, and 7 against H1N1 and H3N2 Viruses

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<th>H3N2</th>
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<td></td>
<td>CC50 (μM)</td>
<td>IC50 (μM)</td>
</tr>
<tr>
<td>1</td>
<td>&gt;20.0</td>
<td>0.23 ± 0.05</td>
</tr>
<tr>
<td>6</td>
<td>0.54 ± 0.14</td>
<td>2.35</td>
</tr>
<tr>
<td>7</td>
<td>0.24 ± 0.01</td>
<td>0.44</td>
</tr>
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</table>

“Cytotoxicity (CC50) and antiviral activity (IC50) were determined by the CPE inhibition assay on MDCK cells. Selectivity index (SI) is the ratio of CC50 to IC50. Data are expressed as means ± SD of three independent experiments. Tamiflu was used as the positive control, with IC50 values of 16.9 and 18.5 nM, respectively.
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Cytotoxicity was assayed with the CCK-8 (Dojindo, Japan) method. Cell lines K562, MCF-7, A549, U937, HeLa, DU145, HL60, and HT29 were purchased from Shanghai Cell Bank, Chinese Academy of Sciences. Cells were routinely grown and maintained in RPMI or DMEM media with 10% fetal bovine serum and with 1% penicillin/streptomycin. All cell lines were incubated in a Thermofluorometric detection. The MIC was defined as the compound concentration required to inhibit influenza virus yield at 48 h postinfection by 50%. Tami
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The antiviral activities against H1N1 and H2N2 were evaluated by the CPE inhibition assay. Confluent MDCK cell monolayers were incubated with influenza virus at 37 °C for 1 h. After removing the virus, cells were maintained in infecting media (RPMI 1640, 4 μg/mL of trypsin) containing different concentrations of test compounds. After 48 h incubation at 37 °C, the cells were fixed with 100 μL of 4% formaldehyde for 20 min at room temperature. After removal of the formaldehyde, the cells were stained with 0.1% crystal violet for 30 min. The plates were washed

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The antiviral activities against H1N1 and H2N2 were evaluated by the CPE inhibition assay. Confluent MDCK cell monolayers were incubated with influenza virus at 37 °C for 1 h. After removing the virus, cells were maintained in infecting media (RPMI 1640, 4 μg/mL of trypsin) containing different concentrations of test compounds. After 48 h incubation at 37 °C, the cells were fixed with 100 μL of 4% formaldehyde for 20 min at room temperature. After removal of the formaldehyde, the cells were stained with 0.1% crystal violet for 30 min. The plates were washed

397 Cytotoxicity was assayed with the CCK-8 (Dojindo, Japan) method. Cell lines K562, MCF-7, A549, U937, HeLa, DU145, HL60, and HT29 were purchased from Shanghai Cell Bank, Chinese Academy of Sciences. Cells were routinely grown and maintained in RPMI or DMEM media with 10% fetal bovine serum and with 1% penicillin/streptomycin. All cell lines were incubated in a Thermofluorometric detection. The MIC was defined as the compound concentration required to inhibit influenza virus yield at 48 h postinfection by 50%. Tami
eralen was used as the positive control, with IC50 values of 16.9 and 18.5 nM, respectively.