New naphthopyrones from marine-derived fungus *Aspergillus niger* 2HL-M-8 and their in vitro antiproliferative activity

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A new cytotoxic dimeric naphthopyrone, aurasperone H (1), together with eight related known polyketides (2–9) was isolated from a marine-derived fungus *Aspergillus niger* 2HL-M-8. The structure of new compound 1 was elucidated on the basis of its spectroscopic data (1D, 2D NMR and CD). Compound 1 exhibited moderate inhibitory activity against the human lung adenocarcinoma A549 and the human leukaemia HL-60 cell lines. Compound 5 displayed significant in vitro antiproliferative activity against HL-60 cell line with an IC\textsubscript{50} value of 0.8 \(\mu\)M.

**Keywords:** *Aspergillus niger*, dimeric naphthopyrones; antiproliferative activity

1. Introduction

Nature is an untapped source of unique and desirable scaffolds for library construction and subsequent drug discovery (Lachance et al. 2012). Marine species have been proven to be an unexplored and prolific source of molecular diversity, which has led to an increased research effort in natural products (Bhatnagar & Kim 2010; Imhoff et al. 2011). Marine-derived fungi, in particular, have become a promising source of new compounds with interest in pharmacology and biotechnology (Pejin et al. 2013; Schueffler & Anke 2014). Numerous marine-derived fungi actually belong to genera well known in the terrestrial environment, such as *Aspergillus*, *Penicillium*, *Cladosporium*, *Phoma* and *Fusarium* (Höller et al. 2000), and the genus *Aspergillus* is the major contributor to secondary metabolites of marine fungal origin (Lee et al. 2013).

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Aspergillus, a genus of filamentous fungi, is renowned for its medical and commercial importance (Sanchez et al. 2012). The genus Aspergillus produces a variety of natural products; major groups of secondary metabolites including polyketides, ribosomal and nonribosomal peptides, and terpenoids (Bromann et al. 2014). In our present investigation into the antineoplastic metabolites of marine micro-organism, a new dimeric naphthopyrone, aurasperone H (1), and eight known polyketides, were obtained from marine-derived fungus Aspergillus niger 2HL-M-8, which was isolated from the mud sample collected on intertidal zone in Huludao coastline, Liaoning Province, China. This paper reports the isolation and structural elucidation of 1, as well as in vitro antiproliferative activities of 1–9 against the human A549 lung adenocarcinoma, the human MGC-803 gastric cancer and the human HL-60 leukaemia cell lines.

2. Results and discussion

The crude extract of the fermentation culture of the fungus A. niger 2HL-M-8 was subjected to various column chromatography (CC) methods to afford one novel (1) and eight known polyketides (2–9) (Figure 1). Compounds 2–9 were identified as isoaurasperone A (2) (Xiao et al. 2014), fonsecinones A–D (3–6) (Priestap 1984), asperpyrones B (7) and C (8) (Akiyama et al. 2003), flavasperone (9) (Sakurai et al. 2002), respectively, by comparison of their NMR (and CD) data with those reported.

Compound 1 was obtained as a yellow amorphous powder, which was assigned a molecular formula of C\textsubscript{34}H\textsubscript{30}O\textsubscript{11} from its HR-ESI-MS data (m/z 615.1861 [M + H]\textsuperscript{+}, calcd. for C\textsubscript{34}H\textsubscript{31}O\textsubscript{11} 615.1860). The \textsuperscript{1}H NMR spectrum exhibited two meta-coupled aromatic proton signals at δ\textsubscript{H} 6.43 (1H, d, J = 2.1 Hz) and 6.26 (1H, d, J = 2.1 Hz), and four aromatic or olefinic proton signals at δ\textsubscript{H} 7.16 (1H, s), 7.00 (1H, s), 6.06 (1H, s) and 6.03 (1H, s), as well as two intramolecularly hydrogen-bonded phenolic hydroxyl proton signals at δ\textsubscript{H} 15.21 (1H, s) and 14.76 (1H, s), which were characteristic of typical linear naphtho-γ-pyrones. In the high field region, two methyl proton signals at δ\textsubscript{H} 2.42 (3H, s) and 1.09 (3H, d, J = 6.2 Hz), along with four methoxy proton signals at δ\textsubscript{H} 3.80 (3H, s), 3.66 (3H, s) and 3.43 (3H, s), were observed. The \textsuperscript{13}C NMR and HSQC spectra revealed that compound 1 contained 34 carbons, including two methyl groups, four methoxy groups, one sp\textsuperscript{3} methine, one sp\textsuperscript{3} methylene, six sp\textsuperscript{2} methines, 20 quaternary sp\textsuperscript{2} carbons, with two being conjugated carbonyls. The above evidences were indicative of a dimer consisting of two linear naphtho-γ-pyrones monomers (Akiyama et al. 2003; Zhang et al. 2007).

The \textsuperscript{1}H and \textsuperscript{13}C NMR data were assigned by the HSQC and HMBC spectra. Compound 1 showed similar \textsuperscript{1}H and \textsuperscript{13}C NMR data to those of isoaurasperone A. The only difference was that 2-hydroxy propyl signals (2.53 m, 43.6; 3.96 m, 64.5; 1.09 d, 22.9) replaced a methyl signal. 2-Hydroxy propyl group was verified by the long-range correlations from H-3\textsubscript{g} C\textsubscript{64.6} and C-2\textsubscript{d} C\textsubscript{43.6} and C-2\textsubscript{00} C\textsubscript{22.9}. The C-7-linked linear rubrofusarin B moiety was indicated by the HMBC correlations between H-3\textsubscript{g} C\textsubscript{7.16}, C-7\textsubscript{a} C\textsubscript{7.00}, C-8\textsubscript{a} C\textsubscript{6.06}, C-9\textsubscript{a} C\textsubscript{6.03}, C-4\textsubscript{a} C\textsubscript{6.26}, C-5\textsubscript{a} C\textsubscript{7.16}, and from H-1\textsubscript{g} C\textsubscript{15.21}, and C-2\textsubscript{00} C\textsubscript{14.76}. The location of 8-OCH\textsubscript{3} to C-8 was suggested by the HMBC correlations of H-1\textsubscript{g} C\textsubscript{15.21}, and from H-3\textsubscript{g} C\textsubscript{6.06} and C-4\textsubscript{a} C\textsubscript{6.03}, and from 8-OCH\textsubscript{3} C\textsubscript{8.03}, suggested the presence of C-10'-linked 2'-substituted linear rubrofusarin B moiety, which was supported by the NOESY correlations of 6'-OCH\textsubscript{3} C\textsubscript{7.16}, 7'-OCH\textsubscript{3} C\textsubscript{6.06} and 8'-OCH\textsubscript{3} C\textsubscript{6.03}. The location of 2-hydroxy propyl was assigned at C-2' by the HMBC correlations between H-3' and C-11', and between H-1' and C-2'. Thus, the connection between the two monomers of 1 was...
determined at C-7 and C-10', which was supported by the NOE correlation from H-9' to 6-OCH₃.

The absolute configuration of compound 1 was established by the circular dichroism (CD) method with comparison to the related literature of bis(naphtho-γ-pyrone) (Mason et al. 1974; Endo & Naoki 1980; Koyama et al. 1987). Compound 1 shows a positive Cotton effect at 272 nm and negative Cotton effect at 288 nm, which is contrary to aurasperone A with S configuration (Shaaban et al. 2012), indicating that the biaryl axis configuration of 1 was R.
[Rh$_2$(OCOCF$_3$)$_4$], as a convenient and practical method, was widely applied to the determination of the absolute stereochemistry of chiral secondary alcohols in the recent years (Liu et al. 2014; Yin et al. 2014). The absolute configuration of C-2 at the 2-hydroxy propyl side chain may be deduced via the CD data of the in situ formed [Rh$_2$(OCOCF$_3$)$_4$] complex (Gerards & Snatzke 1990; Frelek & Szczep 1999), with the inherent contribution subtracted. Unfortunately, the Rh complex of 1 did not display obvious Cotton effect at 350 nm, so the absolute configuration of C-2 was not determined.

Investigation of antiproliferative activities of polyketides 1–9 was carried out using an MTT assay on three human cancer cell lines: A549, MGC-803 and HL-60. Compound 1 exhibited moderate inhibitory activity against A549 and HL-60 cells with IC$_{50}$ values of 67.1 and 11.5 µM, respectively, and almost no activity against the MGC-803 cell line (IC$_{50}>100$ µM). The unique side chain may contribute to its moderate activities. Compounds 2–4 and 6–9 showed no obvious antiproliferative activities (IC$_{50}>100$ µM) against the three selected human cancer cells. However, compound 5 displayed significant in vitro antiproliferative activity against A549, HL-60 and MGC-803 cell lines with IC$_{50}$ values of 2.8, 0.8 and 2.2 µM, respectively. We supposed that the double bond at C-2' and C-3' may affect the activity, and the cyclisation mode of compound 5 may also contribute to significant activity. The morphological changes of HL-60 cells upon different concentration of 5 were observed (see Figure S8 in supplementary material). Moreover, dimeric naphthopyrones have a broad-range of biological activities such as cytotoxic, antitumour and antimicrobial properties. Among eight known polyketides 2–9, isoaurasperone A (2), fonsecinone A (3) and asperpyrone (7) showed antimicrobial activities according to the literature (Lu et al. 2014).

3. Experimental section

3.1. General experimental procedures

The 1D and 2D NMR spectra were recorded on a Bruker ARX-300 or AV-600 NMR spectrometer, with TMS as an internal standard. HR-ESI-MS was recorded on a Varian QFT-ESI mass spectrometer. Semi-preparative high-performance liquid chromatography (HPLC) was carried out using an RP-C18 column (YMC ODS-A, 10 mm × 250 mm, 5 µm), a Hitachi L-2130 pump and a Hitachi L-2400 UV detector. The chromatographic silica gel (200–300 mesh) was produced by Qingdao Haiyang Chemical Co. Ltd., China and Sephadex LH-20 was purchased from GE Healthcare. ODS (50 µm) was produced by YMC Co. Ltd, Japan.

3.2. Fungal material

The fungal strain, A. niger 2HL-M-8, was isolated from the mud sample collected on the intertidal zone in Huludao coastline, Liaoning Province, China, in 2011. The fungus was identified by the biological test and ITSrDNA sequencing test of strains (Samson et al. 2014).

3.3. Fermentation and extraction

The fungus was cultivated in the condition of stilling culture at room temperature. The liquid medium was composed of 20.0 g D-mannitol, 20.0 g D-glucose, 5.0 g yeast extract, 10.0 g peptone, 0.5 g KH$_2$PO$_4$, 0.3 g MgSO$_4$, 1.0 g corn syrup and 33 g sea salt, which were dissolved in 1000 mL of distilled water. After 28 days of cultivation, the fermented broth (60 L) was filtered through cheesecloth to be separated into the supernatant and the mycelia. The supernatant was concentrated to 10 L, thereafter fractionated with EtOAc and $n$-BuOH successively. The mycelia were extracted with acetone under ultrasonic condition and desalted by methanol. The EtOAc
3.4. Separation and purification

The combined crude extract was applied to a CC on silica gel and eluted using petroleum ether: acetone (100:0 to 100:100) gradient system to give nine fractions. Fraction 5 (100:30) was recrystallised with MeOH and subjected to semi-preparative HPLC eluted with 80% methanol in water to yield compounds 2 \((t_R = 21 \text{ min}, 6.5 \text{ mg})\), 6 \((t_R = 27 \text{ min}, 3.2 \text{ mg})\), 4 \((t_R = 24 \text{ min}, 5.7 \text{ mg})\) and 7 \((t_R = 30 \text{ min}, 4.4 \text{ mg})\). Fraction 7 (100:30 to 100:50) was subjected to Sephadex LH-20 CC and semi-preparative HPLC eluted with 73% methanol in water to yield compounds 5 \((t_R = 22 \text{ min}, 3.2 \text{ mg})\) and 8 \((t_R = 29 \text{ min}, 3.0 \text{ mg})\). Further semi-preparative HPLC to the subfraction \((t_R = 16 \text{ min})\) eluted with 70% methanol in water yielded 3 \((t_R = 15 \text{ min}, 4.2 \text{ mg})\).

3.4.1. Aurasperone H \((1)\)

Yellow amorphous powder; HR-ESI-MS \(m/z: 615.1861 \ [M + H]^+ \) (calcd for C\(_{34}H_31O_{11}\) 615.1860). \(^1\)H NMR data (CDCl\(_3\), 600 MHz), \(\delta: 15.21 (1\text{H}, s, 5'-\text{OH}), 14.76 (1\text{H}, s, 5-\text{OH}), 7.16 (1\text{H}, s, H-10), 7.00 (1\text{H}, s, H-9), 6.43 (1\text{H}, d, J = 2.1 \text{ Hz}, H-7'), 6.26 (1\text{H}, d, J = 2.1 \text{ Hz}, H-9'), 6.06 (1\text{H}, s, H-3), 6.03 (1\text{H}, s, H-3'), 4.03 (3\text{H}, s, 6'-OCH\(_3\)), 3.96 (1\text{H}, m, H-2'), 3.80 (3\text{H}, s, 8-\text{OCH}_3), 3.66 (3\text{H}, s, 8'-\text{OCH}_3), 2.89 (1\text{H}, d, J = 5.3 \text{ Hz}, 2''-\text{OH}), 2.53 (2\text{H}, m, H-1'), 2.42 (3\text{H}, s, 2-\text{CH}_3), 1.09 (3\text{H}, d, J = 6.2 \text{ Hz}, H-3'). \(^{13}\)C NMR data (CDCl\(_3\), 150 MHz), \(\delta: 184.5 (\text{C}-4'), 184.4 (\text{C}-4), 168.1 (\text{C}-2'), 167.6 (\text{C}-2), 162.9 (\text{C}-5'), 161.9 (\text{C}-5), 161.5 (\text{C}-8'), 161.1 (\text{C}-6'), 159.8 (\text{C}-8), 158.3 (\text{C}-6), 153.4 (\text{C}-10a), 150.6 (\text{C}-10'a), 140.5 (\text{C}-9'a), 140.4 (\text{C}-9a), 117.3 (\text{C}-7), 111.1 (\text{C}-5a), 108.6 (\text{C}-3'), 108.4 (\text{C}-5'a), 107.4 (\text{C}-3), 104.8 (\text{C}-10'), 104.8 (\text{C}-4a), 104.6 (\text{C}-4'a), 101.7 (\text{C}-9), 101.3 (\text{C}-10), 96.9 (\text{C}-7'), 96.5 (\text{C}-9'), 64.6 (\text{C}-2''), 62.2 (6'-\text{OCH}_3), 56.2 (6'-\text{OCH}_3), 55.9 (8-\text{OCH}_3), 55.2 (8'-\text{OCH}_3), 43.6 (\text{C}-1''), 22.9 (\text{C}-3''), 20.8 (2-\text{CH}_3).\) CD (CDCl\(_3\)) \(\lambda_{\text{max}}: 288 \text{ nm} (\Delta\varepsilon = -6.66), 272 \text{ nm} (\Delta\varepsilon = +6.43).\)

3.5. In vitro MTT assay

The MTT assay (Mosmann 1983) was employed in in vitro cytotoxicity assay, which was performed in 96-well plates. A549 cells at the log phase of their growth cycle \((5 \times 10^5 \text{ cells mL}^{-1})\) were added to each well \((100 \mu\text{L per well})\) and treated in three replicates at various concentrations of the samples. The cells were then incubated for 24 h at 37°C in a humidified atmosphere of 5% CO\(_2\). After 72 h, 20 \(\mu\text{L}\) of MTT solution \((5 \text{ mg mL}^{-1})\) per well was added to each cultured medium before incubating for another 4 h. Dimethyl sulfoxide (DMSO) was then added to each well \((150 \mu\text{L per well})\). After 10 min at room temperature, the OD of each well was measured using a microplate reader (Bio-Rad 550) at a wavelength of 490 nm. In these experiments, the negative reference agent was 0.1% DMSO, and paclitaxel (Tai Chi Pharmaceutical Co., Ltd., Chongqing, China) was used as the positive reference at a concentration of 10 mg mL\(^{-1}\). The same method was used for HL-60 and MGC-803 cell lines.

4. Conclusion

In conclusion, the chemical investigation of the culture broth of marine-derived fungus \(A. \text{niger} \ 2HL-M-8\) led to the isolation of one novel \((1)\) and eight known naphthopyrones \((2-9)\). The structures of these compounds were elucidated on the basis of their spectroscopic data.
(1D, 2D NMR and CD) and by comparison with the literature data. The result of in vitro antiproliferative activity assay indicated that compound 1 showed moderate activity against A549 and HL-60 human cancer cell lines. Compound 5 exhibited potent in vitro antiproliferative activities against all tested cell lines (A549, HL-60 and MGC-803), which deserves further investigation.

Supplementary material
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No potential conflict of interest was reported by the authors.

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Note
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