

A New Azaphilone Derivative from the Co-culture of *Aspergillus versicolor* and *Aspergillus chevalieri*

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Abstract: A new azaphilone derivative, namely aspergiphilone A (**1**), along with seven known compounds including lunatinin (**2**), (2*S*,3*S*)-5,6-dihydroxy-2,6-dimethyl-3-(2-oxopentyl)-2-cyclohexen-1-one (**3**), sterigmatocystin (**4**), gyantrypine (**5**), cottoquinazoline A (**6**), preechinulin (**7**), and (–)-neoechinulin (**8**), was obtained from co-cultures of two marine-derived fungi, *Aspergillus versicolor* IMB17-055 and *A. chevalieri* IMB18-208. Their structures were elucidated through MS, 1D, and 2D NMR analyses, combined with ECD calculations. Their inhibitory activity against mycobacterial CYP121, as well as their antimicrobial and cytotoxic activities, has been reported.

Keywords: *Aspergillus*; azaphilone analogue; co-cultivation; secondary metabolites; marine fungus. © 2025 ACG Publications. All rights reserved.

1. Fungal Source

The fungal strains *Aspergillus versicolor* IMB17-055 and *A. chevalieri* IMB18-208 were isolated from marine sediments collected from a mangrove swamp in Sanya, Hainan province, China (109°51'08.0"E, 18°24'09.0"N, –1 m in depth) and Xieyang Island, Guanxi province, China, (109°16'01.0"E, 20°54'56.0"N, –20 m in depth), respectively. The fungi were identified based on their morphological characteristics and internal transcribed spacer (ITS) gene sequences (GenBank accession nos. MN294468 and MN294469). The strains were deposited in the National Laboratory for Screening Microbial Drugs, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences.

2. Previous Studies

Secondary fungal metabolites serve as rich sources for drug discovery [1]. Over the past few decades, approximately 30,000 natural products have been identified from fungi [2], including

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historically important drugs, such as penicillin, cephalosporin, lovastatin, and echinocandin [1-3]. However, recent studies have suggested that many biosynthetic gene clusters in microorganisms remain silent or are expressed at low levels under conventional laboratory conditions [4]. The co-cultivation of microorganisms is a natural strategy that can mimic complex microbial communities and interspecies communication. This approach has been proven effective in discovering novel bioactive secondary metabolites by activating the expression of cryptic biosynthetic gene clusters [4-5]. In our previous work, the co-cultivation of two marine-derived fungi, *A. versicolor* IMB17-055 and *A. chevalieri* IMB18-208, revealed the presence of new metabolites that were not detected in their individual monocultures, leading to the discovery of a class of novel antifungal antibiotics, namely burnetramic acids A–E, from the bioactive fractions of the extracts [6]. Other metabolites from the co-cultures of the two *Aspergillus* species have not been fully explored.

3. Present Study

A further chemical investigation revealed that a new azaphilone derivative aspergiphilone A (**1**), along with seven known compounds including lunatinin (**2**), (2*S*,3*S*)-5,6-dihydroxy-2,6-dimethyl-3-(2-oxopentyl)-2-cyclohexen-1-one (**3**), sterigmatocystin (**4**), glyantrypine (**5**), cottoquinazoline A (**6**), preechinulin (**7**) and (–)-neoechinulin (**8**) were obtained from the polar fractions of the co-culture extracts of *A. versicolor* IMB17-055 and *A. chevalieri* IMB18-208.

Co-cultivation, fermentation, and extraction were performed as previously described [6]. The obtained extracts (54 g) were subjected to flash chromatography over a reversed-phase (RP) C₁₈ using a stepwise gradient of MeOH-H₂O (10–100%) to yield 14 fractions (F₁–F₁₄). Fraction F₇, eluted by 70% MeOH, was chromatographed on a Sephadex LH-20 column using MeOH-H₂O (9:1). Further purification by preparative C₁₈ HPLC (Capcell PAK C18 MGII 5 μM, 20 mm × 250 mm) with 40% MeOH containing 0.1% formic acid yielded compounds **1** (1 mg), **2** (1 mg), **5** (1.2 mg), **6** (1 mg), **7** (0.5 mg), and **8** (1 mg). Fractions F₈ and F₉, eluted with 70% MeOH, were chromatographed on a Sephadex LH-20, eluted with MeOH-H₂O (8:2). Further purification by preparative C₁₈ HPLC with 40% MeCN-H₂O containing 0.1% formic acid yielded **4** (30 mg). Fraction F₃, eluted with 50% MeOH, was separated using a Sephadex LH-20 column, eluted with MeOH-H₂O (8:2). Subsequently, it was purified using preparative C₁₈ HPLC with 25% MeCN-H₂O containing 0.1% formic acid to give **3** (9 mg).

Aspergiphilone A (1): $[\alpha]_D^{20}$ –21.0 (*c* 0.06, MeOH); UV (MeOH) λ_{\max} (log ϵ) 380 (4.11), 289 (3.78); ECD (*c* 1.48 × 10^{–3} M, MeOH) λ_{\max} ($\Delta\epsilon$) 216 (–0.34), 236 (+0.21), 264 (–0.27), 297 (+0.21), 333 (–0.25), 380 (+0.63) nm; ¹H NMR (DMSO-*d*₆, 600 MHz) and ¹³C NMR (DMSO-*d*₆, 150 MHz) data are shown in Table S1. HR-ESI-MS: *m/z* 269.0577 [M + H]⁺ (calculated for C₁₃H₁₄ClO₄: 269.0575).

DFT Calculation: A conformational search for **1** was carried out using Spartan 14 software with a MMFF94 force field. The geometry of the obtained conformers was optimized at the B3LYP/6-31+G (d, p) level of theory in gas using the Gaussian 09 program. The optimized conformers within 2 kcal/mol were subjected to ECD calculations using a TD-DFT methodology at the CAM-B3LYP/TZVP level in MeOH using the PCM model. The ECD spectrum of each conformer was simulated using SpecDis [7] for a band width of 0.30 eV. The final ECD spectra were generated by averaging the calculated data of the lowest-energy conformers for each structure according to their Boltzmann distribution. The ¹³C NMR shielding constants of **1** were computed using the GIAO method at the B3LYP/6-31 G (2d, p) level with the PCM solvent continuum model in DMSO-*d*₆. [8]

Biological Activity Assay: The enzyme inhibition assay of *Mycobacterium tuberculosis* CYP121 was performed in 100 μL of a PBS buffer (pH 7.2). Compounds **1–5** (50 μM) were incubated with 10 μM CYP121 for 30 min at 37 °C. After incubation, an electron transfer system—comprising 10 μM Ferredoxin reductase, 10 μM Ferredoxin, and 1.2 mM NADPH—was added. The reaction was started with the addition of 400 μM cyclo(L-Tyr-L-Tyr) (cYY) and stopped after 40 min by adding 100 μL of

MeOH with chloramphenicol (final concentration 100 μM) as the internal standard. Econazole was used as a positive control. The reaction mixture was subjected to a LC-MS analysis using water containing 0.1% formic acid (A) and acetonitrile (B) as the solvent, at a flow rate of 0.3 $\text{mL}\cdot\text{min}^{-1}$. The extracts were eluted using a linear gradient from 5% to 95% B for 10 min and washed with 95% solvent B for 2 min. The inhibition rate was calculated by quantifying the ratio of the peak areas of the products.

The minimum inhibitory concentrations (MIC) of compounds **1–3** against *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 29213, and *Escherichia coli* ATCC 25922 were determined under the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [9]. The concentrations of compounds ranged from 128 to 0.0625 $\mu\text{g}/\text{mL}$. Amphotericin B and amikacin were used as positive controls for fungi and bacteria, respectively. Cytotoxicities against human colon cancer HCT8, non-small cell lung carcinoma H460, and pancreatic cancer Hup-T3 cell lines, were evaluated using the CCK8 assay method as described previously [6].

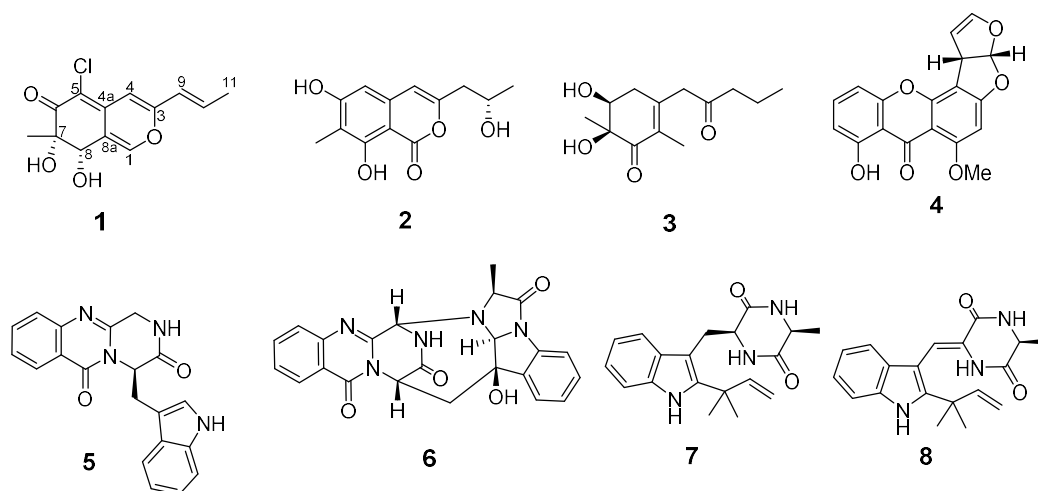


Figure 1. Chemical structures of compounds **1–8**

Aspergiphilone A (**1**) was isolated as a yellow, amorphous powder. The molecular formula of **1** was deduced as $\text{C}_{13}\text{H}_{13}\text{ClO}_4$ on the basis of its HR-ESI-MS data (m/z 269.0577 $[\text{M}+\text{H}]^+$, Figure S1) with a characteristic monochlorinated isotopic pattern (abundance ratio of 3:1). The ^1H NMR spectrum (Figure S2) displayed the presence of four olefin proton signals [δ_{H} 7.52 (1H, s); 6.57 (1H, dq, $J = 16.2, 7.2$ Hz); 6.51 (1H, m); and 6.39 (1H, d, $J = 16.2$ Hz)], an oxygenated methine proton signal [δ_{H} 4.34 (1H, s)], and two methyl proton signals [δ_{H} 1.89 (3H, d, $J = 7.2$) and 1.09 (3H, s)]. The ^{13}C NMR and HSQC spectra of **1** (Figures S3–S4) revealed the presence of 13 carbon signals, including one carbonyl signal (δ_{C} 190.9), eight sp^2 carbons (δ_{C} 103.4–156.8), one oxygenated sp^3 quaternary carbon signal (δ_{C} 76.1), one sp^3 oxymethine signal (δ_{C} 70.8), and two methyl groups (δ_{C} 19.2, 18.3). The UV and NMR data (Table S1) were similar to those of isochromophilone V [10], indicating that **1** is an azaphilone analog. An analysis of the ^1H - ^1H COSY correlations (Figure S5) revealed a spin system for the propenyl moiety. In HMBC spectrum (Figures S6–S7), the correlations of H-1 (δ_{H} 7.52) with C-3, C-4a, and C-8a, along with those of H-4 (δ_{H} 6.51) with C-3 and C-8a, combined with the chemical shifts of these carbons indicated the presence of a dihydropyran ring. In addition, the HMBC correlations of H-8 (δ_{H} 4.32) with C-1, C-4a, C-6, and C-7; those of the methyl singlet at δ_{H} 1.09 with C-6, C-7, and C-8; and that of H-4 (δ_{H} 6.51) with C-5 indicated the existence of 7,8-dihydro-isochromene-6-one core structure. Finally, the HMBC correlation of H-9 with C-3 and C-4 confirmed that the propenyl moiety was substituted at C-3 of the isochromene ring.

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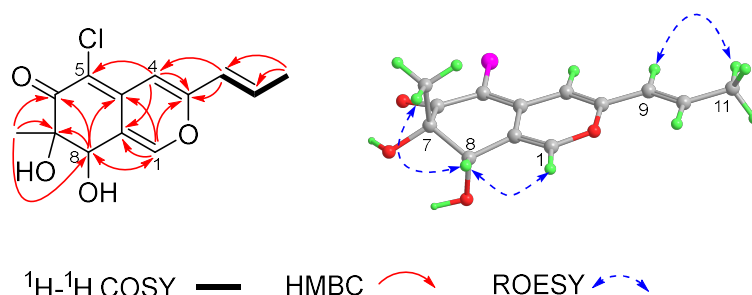


Figure 2. Key ^1H - ^1H -COSY, HMBC, and ROESY correlations of **1**

In the ROESY spectrum, the correlation between H-8 and CH₃-7 indicated that they were oriented on the same side of the ring (Figure S8). The *cis* relative configuration between C-7 and C-8 was further confirmed by the GIAO NMR calculation (Table S4 and Figure S12–S13). The large coupling constants of H-9 and H-10 ($J = 16.2$ Hz) suggest a *trans* geometry for the double bond of the propenyl group. To determine the absolute configuration of compound **1**, two possible enantiomers ((*7S*, *8S*-**1**) and (*7R*, *8R*-**1**)) were subjected to ECD calculations using the TD-DFT method. As shown in Figure 3, the experimental ECD spectrum of **1** was similar to that calculated for the isomers with *7S* and *8S* configurations. Therefore, the absolute configurations of **1** were determined to be *7S* and *8S*. Consequently, the structure of **1** was determined and named as aspergiphilone A.

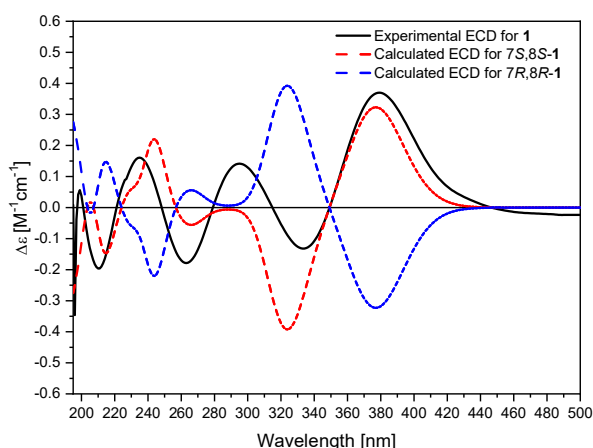


Figure 3. Experimental and calculated ECD spectra of **1**

The known compounds (**2–8**) were identified by comparing the MS, NMR, and UV data (Supporting Information) with those reported in literature [11–18].

There is a critical need for new antitubercular drugs with novel modes of action owing to the emergence of multi- and pan-drug-resistant *Mycobacterium tuberculosis* [19]. The cytochrome P450 enzyme CYP121 has recently been regarded as an attractive target for anti-TB therapeutics [20–21]. Compounds **1–5** were evaluated for their inhibitory activity against CYP121, and the results showed that they were inactive at a concentration of 50 μM . Compounds **1–3** were also tested for their antimicrobial activities against *S. aureus*, *E. coli*, and *C. albicans* and cytotoxicities against the human cancer cell lines H460, HCT8, and Hup-T3. However, all compounds exhibited no inhibitory activities ($\text{MIC} > 128 \mu\text{g}\cdot\text{mL}^{-1}$ or $\text{IC}_{50} > 100 \mu\text{M}$).

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Supporting Information

Supporting Information accompanies this paper on <http://www.acgpubs.org/journal/records-of-natural-products>

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