New Dihydroisocoumarin Root Growth Inhibitors From the Sponge-Derived Fungus Aspergillus sp. NBUF87

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Six new dihydroisocoumarins, aspergimarins A–F (1–6), were discovered together with five known analogs (7–11) from a monoculture of the sponge-derived fungus Aspergillus sp. NBUF87. The structures of these compounds were elucidated through comprehensive spectroscopic methods, and absolute configurations were assigned after X-ray crystallography, use of the modified Mosher’s method, and comparison of electronic circular dichroism (ECD) data with literature values for previously reported analogs. Compounds 1–11 were evaluated in a variety of bioassays, and at 100 µM, both 1 and 5 showed significant inhibitory effects on the lateral root growth of Arabidopsis thaliana Columbia-0 (Col-0). Moreover, at 100 µM, 5 also possessed notable inhibition against the primary root growth of Col-0. Meanwhile, 1–11 were all found to be inactive in vitro against acetylcholinesterase (AChE) (IC₅₀ > 100 µM), four different types of human-derived cancer cell lines (IC₅₀ > 50 µM), as well as methicillin-resistant Staphylococcus aureus and Escherichia coli (MIC > 50 µg/mL), and Plasmodium falciparum W2 (EC₅₀ > 100 µg/mL), in phenotypic tests.

Keywords: dihydroisocoumarin, root growth inhibitor, sponge-derived fungus, Aspergillus sp., electronic circular dichroism

INTRODUCTION

Sponges are among the most primitive multicellular invertebrates and harbor vast microbial populations, owing largely to the unique filter-feeding physiology that is full of pores and channels (Hentschel et al., 2006; Webster and Taylor, 2012). As a result of the long-standing interaction and coevolution with sponges, marine symbiotic microorganisms have differentiated from those of terrestrial origins in terms of their biosynthetic pathways that lead to the production of structurally interesting and biologically active compounds (Thomas et al., 2010; Fan et al., 2012). Therefore, sponge-associated microbes have become an exciting area of drug discovery research (Thomas et al., 2010; Pita et al., 2016). Endophytic fungi that are associated with sponges, especially members of the genus Aspergillus, have been recognized as a source of structurally diverse natural products with...
biological activities that provide value for drug discovery (Blunt et al., 2018; Zhang et al., 2018). In the past decade, the secondary metabolites from sponge-derived Aspergillus fungi have been reported from many classes, including polyketides (Wang et al., 2014; Kong et al., 2015), terpenoids (Liu et al., 2009; Li D. et al., 2012), alkaloids (Zhou et al., 2013, 2014), diketopiperazines (Ahmed et al., 2017), and peptides (Lee et al., 2011). Many of these metabolites have been shown to exhibit strong antitumor, antibacterial, antiviral, and other bioactivities.

Isocoumarins and 3,4-dihydroisocoumarins, subclasses of polyketide compounds, are also lactone-containing natural products that are abundantly produced among fungi, bacteria, liverworts, lichens, as well as some higher plants (Elsebai and Ghabbour, 2016; Saeed, 2016; Hussain and Green, 2017; Chen M. et al., 2019). Moreover, these compounds have been isolated from marine sponges, insect pheromones, and venoms (Saeed, 2016). Almost 400 isocoumarins and dihydroisocoumarins have been reported to date, and these compounds have been found to be of broad interest across many pharmacological applications (Saeed, 2016; Chen M. et al., 2019). For example, isocoumarin derivatives from some marine-derived fungi are found to possess a wide range of biological properties including enzyme inhibitory (Kim et al., 2015; Chen S. et al., 2016; Wieze et al., 2016; Cai et al., 2018), cytotoxic (Wang et al., 2019; Wu et al., 2019), antibacterial (Li S. et al., 2012; Lei et al., 2017; Chen Y. et al., 2018; Wang et al., 2019), antiproliferative (Tsukada et al., 2011), anti-food allergic (Niu et al., 2018), as well as anti-inflammatory (Kim et al., 2015; Chen Y. et al., 2018; Liu et al., 2018) activities.

As part of a continuing research program investigating the biologically active secondary metabolites from sponge-derived fungi (Ding et al., 2018; Huang et al., 2019; Li W. et al., 2019), a detailed chemical investigation was initiated on the culture of fungus Aspergillus sp. NBUF87. The fungus was isolated from a South China Sea marine sponge of the genus Hymeniacidon. The EtOAc extract of the culture of fungus Aspergillus sp. NBUF87 exhibited inhibitory effects on the root growth of Arabidopsis thaliana Columbia-0 (Col-0), a typical model organism for studying plant growth and development. The separation and purification of the bioactive extract led to the discovery of six new dihydroisocoumarin compounds (1–6) and five known analogs (7–11) (Figure 1). Herein, the detailed isolation and structure elucidation of these dihydroisocoumarin derivatives, together with the evaluation of their inhibitory effects against the root growth of Col-0 and a preliminary broader biological activity screening are described.

**MATERIALS AND METHODS**

**General Experimental Procedures**

Optical rotation measurements were conducted with a JASCO P-2000 digital polarimeter. IR and UV spectra were obtained with a Thermo Scientific Nicolet iS5 FT-IR spectrometer and a Thermo Scientific Evolution 201 spectrophotometer, respectively. Electronic circular dichroism (ECD) spectra were collected on a JASCO J-1500 spectrophotometer. 1D and 2D NMR spectra were recorded in DMSO-<sup>d</sup><sub>6</sub> (or CDCl<sub>3</sub>) with a Palo Alto Varian 600 MHz spectrometer, using standard pulse sequences. HRESIMS data were collected on an Agilent Technologies 6224 TOF MS. X-ray single-crystal diffraction data were acquired using an Agilent Gemini Ultra diffractometer with Cu Kα radiation (λ = 1.54178 Å). Medium-pressure liquid chromatography (MPLC) was performed using a Bonna-Agela FLEXA purification instrument. Column chromatography (CC) was carried out with silica gel (200–300 mesh, Qingdao) and Amersham Biosciences Sephadex LH-20. Reversed-phase HPLC (RP-HPLC) was conducted using a Waters 1525 binary HPLC pump equipped with a Waters 2996 photodiode array detector and a YMC-Pack C18 column (YMC, 20 × 250 mm, 5 μm).

**Fungal Material**

The fungus Aspergillus sp. NBUF87 was isolated from the sponge Hymeniacidon sp. obtained from the Paracel Islands in the South China Sea, and was determined as being Aspergillus sp. by its morphology and gene sequence (ITS rDNA region) analyses (GenBank accession no. MH595747.1). The strain specimen was deposited in PDB medium to the repository conserved at the College of Food and Pharmaceutical Sciences, Ningbo University, China.

**Fermentation, Extraction, and Isolation**

Spores of Aspergillus sp. NBUF87 were initially inoculated into Erlenmeyer flasks (1 L) containing 400 mL of the seed medium (potato dextrose broth powder 26 g/L and sea salt 35 g/L dissolved in distilled water) and were grown on a shaker (150 r/min) for 100 h at 28°C. The subsequent amplified fermentation was conducted in 105 × 1 L Erlenmeyer flasks, each containing solid rice medium (rice 120 g, sea salt 6.3 g, purified H₂O 180 mL), followed by inoculation with 30 mL of the seed culture. After fermentation at room temperature under static conditions for 6 weeks, the fermented substrate was repeatedly extracted with EtOAc. Removal of EtOAc under reduced pressure yielded 54 g of crude extract, which was subjected to vacuum liquid chromatography (VLC) on silica gel column eluting with a petroleum ether/EtOAc stepwise gradient system (from 1:0 to 1:1) to generate seven fractions (Fr. 1–7).

Fraction 4 (3.9 g) was subjected to Sephadex LH-20 gel filtration chromatography, eluted with isocratic CH₂Cl₂/CH₃OH (1:1, v/v) to furnish five sub-fractions (Fr. 4A–4F). The further separation of Fr. 4E (1.8 g) was conducted by reversed-phase ODS MPLC with a gradient elution of CH₂OH/H₂O (from 25 to 100% CH₂OH, flow rate 20 mL/min, 180 min, UV detection at 210 nm) to afford 60 test tube sub-fractions. Subsequently, aspergimarin A (1, 15.3 mg, t<sub>R</sub> 33.4 min) was purified from Fr. 4E-20 by semi-preparative RP-HPLC [YM-C-Pack C18 column (YM-C, 20 × 250 mm, 5 μm), UV detection at 210 and 246 nm] with 28% CH₃CN/H₂O at 2 mL/min. Aspergimarin E (5, 3.6 mg, t<sub>R</sub> 40.2 min), compounds 10 (4.3 mg, t<sub>R</sub> 72.3 min) and 11 (4.7 mg, t<sub>R</sub> 58.2 min) were purified from Fr. 4E-19 by semi-preparative RP-HPLC with 24% CH₃CN/H₂O at 2 mL/min. Aspergimarin F (6, 3.4 mg, t<sub>R</sub> 57.3 min) was purified from Fr. 4E-18 by semi-preparative RP-HPLC with 24% CH₃CN/H₂O at 2 mL/min. Fr. 4B (1.0 g) was conducted by MPLC (30–90% CH₂OH/H₂O, flow rate 20 mL/min, 120 min, UV detection at 210 nm) to produce 10
Fraction 5 (4.8 g) was separated by Sephadex LH-20 gel filtration chromatography utilizing an isocratic elution gradient of CH$_2$Cl$_2$—CH$_3$OH (1:1, v/v) to afford three sub-fractions (Fr. 5A–5C). The further separation of Fr. 5B (2.5 g) was conducted by RP-MPLC (ODS, 15–100% CH$_3$OH/H$_2$O, flow rate 20 mL/min, 140 min, UV detection at 210 nm) to give 35 tubes. Aspergimarin B (2, 2.5 mg, t$_R$ 31.4 min) and aspergimarin C (3, 3.8 mg, t$_R$ 22.9 min) were further purified from Fr.5B-23 by semi-preparative RP-HPLC with 30% CH$_3$CN/H$_2$O at 4 mL/min. Fr. 5A (0.9 g) was separated by MPLC (ODS, 25–100% CH$_3$OH/H$_2$O, flow rate 20 mL/min, 150 min, UV detection at 210 nm) to obtain 38 tubes. Fr.5A-15 was further purified by semi-preparative RP-HPLC with 25% CH$_3$CN/H$_2$O at 4 mL/min to afford aspergimarin D (4, 6.3 mg, t$_R$ 31.0 min). Fr. 5A-14 was purified by semi-preparative RP-HPLC with 22% CH$_3$CN/H$_2$O at 4 mL/min to afford compound 8 (26.1 mg, t$_R$ 31.6 min) (Supplementary Figures S11–S40).

Aspergimarin A (1): white, crystals; mp 177.9–178.8 °C; [α]$_D$ 34.0 (c 0.2, CHCl$_3$); UV (MeOH) $\lambda_{max}$ (log ε): 219 (4.14), 248 (3.77), 346 (3.58) nm; CD (c = 0.68 mM, MeOH) $\lambda_{max}$ (Δε): 223 (+ 2.15), 237 (−1.93), 245 (−1.10), 259 (−6.32) nm; IR (KBr) $\nu$max: 3205, 1648, 1483, 1354, 1281, 820, 795, 700 cm$^{-1}$; $^1$H and $^{13}$C NMR data, see Table 1; HRESIMS m/z 265.1082 [M – H]$^–$ (calcd for C$_{14}$H$_{12}$O$_5$, 265.1081).

Aspergimarin B (2): brown, oil; [α]$_D$ 20.6 (c 0.2, MeOH); UV (MeOH) $\lambda_{max}$ (log ε): 211 (4.24), 245 (3.66) nm; CD (c = 0.33 mM, MeOH) $\lambda_{max}$ (Δε): 225 (+ 0.88), 235 (−1.54), 243 (−0.81), 257 (−5.77) nm; IR (KBr) $\nu$max: 3226, 2929, 1723, 1675, 1469, 1379, 1207, 829 cm$^{-1}$; $^1$H and $^{13}$C NMR data, see Table 1; HRESIMS m/z 365.1232 [M – H]$^–$ (calcd for C$_{18}$H$_{12}$O$_8$, 365.1242).

Aspergimarin C (3): brown, oil; [α]$_D$ 24.5 (c 0.3, MeOH); UV (MeOH) $\lambda_{max}$ (log ε): 219 (4.27), 344 (3.63) nm; CD (c = 0.45 mM, MeOH) $\lambda_{max}$ (Δε): 229 (+ 0.86), 238 (−1.44), 243 (−0.32), 257 (−5.37) nm; IR (KBr) $\nu$max: 3368, 2921, 1671, 1469, 1378, 1287, 1206, 1123 cm$^{-1}$; $^1$H and $^{13}$C NMR data, see Table 1; HRESIMS m/z 435.1416 [M + K]$^+$ (calcd for C$_{20}$H$_{28}$KO$_8$, 435.1415).

Aspergimarin D (4): yellow, oil; [α]$_D$ 22.2 (c 0.5, MeOH); UV (MeOH) $\lambda_{max}$ (log ε): 214 (4.16), 312 (3.24) nm; CD (c = 0.68 mM, MeOH) $\lambda_{max}$ (Δε): 221 (−6.98), 241 (−0.28), 256 (−2.47), 284 (−0.48), 311 (−1.61) nm; IR (KBr) $\nu$max: 3402, 2935, 1717, 1489, 1455, 1418, 1259, 1131, 1052, 963 cm$^{-1}$; $^1$H and $^{13}$C NMR data, see Table 2; HRESIMS m/z 295.1537 [M + H]$^+$ (calcd for C$_{14}$H$_{18}$O$_5$, 295.1540).

Aspergimarin E (5): colorless, oil; [α]$_D$ 24.5 (c 0.3, MeOH); UV (MeOH) $\lambda_{max}$ (log ε): 209 (4.17), 246 (3.52), 312 (3.42) nm; CD (c = 0.56 mM, MeOH) $\lambda_{max}$ (Δε): 206 (−8.37), 218 (−0.47), 224 (−1.04), 243 (± 3.53), 264 (−0.71), 280 (± 0.01), 311 (−0.53) nm; IR (KBr) $\nu$max: 3367, 2920, 1672, 1461, 1230, 1117, 823 cm$^{-1}$; $^1$H and $^{13}$C NMR data, see Table 2; HRESIMS m/z 289.1034 [M + Na]$^+$ (calcd for C$_{13}$H$_{18}$NaO$_5$, 289.1046).

Aspergimarin F (6): yellow, amorphous powder; [α]$_D$ 24.5 (c 0.2, MeOH); UV (MeOH) $\lambda_{max}$ (log ε): 221 (4.14), 256 (3.71), 332 (3.44) nm; CD (c = 0.57 mM, MeOH) $\lambda_{max}$ (Δε): 208 (−8.75), 240 (± 2.67), 266 (−1.47) nm; IR (KBr) $\nu$max: 3360, 2920, 2849, 2361, 1707, 1664, 1452, 1272, 1135, 668 cm$^{-1}$; $^1$H and $^{13}$C NMR data, see Table 2; HRESIMS m/z 287.0879 [M + Na]$^+$ (calcd for C$_{14}$H$_{16}$NaO$_5$, 287.0890).

**X-Ray Crystal Structure Analysis of 1**

A single crystal of 1 was obtained from 90% CH$_3$OH/H$_2$O. Crystal X-ray diffraction data was collected on an Agilent Gemini Ultra diffractometer with Cu Kα radiation (λ = 1.54178 Å). The structure was solved by direct methods (SHELXS–97) and refined with full-matrix least-squares difference Fourier techniques. All non-hydrogen atoms were refined anisotropically.
and hydrogen atoms were placed in the idealized geometrical positions and refined isotropically with a riding model. Crystallographic data for 1 have been deposited in the Cambridge Crystallographic Data Center as supplementary publication no. CCDC 1919904. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, United Kingdom (fax: +44-(0)1223-336033, or e-mail: deposit@ccdc.cam.ac.uk).

Crystal data of aspergimarin A (1): C14H18O5, Mr = 266.28, monoclinic, a = 4.9195(3) Å, b = 24.7343(17) Å, c = 5.6478(4) Å, α = γ = 90°, β = 101.812(2)°, V = 672.92(8) Å³, space group P2₁, Z = 2, Dc = 1.314 mg/m³, μ = 0.829 mm⁻¹, and F(000) = 284. Crystal size: 0.200 × 0.170 × 0.130 mm³. Independent reflections: 2294 (Rint = 0.0377). Final R indices \([I > 2 \sigma(I)]\), R₁ = 0.0717, wR₂ = 0.2076. Goodness of fit on \(F^2\) was 1.109.

### Table 1

| Position | \(\delta_H\) (\(\delta_H\) in Hz) | \(\delta_C\), type | \(\delta_H\) (\(\delta_H\) in Hz) | \(\delta_C\), type | \(\delta_H\) (\(\delta_H\) in Hz) | \(\delta_C\), type |
|----------|-------------------------------|----------------|-------------------------------|----------------|-------------------------------|----------------|
| 1        | 169.5, C                      |                | 169.5, C                      |                | 169.5, C                      |                |
| 3        | 4.59, m                       | 79.4, CH       | 4.59, m                       | 79.2, CH       | 4.59, m                       | 79.2, CH       |
| 4        | 3.06, dd (16.9, 3.4) 2.60, dd | 26.3, CH₂      | 3.05, dd (16.9, 3.4) 2.60, dd | 26.4, CH₂      | 3.05, dd (16.9, 3.4) 2.60, dd | 26.3, CH₂      |
| 4a       | 124.5, C                      |                | 124.0, C                      |                | 124.5, C                      |                |
| 5        | 146.6, C                      |                | 145.7, C                      |                | 145.6, C                      |                |
| 7        | 7.06, d (8.9)                 | 123.9, CH      | 7.06, d (8.9)                 | 124.0, CH      | 7.06, d (8.9)                 | 123.9, CH      |
| 8        | 6.72, d (8.9)                 | 115.1, CH      | 6.72, d (8.9)                 | 115.2, CH      | 6.72, d (8.9)                 | 115.2, CH      |
| 8a       | 153.9, C                      |                | 153.9, C                      |                | 153.9, C                      |                |
| 1'       | 1.78, m 1.68, m               | 34.3, CH₂      | 1.76, m 1.70, m               | 33.9, CH₂      | 1.74, m                       | 33.9, CH₂      |
| 2'       | 1.46, m                       | 20.8, CH₂      | 1.44, m                       | 20.3, CH₂      | 1.46, m                       | 20.3, CH₂      |
| 3'       | 1.36, m                       | 38.6, CH₂      | 1.55, m                       | 34.9, CH₂      | 1.55, m                       | 34.9, CH₂      |
| 4'       | 3.60, m                       | 65.6, CH       | 4.83, m                       | 70.2, CH       | 4.83, m                       | 68.8, CH       |
| 5'       | 1.05, d (6.2)                 | 23.7, CH₃      | 1.16, d (6.3)                 | 19.8, CH₃      | 1.17, d (6.2)                 | 19.8, CH₃      |
| 5′       |                                |                | 171.8, C                      |                | 170.4, C                      |                |
| 2''      |                                |                |                                |                | 2.45, m 1.24, m              | 2.38, m        |
| 3''      |                                |                |                                |                | 2.46, m                       | 29.1, CH₂      |
| 3''-OH   | 10.38, s                      |                | 10.37, s                      |                | 10.37, s                      |                |
| 5''-OH   |                                |                |                                |                | 4.53, s                       |                |
|          |                                |                |                                |                | 4.36, s                       |                |

s, singlet; d, doublet; dd, doublet of doublets; m, multiplet.

Preparation of MTPA Esters of 3–5 for Modified Mosher’s Analysis

Under an atmosphere of nitrogen, pyridine-\(d_5\) (500 μL) and (R)-MTPA-Cl (8 μL) was sequentially added to an EP tube containing compounds 3, 4, or 5 (1.0 mg), separately. The mixture was shaken at 28 °C for 12 h and then purified by RP-HPLC to obtain the (S)-MTPA esters 3a, 4a, and 5a. By the same procedure, the (R)-MTPA esters 3b, 4b, and 5b were obtained using (S)-MTPA-Cl as a reagent. Key \(^1\)H NMR signals used for configurational assignments were determined by respective \(^1\)H—\(^1\)H COSY correlations and the already completed full assignments of \(^1\)H NMR data for 3, 4, and 5 (see Supplementary Figures S61–S72).

The C-3′ absolute configuration of 3 were established as \(R\) on the basis of the \(\Delta\) values (\(\Delta\delta_{H-2,4′} = -0.01\); \(\Delta\delta_{H-2,4′} = +0.03\); \(\Delta\delta_{H-2,5′} = +0.01\)) of the (S)- and (R)-MTPA esters (3a and 3b), 3a: H-2′ (\(\delta_H = 2.37\)), H-4′ (\(\delta_H = 1.89\)), H-5′ (\(\delta_H = 4.45\)); 3b: H-2′ (\(\delta_H = 2.38\)), H-4′ (\(\delta_H = 1.86\)), H-5′ (\(\delta_H = 4.44\)).

The C-4′ absolute configuration of 4 was established as \(S\) on the basis of the \(\Delta\) values (\(\Delta\delta_{H-3,5′} = +0.05\); \(\Delta\delta_{H-3,5′} = -0.01\)) of the (S)- and (R)-MTPA esters (4a and 4b), 4a: H-3′ (\(\delta_H = 1.64, 1.27\)), H-5′ (\(\delta_H = 1.21\)); 4b: H-3′ (\(\delta_H = 1.59, 1.22\)), H-5′ (\(\delta_H = 1.31\)).

Both C-4 and C-4′ absolute configuration of 5 were established as \(S\) on the basis of the \(\Delta\) values (\(\Delta\delta_{H-3,5′} = +0.17\); \(\Delta\delta_{H-3,5′} = -0.19\); \(\Delta\delta_{H-6,5′} = -0.02\); \(\Delta\delta_{H-6,5′} = +0.08\); \(\Delta\delta_{H-6,5′} = +0.13\); \(\Delta\delta_{H-6,5′} = +0.03\); \(\Delta\delta_{H-6,5′} = +0.07\); \(\Delta\delta_{H-6,5′} = +0.08\); \(\Delta\delta_{H-6,5′} = -0.07\)) of the (S)- and (R)-MTPA esters (5a and 5b), 5a: H-3 (\(\delta_H = 4.65\)), H-5 (\(\delta_H = 7.27\)), H-6 (\(\delta_H = 7.63\)), H-1′ (\(\delta_H = 1.66, 1.53\)), H-2′ (\(\delta_H = 1.49\)), H-3′ (\(\delta_H = 1.60, 1.32\)), H-5′ (\(\delta_H = 1.25\)); 5b: H-3 (\(\delta_H = 4.48\)), H-5 (\(\delta_H = 7.46\)), H-6 (\(\delta_H = 7.65\)), H-1′ (\(\delta_H = 1.58, 1.40\)), H-2′ (\(\delta_H = 1.46\)), H-3′ (\(\delta_H = 1.53, 1.24\)), H-5′ (\(\delta_H = 1.32\)).
**TABLE 2** | $^1$H (600 MHz) and $^{13}$C (150 MHz) NMR data of 4–6.

| Position | 4 (DMSO-$d_6$) | 5 (CDCl$_3$) | 6 (DMSO-$d_6$) |
|----------|----------------|----------------|----------------|
| Position | $^1$H (J in Hz) | $^1$C, type | $^1$H (J in Hz) | $^1$C, type | $^1$H (J in Hz) | $^1$C, type |
| 1        | 161.5, C       | 168.7, C       | 169.8, C       |
| 3        | 4.37, m        | 78.1, CH        | 4.47, m        | 83.6, CH        | 4.61, m        | 79.9, CH        |
| 4        | 2.91, dd (16.1, 3.1) | 32.7, CH$_2$ | 4.71, d (7.5) | 67.5, CH        | 2.91, dd (16.2, 3.3) | 31.4, CH$_2$ |
| 4a       | 2.08, s        | 60.7, CH$_3$    | 10.97, s       |

$s$, singlet; $d$, doublet; $dd$, doublet of doublets; $t$, triplet; $m$, multiplet.

**Plant Growth Response Assays**

*Arabidopsis thaliana* Col-0, a model organism for plant growth and development, was used to test each isolated compound according to a previously described protocol (Li X. et al., 2018; Santos et al., 2012). Plants grown in 2% ($v/v$) DMSO were used as the negative control. Seeds of Col-0 incubated in 1 µM of 6-benzylaminopurine (BAP) were selected as the positive control. Test samples were dissolved in 2% ($v/v$) DMSO at various test concentrations for the experiment.

**In vitro AChE Activity Assays**

For compounds 1–11, the *in vitro* acetylcholinesterase (AChE) activity was assessed by the colorimetric method in 96-well plates according to a previously reported method (Santos et al., 2012). Donepezil was selected as the positive control with IC$_{50}$ value of 11.9 nM (Chen H. et al., 2018).

**In vitro Cancer Cell Cytotoxicity Assays**

The *in vitro* cytotoxic activities of all isolated compounds against four human cancer cell lines (CCRF-CEM, MDA-MB-231, HCT-116, and AGS) were evaluated by the MTT method as previously described (Gu et al., 2018). The method described by Gu et al. (2018). Ciprofloxacin was selected as a positive control against the above-mentioned bacteria with MIC values of 0.5 µg/mL.

**In vitro Antimalarial Activity Assays**

The *in vitro* antimalarial activity of the compounds was evaluated against the parasite (*Plasmodium falciparum* W2), which was cultured continuously according to a previously described method (Lazaro et al., 2006). Chloroquine, atovaquone, and artemisinin were used as positive controls against the above parasite with EC$_{50}$ values of 112, 2.5, and 160 nM, respectively.

**RESULTS AND DISCUSSION**

**Structure Elucidation**

Compound 1 was isolated as white crystals. The molecular formula of 1 was determined to be C$_{14}$H$_{18}$O$_5$ based on an ion peak observed in HREIMS spectrum ($m/z$ [M - H]$^-$ 265.1082, calcld for C$_{14}$H$_{17}$O$_5$, 265.1081), and this implied the compound has six degrees of unsaturation. The $^1$H NMR data of 1 (Table 1) demonstrated two aromatic signals ([δ$_H$ 7.07, d, $J = 8.9$ Hz, H-6] and (δ$_H$ 6.72, d, $J = 8.9$ Hz, H-7]) that were suggestive of two ortho protons. In addition, one phenolic hydroxy group at δ$_H$ 10.38 (1H, s, 8-OH), two oxygenated methine groups at δ$_H$ 4.59 (1H, m, H-3) and 3.60 (1H, m, H-4'), four methylene groups at δ$_H$ 3.06 (1H, dd, H-4a), 2.60 (1H, dd, H-4b), 1.78 (1H, m, H-1a), 1.68 (1H, m, H-1b), 1.46 (2H, m, H-2'), and 1.36 (2H, m, H-3'), and one methyl group at δ$_H$ 1.05 (3H, d, H-5') were observed in the $^1$H NMR spectrum of 1. The $^{13}$C NMR and DEPT spectra together for 1 indicated 14 carbon

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signals (Table 1), including a carbonyl at δC 169.5 (C-1), six aromatics at δC 153.9 (C-8), 146.6 (C-5), 124.5 (C-4a), 123.9 (C-6), 115.1 (C-7), and 108.2 (C-8a), and seven alkyl carbons that were two oxygenated methines at δC 79.4 (C-3) and 65.6 (C-4′), four hydrocarbon methylenes at δC 38.6 (C-3′), 34.3 (C-1′), 26.3 (C-4), and 20.8 (C-2′), and a methyl at δC 23.7 (C-5′). In total, the NMR data suggested the presence of a dihydroisocoumarin skeleton contained in 1. The 1H−1H COSY correlations of H-5′/H-4′/H-3′/H-2′/H-1′ further indicated a continuous spin system in the molecule, identified as -CH2-CH2-CH2-CH(OH)-CH3 (Figure 2). Furthermore, according to the key HMBC correlations presented in Figure 2, including from H-2′ to C-3, from H-4 to C-1′, C-3, C-5, C-4a, and C-8a, as well as from 8-OH to C-7, C-8, and C-8a, the planar structure of 1 was established as shown. Finally, on the basis of X-ray single-crystal diffraction analysis (Figure 3), the absolute configuration of 1 was established as 3R,4′S, and this new molecule was given the trivial name aspergimarin A.

Compound 2 was obtained as a brown oil, and the molecular formula of C18H22O8 was assigned to this molecule by the anion HRESIMS peak at m/z 365.1232 [M−H]− (calcd for C18H21O8, 365.1242). The 1H and 13C NMR spectroscopic data of 2 (Table 1) resembled those of 1, and it was determined that the core structure of an oxygenated hydrocarbon-extended dihydroisocoumarin was shared between these molecules. The 1H−1H COSY correlation between H-2′′ and H-3′′, along with key HMBC correlations from H-3′′ (δH 2.46) to C-1′′ (δC 171.8) and C-4′′ (δC 173.5) (Figure 2), indicated the existence of the linear chain -OCO-CH2-CH2-CO2H in the structure of 2. Furthermore, according to the same biosynthetic pathway with 1 based on and the key HMBC correlation observed from H-4′ (δH 4.83) to C-1′′, the planar structure of 2 was established. The absolute configuration for 2 was suggested as being 3R,4′S based on the biosynthetic logic that it would match that of 1. The CD spectra of 1 and 2 (Figure 4) are able to be overlapped, with the same Cotton effects observed, which further support the configurational assignment. Accordingly, the resolved structure of compound 2 was afforded the trivial name aspergimarin B.

Compound 3 was also obtained as a brown oil, and its molecular formula of C20H28O8 was determined by the potassium cation adduct peak in the HRESIMS spectrum at m/z 435.1416 [M+K]+ (calcd for C20H28K2O8, 435.1416). The CD spectrum of 3 (Figure 4) and the 1H and 13C NMR spectroscopic data (Table 1) were similar to those of both 1 and 2, indicating
that this molecule is another oxygenated hydrocarbon-extended dihydroisocoumarin analog with the same absolute configuration at C-3 and C-4'. It was determined from a $^{1}H-^{1}H$ COSY correlation of H-4'/H-5' and the key HMBC correlations from H-2'/ (δ_H 2.38) to C-1'' (δ_C 170.4) and C-4'' (δ_C 43.7), as well as from 3''-OH (δ_H 4.53) to C-2'' (δ_C 46.9), C-3'' (δ_C 69.8) and C-6'' (δ_C 27.4) (Figure 2), that there is a different secondary carbon side chain [-OCO-CH2-C(CH3)(OH)-CH2-CH2OH] in the molecule of 3 as compared to 2. Furthermore, the key HMBC correlation observed from H-4' (δ_H 4.83) to C-1'' allowed for the planar structure of 3 to be completed. Since the biosynthetic logic of 3 with relation to 1 and 2, together with the matching CD data of these molecules allowed the partial absolute configuration to be assigned as 3R,4'S, only one stereocenter remained uncertain. Using the modified Mosher’s method (Figure 5) (Gu et al., 2018) the absolute configuration of C-3'' of 3 was established as being R. Therefore, the absolute configuration of 3 was determined to be 3R,4'S,3'R.

Compound 4 was obtained as a yellow oil, and the molecular formula of this molecule was determined to be C_{16}H_{22}O_{5} was based on a peak observed in the HRESIMS spectrum at m/z 295.1537 [M + H]^+ (calcd for C_{16}H_{23}O_{5}, 295.1540). The data of 4 from spectroscopic analysis, including UV, IR, and NMR, were extremely similar to those reported for the known compound, penicimarin C (9) (Qi et al., 2013). The exceptions noted were determined to be due to the substitution of one methoxy group for a proton at C-7. The placement of the additional methoxy group at C-7 was determined from key HMBC correlations observed from H-5 (δ_H 7.06) to C-4 (δ_C 32.7), C-7 (δ_C 152.2), and C-8a (δ_C 119.0), and from 7-O-CH_3 (δ_H 3.81) to C-7, together with the $^{1}H-^{1}H$ COSY correlation of H-5/H-6 (δ_H 7.28) (Figure 2). Accordingly, the planar structure of 4 was unambiguously established as shown. Compared to the CD data of 1 and some previously described values for dihydroisocoumarins (Choukchou-Braham et al., 1994), the observed CD spectrum of 4 (Figure 4) indicated the R configuration at C-3. The absolute configuration at the side chain was suggested as 4'S to match that of 1 according to biosynthetic logic, and this was confirmed by use of the modified Mosher’s method (Figure 5). Thus, the absolute configuration of 4 was determined to be 3R,4'S.

Compound 5 was isolated as a colorless oil, and its molecular formula was established as being C_{14}H_{18}O_{5} according to the associated sodiated molecular ion peak in the HRESIMS spectrum at m/z 289.1034 [M + Na]^+ (calcd for C_{14}H_{18}NaO_{5}, 289.1046). This formula for 5 corresponds to an additional OH with respect to 1, and the $^{1}H$ and $^{13}C$ NMR data (Table 2) suggested shared carbon skeletons with the substitution of an additional hydroxy group. From the chemical shift differences calculated between 5 and 1, the additional hydroxy group of 5 was suggested to be at C-4. This assignment was further supported by key HMBC correlations from H-4 (δ_H 4.71) to C-5 (δ_C 116.3) and from H-5 (δ_H 7.03) to C-4 (δ_C 67.5), together with the $^{1}H-^{1}H$ COSY correlation of H-4/H-3 (δ_H 4.47) (Figure 2). The absolute configuration at C-3 was determined to be R by the comparison of CD data with 1 and 4 (Figure 4). The absolute configuration of chiral centers at both C-4 and C-4' in 5 was established as being S by use of the modified Mosher’s method (Figure 5). Therefore, the absolute configuration of 5 was determined to be 3R,4S,4'S.

![Figure 4](image-url) | Experimental ECD spectra of 1–6, collected in MeOH.

![Figure 5](image-url) | Modified Mosher’s analysis for 3–5. Values shown denote Δλ (δS – δR) (ppm) for the MTPA esters of 3–5.
Compound 6 was isolated as a yellow amorphous powder, and its molecular formula was determined to be C$_{14}$H$_{16}$O$_{5}$ by an associated sodiated molecular ion peak in the HRESIMS spectrum at m/z 287.0879 [M + Na]$^+$ (calcd for C$_{14}$H$_{16}$NaO$_{5}$$^+$, 287.0890). The spectroscopic data of 6, including UV, IR, and NMR, resembled those previously reported for the known molecule penicilloxalone B (10) (Ren et al., 2019). From the $^1$H and $^{13}$C NMR data, it was obvious that the aromatic substitution patterns of 6 and 10 differed, with 6 bearing protons at C-5 and C-6 while 10 has protons at C-6 and C-7. Furthermore, the key HMBC correlations observed from H-5 ($\delta$H 6.63) to C-4 ($\delta$C 31.4), C-7 ($\delta$C 144.4), and C-8a ($\delta$C 108.4) together with the $^1$H→$^1$H COSY correlation of H-5/H-6 ($\delta$H 7.00) (Figure 2) corroborated that the two phenolic hydroxy groups of 6 were situated at C-7 and C-8 ($\delta$C 150.0). The absolute configuration of 6 was determined to be 3R, the same as for 10, by comparison of the observed CD spectrum for this molecule with reported data for 10 (Figure 4).

Five additional compounds isolated from Aspergillus sp. NBUF87 in the course of this study were determined, by comparison of the spectroscopic and spectrometric data of each with reported values, to be the known isocoumarin derivatives aspergilamin B (7) (Li S. et al., 2012), penicimar B (8) (Qi et al., 2013), penicimar C (9) (Qi et al., 2013), penilloxalone B (10) (Ren et al., 2019), and (R)-3-(3-hydroxypropyl)-8-hydroxy-3,4-dihydroisocoumarin (11) (Sun et al., 2017). The absolute configurations for 7-9 were established to be 3R, 4'S because the CD spectra (see Supplementary Figures S61, S73), and optical rotation data matched literature values (Li S. et al., 2012; Qi et al., 2013). The absolute configuration of 10 and 11 was also determined to be 3R because the optical rotation data matched literature reported values for these molecules (Sun et al., 2017; Ren et al., 2019).

**Effects of Compounds 1–11 on Plant Growth of Arabidopsis thaliana Columbia-0**

The isolated compounds 1–11 were subjected to bioassays for testing plant growth response using A. thaliana Col-0, a model plant growth organism. At 100 µM, for both 1 and 5, root growth inhibitory activity was observed against Col-0. Interestingly, while 1 showed only significant inhibitory effect on the lateral root growth of Col-0, 5 caused notable inhibition of both lateral root and primary root growth, as shown in Figure 6. Compounds 2–4 and 6–11 did not show any obvious activity in the same plant growth response assay at 100 µM.

**Results of Compounds 1–11 Against Four Additional Bioassays**

All compounds isolated in this study (1–11) were also tested for their in vitro inhibitory activity of AChE in a biochemical assay, and phenotypic tests for cytotoxicity against four human-derived cancer cell lines, namely, CCRF-CEM (acute lymphoblastic leukemia T lymphocytes), MDA-MB-231 (breast cancer), HCT-116 (colon cancer), and AGS (gastric adenocarcinoma), and antibacterial activity toward methicillin-resistant S. aureus ATCC43300 and E. coli ATCC25922, and antimalarial activity against P. falciparum W2. None of these compounds exhibited inhibition of AChE (IC$_{50} > 100$ µM), cytotoxic activities against any of the cell lines tested (IC$_{50}$ > 50 µM), antibacterial activities toward the two bacteria (MIC > 50 µg/mL), or antimalarial activity against the parasite (EC$_{50} > 100$ µg/mL).

**CONCLUSION**

In summary, six new dihydroisocoumarin derivatives, aspergimarin A−F (1–6) were obtained along with five known analogs (7–11) from the fermentation of a fungus Aspergillus sp. NBUF87, isolated from the sponge Hymeniacidon sp. collected from the Paracel Islands in the South China Sea. Structurally, compounds 2 and 3 are esters of the C-4’ hydroxy group in the C-3 side chain of 1, representing relatively rare isocoumarin derivatives according to previous literature reports (Saeed, 2016). In a plant growth response assay using A. thaliana Col-0 as a model organism, only 1 and 5 showed inhibitory activity against root growth, while the others were inactive at up to 100 µM. This finding indicates that the substitution pattern of hydroxy groups in these dihydroisocoumarins may play an important role in root growth inhibitory activity, and further studies remain necessary to interrogate this phenomenon. Since none of the isolated compounds, including 1 and 5, were found to be broadly AChE inhibitors, anticancer agents, antibacterial agents, or antimalarial agents, it is proposed that these root growth inhibitors act through more elaborate signaling pathways. Moreover, 1 and 5 were here as the root growth inhibitors of Col-0, suggesting that they have an important impact in agricultural production.
DATA AVAILABILITY STATEMENT
The datasets generated for this study can be found in the Cambridge Structural Database https://www.ccdc.cam.ac.uk/structures/accession1919904.

AUTHOR CONTRIBUTIONS
All authors conceived the research, analyzed the data, contributed to the study, and approved the final version of the manuscript. LH, LD, XL, NW, WC, XW, and JL performed the experiments. LH wrote the manuscript. LD, CN, JL, XY, and SH read and revised the manuscript.

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SUPPLEMENTARY MATERIAL
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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