Peptides and polyketides isolated from the marine sponge-derived fungus Aspergillus terreus SCSIO 41008

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[ABSTRACT] Two new isomeric modified tripeptides, aspergillamides C and D (compounds 1 and 2), together with fifteen known compounds (compounds 3–17), were obtained from the marine sponge-derived fungus Aspergillus terreus SCSIO 41008. The structures of the new compounds, including absolute configurations, were determined by extensive analyses of spectroscopic data (NMR, MS, UV, and IR) and comparisons between the calculated and experimental electronic circular dichroism (ECD) spectra. Butyrolactone I (compound 11) exhibited strong inhibitory effects against Mycobacterium tuberculosis protein tyrosine phosphatase B (MptpB) with the IC50 being 5.11 ± 0.53 μmol·L−1, and acted as a noncompetitive inhibitor based on kinetic analysis.

[KEY WORDS] Aspergillus terreus; Aspergillamides; Polyketides; MptpB inhibitor


Introduction

Marine-derived peptides possess high potential nutraceutical and medicinal applications, due to their wide range of bioactivities, such as antimicrobial, antiviral, antitumor, and antioxidative activities [1-2]. Aspergillamides, a kind of modified tripeptides possessing rare dehydrotryptamine moieties, are mainly isolated from the marine-derived Aspergillus fungi with modest cytotoxicities, demonstrating structural diversity arising from geometric isomerization of double bonds and various categories of amino acid [3-4]. However, no more than ten natural aspergillamides have been reported. Marine sponge-associated microorganisms have been proven to be a prolific source of secondary metabolites with novel structures and interesting bioactivities [5-7]. The fungus Aspergillus terreus, a well-known producer of lovastatin [8], can produce a variety of structural classes, such as butenolides [9-11], terpenoids [12-13], peptides [14-15], and alkaloids [15-16]. Some of the above metabolites exhibit cytotoxic [10, 15, 17], NO inhibitory [9], COX-2 inhibitory [12], and antiviral activities [15].

Tuberculosis (TB) is a prevalent infectious disease caused by Mycobacterium tuberculosis (Mtb), ranking as the second deadliest communicable disease worldwide. The Mtb protein tyrosine phosphatase B (MptpB), which is secreted by Mtb into the host cell and attenuates host immune defenses by manipulating the host-signaling pathways [18], has been proven to be an important virulence factor. Recently, there has been an urgent need for finding new MptpB inhibitors with a different mode of action from currently applied drugs [19]. As our ongoing efforts for searching novel and bioactive secondary metabolites from marine microorganisms [20-22], the Aspergillus terreus SCSIO 41008 was selected for chemical investigations, owing to an interesting HPLC-UV profile for its extract cultured in a rich nutrient-culture medium by a fermenter.

Results and Discussion

Compound 1 (Fig. 1) was obtained as colorless oil. It gave a molecular formula of C28H34N4O4 as determined by the
Fig. 1 Structures of compounds 1–17

Fig. 2 Key HMBC, 1H-H COSY and NOESY correlations of compounds 1 and 2

The Z configuration of Δ⁹ was assigned by the cis-coupling constant ($J_{H9,H10} = 9.8$ Hz) as well as the NOE correlations between H-9 and H-10. The gross structure of compound 1 was thus established and was assigned the trivial name aspergillamide C.

Compound 2 was also isolated as colorless oil and shared the same molecular formula as 1 according to the HR-ESI-MS data. The highly similar spectroscopic features, including NMR, UV, and IR data, illustrated that their structures were very closely related. The major difference in the ¹H NMR spectrum was that the large trans-coupling constant between H-9 and H-10 was 14.0 Hz in 2, instead of 9.8 Hz in 1, implying the E configuration of the Δ⁹ double bond in 2. Accordingly, compound 2, the Δ⁹ double bond isomer of 1, was elucidated as shown in Fig. 1 and was accorded the trivial name aspergillamide D.

It proved difficult to incontrovertibly isolate the cis- and trans-isomer of the Δ⁹ double bond among aspergillamides, while the trans-isomer was a more stable conformation [3-4, 23]. Thus compound 1 was likely obtained as an artifact derived from 2 under the light during the isolation process. Both compounds 1 and 2 were assigned as trans-amide relationships based on the NOE correlation of N-Me (H3-18) and α-proton (H-20), which was also supported by the similar ¹H NMR data of the α-proton among these trans-amide isomers (1–6) [3]. Moreover, the absolute configurations of compounds 1 and 2 were determined by specific rotations and ECD data in comparison with those of the co-isolated siblings (3–6), in combination with biogenetic point of view. These similar specific rotations among compounds 1–6 as well as the nearly identical experimental ECD curves of them (Fig. 3) suggested that compounds 1 and 2 shared the same configurations of 12S, 20S with 3–6 of the same biosynthetic origin. In addition, the experimental ECD curves of both 1 [227 nm (negative), 209 nm (positive)] and 2 [227 nm (negative), 201 nm

quasi-molecular ion peak at $m/z$ 513.2482 [M + Na]$^+$ (Calcd. 513.2478) observed from the HR-ESI-MS data. The ¹H NMR spectrum (Table 1) along with HSQC experiment of 1 showed eleven aromatic or olefinic (δ_H 6.16–7.63), three methinic [δ_H 4.85 (overlapped, H-12), 4.73 (d, $J = 11.2$ Hz, H-20), 2.04 (m, H-21)], two methylenic [δ_H 2.83 (dd, $J = 9.1$, 13.3 Hz, H-13a), 2.78 (dd, $J = 6.3$, 13.3 Hz, H-13b), 1.40 (m, H-22a), 0.98 (m, H-22b)], and four methyl [two singlets (δ_H 2.63 for H3-18 and δ_H 1.93 for H3-26), one doublet (δ_H 0.93, d, $J = 6.3$ Hz, H3-24), and one triplet (δ_H 0.85, t, $J = 6.3$ Hz, H3-23)] proton signals. Apart from the above 20 corresponding hydrogen-bearing carbons, eight carbons remained in the ¹³C NMR spectrum, including three carbonyls and five olefinics (one oxygenated).

The aforementioned NMR characteristics showed great similarity to that of aspergillamide A (3), a modified tripeptide we also obtained from a marine fungus of the genus Aspergillus [3]. The major differences included the presence of hydroxy-substituted aromatic carbon at C-17 (δ_C 157.4) and one 1-methylpropyl group at C-20 instead of an aromatic methine (δ_H/C 7.13/127.9) and an isobutyl group in 3, respectively, which were also supported by the HMBC correlations (Fig. 2) from H-15/H-16 to C-17, from H3-24 to C-20/C-21/C-22, and from H3-23 to C-21/C-22, as well as signals of a spin system of CH3-24/CH-21/CH2-22/CH3-23 and a AA'XX' coupling system observed from the ¹H-¹H COSY data (Fig. 2).
(positive)) showed similar cotton effects to the calculated ECD curve [233 nm (negative), 206 nm (positive)] of (12S, 20S)-2 (Fig. 3), further confirming the above assignments.

However, the absolute configurations at C-21 of 1 and 2 were undetermined by acid hydrolysis experiment due to the variable structures of them.

Table 1  $^1$H NMR (700 MHz) and $^{13}$C NMR (175 MHz) data of compounds 1 and 2 in CD$_3$OD (J in Hz)

<table>
<thead>
<tr>
<th>No.</th>
<th>1</th>
<th>2</th>
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<tbody>
<tr>
<td></td>
<td>$\delta_H$</td>
<td>$\delta_C$, type</td>
</tr>
<tr>
<td>1</td>
<td>137.6, C</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>7.64, s</td>
<td>124.3, CH</td>
</tr>
<tr>
<td>3</td>
<td>113.3, C</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>128.2, C</td>
<td></td>
</tr>
<tr>
<td>5</td>
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<td>119.4, CH</td>
</tr>
<tr>
<td>6</td>
<td>7.08, m</td>
<td>120.5, CH</td>
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<td>9</td>
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<tr>
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<td>11</td>
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<tr>
<td>13</td>
<td>2.83, dd (12.6, 9.1) 2.78, dd (6.3, 12.6)</td>
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<tr>
<td>14</td>
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<tr>
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<td>25.3, CH$_2$</td>
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<td>1.40, m</td>
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<td>0.85, t (7.0)</td>
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<tr>
<td>26</td>
<td>1.93, s</td>
<td>22.1, CH$_3$</td>
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Fig. 3  A): The experimental ECD curves of compounds 1−6; B): Comparison between the experimental ECD curves of compounds 1−2 and the calculated ECD curve of compound 2
The structures of 15 co-isolated known compounds (3–17) were identified by comparison of their spectroscopic data with those reported literatures. They were elucidated as follows: aspergillamide A (3) [10], aspergillamide B (4) [11], cis-L-phenylalaninamide (5) [20], trans-L-phenylalaninamide (6) [24], terretrione B (7) [25], terretrione C (8) [29], cyclo-(L-Pro-L-Phe) (9) [26], brevianamide F (10) [27], butyrolactone I (11) [28], 1,8-dihydroxy-3-methoxy-6-methylanthracene-9,10-dione (12) [29], questin (13) [30], 1-methyl emodin (14) [31], methyl 6-acetyl-4-methoxy-5,7,8-trihydroxynaphthalene-2-carboxylate (15) [32], methyl 6-acetyl-4-methoxy-5, 8-dihydroxynaphthalene-2-carboxylate (16) [32], and (S)-6, 8-dimethoxy-3-methylsichroman-1-one (17) [33], among which 17 was obtained as a new natural product.

All the obtained compounds were evaluated for their MptpB inhibitory activity. Butyrolactone I (11) showed strong inhibitory activity with an IC50 of 5.11 ± 0.53 μmol·L⁻¹, compared to the positive control (oleanolic acid, 22.1 ± 0.72 μmol·L⁻¹). However, the remaining compounds revealed weak inhibitory effects with inhibition rates of < 60% at a concentration of 50 μmol·L⁻¹. Besides, all these compounds (1–17) exhibited weak or no cytotoxic activities towards human glioma U87 cells at a concentration of 10 μmol·L⁻¹ and also showed weak or no protective activity against glutamate-induced toxicity in HT22 cells at a concentration of 10 μmol·L⁻¹. In order to clarify the mechanism of MptpB inhibitory type, the enzyme kinetic experiment in vitro was further carried out. The results (Fig. 4) demonstrated that 11 acted as a noncompetitive inhibitor. While the modified tripeptides were reported with insecticidal activity against brine shrimp, Artemia salina [4], modest cytotoxicity towards the human colon carcinoma cell line HCT-116 was observed [3].

**Fig. 4  Kinetic analysis of the inhibition of MptpB by compound 11**

In summary, ten peptide derivatives (1–10), including two new tripeptides (1 and 2), along with seven known polyketides (11–17), were obtained from the sponge-derived fungus *Aspergillus terreus* SCSIO 41008. The structures of the pair of isomeric naturally uncommon modified tripeptides, aspergillamides C (1) and D (2), were determined by extensive analyses of spectroscopic data and ECD calculation, including their absolute configurations. Our findings would contribute to enriching chemical context of the species *Aspergillus terreus* and expanding the chemical diversity of aspergillamides. Moreover, butyrolactone I (11) displayed notable inhibitory activity against MptpB and acted as a noncompetitive inhibitor, implying its therapeutic potential as an anti-TB drug.

**Experimental**

**General experimental procedure**

Optical rotations were measured with an MCP 500 automatic polarimeter (Anton Paar, Graz, Austria). UV spectra were recorded on a UV-2600 spectrometer (Shimadzu, Kyoto, Japan). CD spectra were measured with a Chirascan circular dichroism spectrometer (Applied Photophysics, Ltd., Leatherhead, UK). IR spectra were measured on IR Affinity-1 spectrometer (Shimadzu, Kyoto, Japan). HR-ESI-MS and ESI-MS spectra data were recorded on a MaXis quadrupole-time-of-flight mass spectrometer and an amaZon SL ion trap mass spectrometer (Bruker, Karlsruhe, Germany), respectively. The NMR spectra including were recorded on an Avance 500 spectrometer (Bruker, Karlsruhe, Germany) or AVANCE III HD 700 spectrometer (Bruker, Karlsruhe, Germany) using TMS as an internal standard. Thin layer chromatography (TLC) and column chromatography were performed on plates pre-coated with silica gel GF254 (10 μm). HR-ESI-MS and ESI-MS spectra data were recorded on a Hi-Back Primaide apparatus using an ODS column (YMC-pack ODS-A, Japan, 10 mm × 250 mm, 5 μm). The artificial sea salt (Guangzhou Haiyi Aquarium Technology Company, Guangzhou, China) was used for fermentation.

**Fungal materials**

The fungal strain, *Aspergillus terreus* SCSIO 41008, was isolated from the marine sponge *Callyspongia sp.*, which was collected from the seaside in Xuen County, Guangdong Province, China, in August 2013. It was identified by its morphological characteristics and ITS gene sequences (GenBank accession No. MF536093), as well as the phylogenetic tree analysis. The strain was stored on MB agar (malt extract 15 g, sea salt 10 g, and agar 15 g) slants at 4 °C and a voucher specimen was deposited in the CAS Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, China.

**Fermentation and extraction**

The strain *A. terreus* SCSIO 41008 grown on MB plates was inoculated into 300 mL of MB medium (malt extract 15 g·L⁻¹ and sea salt 20 g·L⁻¹, PH 7.4) and then incubated for 4 d. The 4.2-L seed culture was successively transferred into a 65-L fermenter containing 40 L of S6 medium (potato 200 g·L⁻¹,
peptone 5 g·L⁻¹, mannitol 20 g·L⁻¹, glucose 20 g·L⁻¹, monosodium glutamate (MSG) 5 g·L⁻¹, yeast extract 3 g·L⁻¹ and sea salt 20 g·L⁻¹, PH 7.4), and then incubated at 28 °C, 135 r·min⁻¹, 12 L·min⁻¹ aseptic air, and 3.0 Mpa for 7 d. During fermentation, 100 mL samples were withdrawn from the fermentor every day and subjected to HPLC analysis.

The whole culture broth (40 L) was harvested and filtered to yield the mycelium cake and liquid broth. The mycelium cake was extracted by ultrasonication using acetone for three times. The acetone solution was evaporated under reduced pressure to afford an aqueous solution. Then the aqueous solution was extracted three times with EtOAc to produce an EtOAc solution. The liquid broth was also extracted with EtOAc for three times. Both EtOAc solutions were combined and concentrated under vacuum to yield a crude gum (50 g).

**Purification**

The EtOAc extract was submitted to silica gel vacuum liquid chromatography (VLC) using step gradient elution with petroleum ether–CH₂Cl₂ (100: 0, 80: 20, 50: 50, 20: 80, 0: 100, V/V) to obtain ten fractions (Fr.s 1-10) based on TLC profiles. Compound 17 (5 mg, tₖ 20 min) was obtained from Fr.3 (1.3 g) followed by ODS chromatography eluting with MeCN–H₂O (10%-100%) and semi-preparative HPLC (27% MeCN:H₂O, 2.5 mL·min⁻¹), respectively. Fr. 4 (2.0 g) was separated into eight subfractions (Fr.s 4-1-4-8) by semi-preparative HPLC (47% MeCN:H₂O, 2.5 mL·min⁻¹), which meanwhile afforded Compound 15 (8 mg, tₖ 28 min) and compound 12 (7 mg, tₖ 42 min). Fr.4-3 was purified by semi-preparative HPLC (41% MeCN–H₂O, 0.04% TFA, 2.5 mL·min⁻¹) to yield 13 (7 mg, tₖ 30 min). Fr.4-4 was also purified by semi-preparative HPLC (57% MeCN–H₂O, 1.8 mL·min⁻¹) to yield 6 (2 mg, tₖ 13 min) and 5 (2 mg, tₖ 19 min). Fr. 4-7 was purified by semi-preparative HPLC (53% MeCN–H₂O, 3 mL·min⁻¹) to yield 4 (6.7 mg, tₖ 16 min) and 3 (9.5 mg, tₖ 22 min). Compound 14 (58 mg, tₖ 19 min) was obtained from Fr. 5 (1.8 g), following ODS chromatography eluting with MeCN–H₂O (10%-100%) and semi-preparative HPLC (63% MeCN–H₂O, 1.3 mL·min⁻¹), respectively. Fr. 6 (1.2 g) was separated into eight subfractions (Fr.s 6-1-6-8) by ODS chromatography eluting with MeCN–H₂O (10%-100%). Compound 9 (11 mg, tₖ 32 min) was obtained from Fr. 6-1 by semi-preparative HPLC (16% MeCN–H₂O, 2.5 mL·min⁻¹). Fr. 6-2 was further purified by semi-preparative HPLC (21% MeCN–H₂O, 2.5 mL·min⁻¹) to yield 10 (5 mg, tₖ 21 min), 7 (21.5 mg, tₖ 23 min), and 8 (55 mg, tₖ 37 min). Fr. 7 (2.5 g) was separated into nine subfractions (Fr.s 7-1-7-9) by ODS chromatography eluting with MeOH–H₂O (10%-100%). Fr. 7-5 was further purified by semi-preparative HPLC (31% MeCN–H₂O, 2.5 mL·min⁻¹) to yield 16 (3 mg, tₖ 31 min). Fr. 7-6 was further purified by semi-preparative HPLC (41% MeCN–H₂O, 2.5 mL·min⁻¹) to yield 2 (6 mg, tₖ 17 min), 1 (4.4 mg, tₖ 29 min), and 11 (700 mg, tₖ 40 min).

Aspergillamide C (1): colorless oil, [α]₂⁰° +54 (c 0.20, MeOH); UV (MeOH) λmax (log ε) 280 (2.83), 220 (3.30), 200 (3.63) nm; CD (0.25 mg·mL⁻¹, MeOH) λmax (Δε) 209 (8.64), 227 (–4.65) nm; IR (film) νmax 3292, 2966, 2873, 1647, 1558, 1506, 1456, 1338 cm⁻¹; HR-ESI-MS m/z 513.2482 [M + Na]⁺ (Calcd. for C₂₉H₃₄N₄NaO₄, 513.2478); For ¹H and ¹³C NMR data, see Table 1.

Aspergillamide D (2): colorless oil, [α]₂⁰° +53 (c 0.21, MeOH); UV (MeOH) λmax (log ε) 280 (2.64), 220 (3.36), 200 (3.65) nm; ECD (0.20 mg·mL⁻¹, MeOH) λmax (Δε) 201 (+10.65), 227 (–6.51) nm; IR (film) νmax 3305, 2968, 2876, 1616, 1539, 1516, 1458, 1089 cm⁻¹; HR-ESI-MS m/z 513.2488 [M + Na]⁺ (Calcd. for C₂₉H₃₄N₄NaO₄, 513.2478); For ¹H and ¹³C NMR data, see Table 1.

**MptpB inhibition assay**

The target enzyme MptpB was prepared according to the reported protocols [¹⁹]. In brief, the inhibition assays were performed using the 96-well microplates (In Vitrogen, Carlsbad, USA) by monitoring the hydrolysis of the fluorogenic phosphatase substrate p-nitrophenyl phosphate (pNPP) (Aladdin) according to the manufacturer’s instruction. IC₅₀ of compounds with more than 60% of inhibitory activity against MptpB was determined at different concentrations using two-fold serial dilution (1.5625–100 µmol·L⁻¹). Oleanolic acid was used as positive control (IC₅₀ 22.1 ± 2.4 µmol·L⁻¹).

To determine the type of inhibition, different inhibitor concentrations (0, 5, and 10 µmol·L⁻¹) and different concentrations of pNPP (0.05, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 mmol·L⁻¹) were performed as explained above. The type of inhibition was determined by fitting data to Lineweaver-Burk plot by double reciprocal of inhibitor concentration versus velocity. All the assays were performed in triplicate to at least three independent experiments.

**Cytotoxicity bioassay**

The obtained compounds (1–17) were also evaluated for their cytotoxic activity against human glioma U87 cells and neuroprotective activity against glutamate-induced cell death in the hippocampal neuronal HT22 cells [³⁴-³⁵]. In brief, the U87 cells (American Type Culture Collection, ATCC; Manassas, VA, USA) and the mouse hippocampal HT-22 cells, provided by Seoul National University (Korea), were cultured in RPMI-1640 (Gibco, New York, USA) and DMEM media (Gibco-BRL, New York, USA), respectively, supplemented with 10% fetal bovine serum (FBS), 100 U·mL⁻¹ of penicillin and 100 µg·mL⁻¹ of streptomycin, at 37 °C in a humidified atmosphere containing 5% CO₂. The cells were sub-cultured once every 2 day. Cell viability was determined using MTT assays according to the reported methods [³⁴]. Briefly, cells were seeded into a 96-well plate at a density of 4 × 10⁴ cells/well in growth medium and cultured to about 60%–70% confluence, prior to the initiation of experimental treatment. Cells were washed three times with PBS and 10 µL of MTT solution (5 mg·mL⁻¹ stock) was added to the cells, and then incubated for 1 h at 37 °C. The medium was removed carefully and 150 µL of DMSO was then added to resolve the blue formazan in living cells. Optical density (OD) value was measured at 570 nm by employing a microplate reader (TECAN A-5002, Austria). All the compounds were prepared at a concentration of 10 µmol·L⁻¹ on the preliminary screen-
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References