

## 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 (MtpB) with the IC<sub>50</sub> being 5.11 ± 0.53 μmol·L<sup>-1</sup>, and acted as a noncompetitive inhibitor based on kinetic analysis.

**[KEY WORDS]** *Aspergillus terreus*; Aspergillamides; Polyketides; MtpB inhibitor

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### 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<sup>[1–2]</sup>. 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<sup>[3–4]</sup>. 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<sup>[5–7]</sup>. The fungus *Aspergillus terreus*, a well-known producer of lovastatin<sup>[8]</sup>, can produce a variety of structural classes, such as butenolides<sup>[9–11]</sup>, terpenoids<sup>[12–13]</sup>, peptides<sup>[14–15]</sup>, and alkaloids<sup>[15–16]</sup>. Some of the above metabolites exhibit cytotoxic<sup>[10, 15, 17]</sup>, NO inhibitory<sup>[9]</sup>, COX-2 inhibitory<sup>[12]</sup>, and antiviral activities<sup>[15]</sup>.

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 (MtpB), which is secreted by Mtb into the host cell and attenuates host immune defenses by manipulating the host-signaling pathways<sup>[18]</sup>, has been proven to be an important virulence factor. Recently, there has been an urgent need for finding new MtpB inhibitors with a different mode of action from currently applied drugs<sup>[19]</sup>. As our ongoing efforts for searching novel and bioactive secondary metabolites from marine microorganisms<sup>[20–22]</sup>, 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 C<sub>28</sub>H<sub>34</sub>N<sub>4</sub>O<sub>4</sub> as determined by the

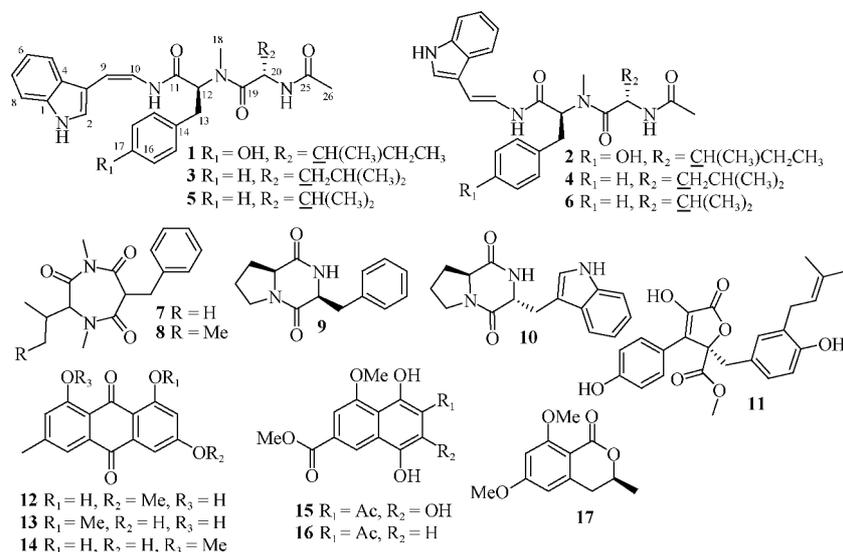
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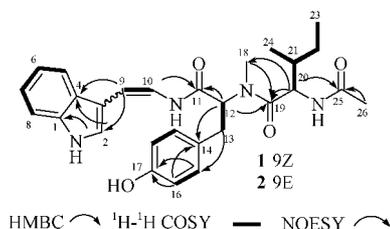
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**Fig. 1** Structures of compounds 1–17

quasi-molecular ion peak at  $m/z$  513.2482  $[M + Na]^+$  (Calcd. 513.2478) observed from the HR-ESI-MS data. The  $^1\text{H}$  NMR spectrum (Table 1) along with HSQC experiment of **1** showed eleven aromatic or olefinic ( $\delta_{\text{H}}$  6.16–7.63), three methinic [ $\delta_{\text{H}}$  4.85 (overlapped, H-12), 4.73 (d,  $J = 11.2$  Hz, H-20), 2.04 (m, H-21)], two methylenic [ $\delta_{\text{H}}$  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 ( $\delta_{\text{H}}$  2.63 for H<sub>3</sub>-18 and  $\delta_{\text{H}}$  1.93 for H<sub>3</sub>-26), one doublet ( $\delta_{\text{H}}$  0.93, d,  $J = 6.3$  Hz, H<sub>3</sub>-24), and one triplet ( $\delta_{\text{H}}$  0.85, t,  $J = 6.3$  Hz, H<sub>3</sub>-23)] proton signals. Apart from the above 20 corresponding hydrogen-bearing carbons, eight carbons remained in the  $^{13}\text{C}$  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 ( $\delta_{\text{C}}$  157.4) and one 1-methylpropyl group at C-20 in **1** instead of an aromatic methine ( $\delta_{\text{H/C}}$  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 H<sub>3</sub>-24 to C-20/C-21/C-22, and from H<sub>3</sub>-23 to C-21/C-22, as well as signals of a spin system of CH<sub>3</sub>-24/CH-21/CH<sub>2</sub>-22/CH<sub>3</sub>-23 and a AA'XX' coupling system observed from the  $^1\text{H}$ - $^1\text{H}$  COSY data (Fig. 2).


**Fig. 2** Key HMBC,  $^1\text{H}$ - $^1\text{H}$  COSY and NOESY correlations of compounds **1** and **2**

The *Z* configuration of  $\Delta^9$  was assigned by the *cis*-coupling constant ( $J_{\text{H-9/H-10}} = 9.8$  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  $^1\text{H}$  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  $\Delta^9$  double bond in **2**. Accordingly, compound **2**, the  $\Delta^9$  double bond isomer of **1**, was elucidated as shown in Fig. 1 and was accorded the trivial name aspergillamide D.

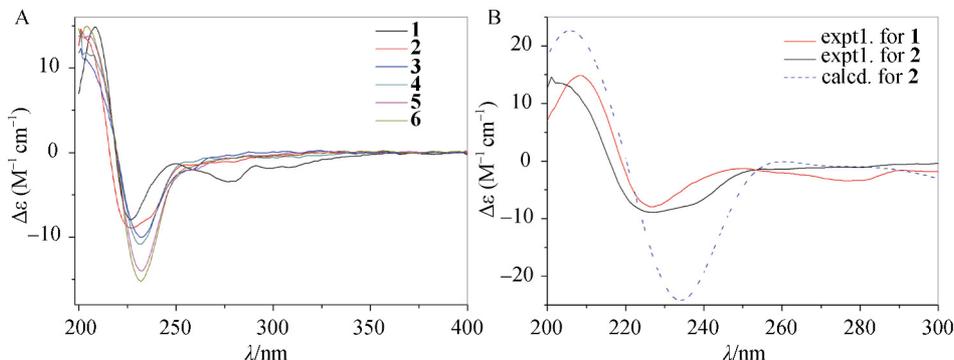
It proved difficult to incontrovertibly isolate the *cis*- and *trans*-isomer of the  $\Delta^9$  double bond among aspergillamides, while the *trans*-isomer was a more stable conformation [3-4, 23]. Thus compound **1** was likely obtained as an artifact derived from **2** under the light during the isolation process. Both compounds **1** and **2** were assigned as *trans*-amide relationships based on the NOE correlation of N-Me (H<sub>3</sub>-18) and  $\alpha$ -proton (H-20), which was also supported by the similar  $^1\text{H}$  NMR data of the  $\alpha$ -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 12*S*, 20*S* 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

(positive)] showed similar cotton effects to the calculated ECD curve [233 nm (negative), 206 nm (positive)] of (1*S*, 20*S*)-**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** <sup>1</sup>H NMR (700 MHz) and <sup>13</sup>C NMR (175 MHz) data of compounds **1** and **2** in CD<sub>3</sub>OD (*J* in Hz)

No.	<b>1</b>		<b>2</b>	
	$\delta_{\text{H}}$	$\delta_{\text{C}}$ , type	$\delta_{\text{H}}$	$\delta_{\text{C}}$ , type
1		137.6, C		138.7, C
2	7.64, s	124.3, CH	7.33, s	124.6, CH
3		113.3, C		113.3, C
4		128.2, C		126.6, C
5	7.63, d (7.7)	119.4, CH	7.81, d (7.7)	120.6, CH
6	7.08, m	120.5, CH	7.10, m	120.5, CH
7	7.16, m	123.1, CH	7.15, m	122.9, CH
8	7.42, d (7.7)	112.4, CH	7.38, d (7.7)	112.5, CH
9	6.16, d (9.8)	105.9, CH	6.60, d (14.0)	110.3, CH
10	6.76, d (9.8)	118.7, CH	7.39, d (14.0)	119.7, CH
11		168.7, C		168.3, C
12	4.85, overlapped	52.9, CH	4.91, overlapped	53.1, CH
13	2.83, dd (12.6, 9.1) 2.78, dd (6.3, 12.6)	38.4, CH <sub>2</sub>	2.96, dd (12.6, 9.1) 2.87, dd (6.3, 12.6)	38.5, CH <sub>2</sub>
14		127.8, C		128.0, C
15	6.87, d (8.4)	131.2 (× 2), CH	6.99, d (8.4)	131.3 (× 2), CH
16	6.64, d (8.4)	116.5 (× 2), CH	6.66, d (8.4)	116.5 (× 2), CH
17		157.4, C		157.6, C
18	2.63, s	30.7, CH <sub>3</sub>	2.76, s	30.9, CH <sub>3</sub>
19		175.4, C		175.0, C
20	4.73, d (11.2)	62.3, CH	4.80, d (11.2)	62.0, CH
21	2.04, m	32.7, CH	2.04, m	33.5, CH
22	1.40, m 0.98, m	25.3, CH <sub>2</sub>	1.41, m 1.01, m	25.4, CH <sub>2</sub>
23	0.85, t (7.0)	10.5, CH <sub>3</sub>	0.86, t (7.0)	10.7, CH <sub>3</sub>
24	0.93, d (6.3)	15.7, CH <sub>3</sub>	0.92, d (6.3)	15.6, CH <sub>3</sub>
25		172.6, C		172.7, C
26	1.93, s	22.1, CH <sub>3</sub>	1.95, s	22.1, CH <sub>3</sub>



**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**)<sup>[3]</sup>, aspergillamide B (**4**)<sup>[3]</sup>, *cis*-L-phenyla-laninamide (**5**)<sup>[24]</sup>, *trans*-L-phenylalaninamide (**6**)<sup>[24]</sup>, terretrione B (**7**)<sup>[25]</sup>, terretrione C (**8**)<sup>[25]</sup>, cyclo-(L-Pro-L-Phe) (**9**)<sup>[26]</sup>, brevianamide F (**10**)<sup>[27]</sup>, butyrolactone I (**11**)<sup>[28]</sup>, 1,8-dihydroxy-3-methoxy-6-methylanthracene-9,10-dione (**12**)<sup>[29]</sup>, questin (**13**)<sup>[30]</sup>, 1-methyl emodin (**14**)<sup>[31]</sup>, methyl 6-acetyl-4-methoxy-5,7,8-trihydroxynaphthalene-2-carboxylate (**15**)<sup>[32]</sup>, methyl 6-acetyl-4-methoxy-5, 8-dihydroxynaphthalene-2-carboxylate (**16**)<sup>[32]</sup>, and (*S*)-6, 8-dimethoxy-3-methylisochroman-1-one (**17**)<sup>[33]</sup>, 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  $IC_{50}$  of  $5.11 \pm 0.53 \mu\text{mol}\cdot\text{L}^{-1}$ , compared to the positive control (oleanolic acid,  $22.1 \pm 2.4 \mu\text{mol}\cdot\text{L}^{-1}$ ). However, the remaining compounds revealed weak inhibitory effects with inhibition rates of  $< 60\%$  at a concentration of  $50 \mu\text{mol}\cdot\text{L}^{-1}$ . Besides, all these compounds (**1–17**) exhibited weak or no cytotoxic activities towards human glioma U87 cells at a concentration of  $10 \mu\text{mol}\cdot\text{L}^{-1}$  and also showed weak or no protective activity against glutamate-induced toxicity in HT22 cells at a concentration of  $10 \mu\text{mol}\cdot\text{L}^{-1}$ . 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*<sup>[4]</sup>, modest cytotoxicity towards the human colon carcinoma cell line HCT-116 was observed<sup>[3]</sup>.

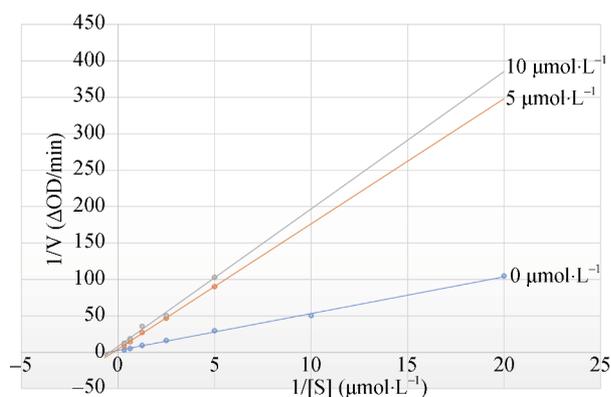


Fig. 4 Kinetic analysis of the inhibition of MptpB by compound **11**

In summary, ten peptide derivatives (**1–10**), including two new tripeptides (**1** and **2**), along with seven known polypeptides (**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 GF<sub>254</sub> (10–40 μm) and over silica gel (200–300 mesh) (Qingdao Marine Chemical Factory, Qingdao, China), C<sub>18</sub> reversed-phase silica gel (RP-18, 150 to 200 mesh, Fuji Silysia Chemical Ltd., Japan), and Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden), respectively. All solvents used were of analytical grade (Tianjin Fuyu Chemical and Industry Factory, Tianjin, China). Semi-preparative high performance liquid chromatography was performed on a Hitachi Primaide apparatus using an ODS column (YMC-pack ODS-A, Japan, 10 mm × 250 mm, 5 μm, 2 mL·min<sup>-1</sup>). The artificial sea salt (Guangzhou Haili 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 Xuwen 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<sup>-1</sup> and sea salt 20 g·L<sup>-1</sup>, PH 7.4 before sterilization) in a 1.0 L Erlenmeyer flask (14 flasks were employed). The flasks were then incubated at 28 °C on a rotary shaker at 200 r·min<sup>-1</sup> 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<sup>-1</sup>,

peptone 5 g·L<sup>-1</sup>, mannitol 20 g·L<sup>-1</sup>, maltose 20 g·L<sup>-1</sup>, glucose 20 g·L<sup>-1</sup>, monosodium glutamate (MSG) 5 g·L<sup>-1</sup>, yeast extract 3 g·L<sup>-1</sup> and sea salt 20 g·L<sup>-1</sup>, PH 7.4), and then incubated at 28 °C, 135 r·min<sup>-1</sup>, 12 L·min<sup>-1</sup> 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-CH<sub>2</sub>Cl<sub>2</sub> (100 : 0, 80 : 20, 50 : 50, 20 : 80, 0 : 100, V/V) to obtain ten fractions (Frs. 1–10) based on TLC profiles. Compound **17** (5 mg, *t<sub>R</sub>* 20 min) was obtained from Fr.3 (1.3 g) followed by ODS chromatography eluting with MeCN-H<sub>2</sub>O (10%–100%) and semipreparative HPLC (27% MeCN/H<sub>2</sub>O, 2.5 mL·min<sup>-1</sup>), respectively. Fr. 4 (2.0 g) was separated into eight subfractions (Frs. 4-1–4-8) by semipreparative HPLC (47% MeCN-H<sub>2</sub>O, 2.5 mL·min<sup>-1</sup>), which meanwhile afforded Compound **15** (8 mg, *t<sub>R</sub>* 28 min) and compound **12** (7 mg, *t<sub>R</sub>* 42 min). Fr.4-3 was purified by semi-preparative HPLC (41% MeCN-H<sub>2</sub>O, 0.04% TFA, 2.5 mL·min<sup>-1</sup>) to yield **13** (7 mg, *t<sub>R</sub>* 30 min). Fr. 4-4 was also purified by semi-preparative HPLC (57% MeCN-H<sub>2</sub>O, 1.8 mL·min<sup>-1</sup>) to yield **6** (2 mg, *t<sub>R</sub>* 13 min) and **5** (2 mg, *t<sub>R</sub>* 19 min). Fr. 4-7 was purified by semi-preparative HPLC (53% MeCN-H<sub>2</sub>O, 3 mL·min<sup>-1</sup>) to yield **4** (6.7 mg, *t<sub>R</sub>* 16 min) and **3** (9.5 mg, *t<sub>R</sub>* 22 min). Compound **14** (58 mg, *t<sub>R</sub>* 19 min) was obtained from Fr. 5 (1.8 g), following ODS chromatography eluting with MeCN-H<sub>2</sub>O (10%–100%) and semi-preparative HPLC (63% MeCN-H<sub>2</sub>O, 1.3 mL·min<sup>-1</sup>), respectively. Fr. 6 (1.2 g) was separated into eight subfractions (Frs. 6-1–6-8) by ODS chromatography eluting with MeCN-H<sub>2</sub>O (10%–100%). Compound **9** (11 mg, *t<sub>R</sub>* 32min) was obtained from Fr. 6-1 by semi-preparative HPLC (16% MeCN-H<sub>2</sub>O, 2.5 mL·min<sup>-1</sup>). Fr. 6-2 was further purified by semi-preparative HPLC (21% MeCN-H<sub>2</sub>O, 2.5 mL·min<sup>-1</sup>) to yield **10** (5 mg, *t<sub>R</sub>* 21 min), **7** (21.5 mg, *t<sub>R</sub>* 23 min), and **8** (55 mg, *t<sub>R</sub>* 37 min). Fr. 7 (2.5 g) was separated into nine subfractions (Frs. 7-1–7-9) by ODS chromatography eluting with MeOH-H<sub>2</sub>O (10%–100%). Fr. 7-5 was further purified by semi-preparative HPLC (31% MeCN-H<sub>2</sub>O, 2.5 mL·min<sup>-1</sup>) to yield **16** (3 mg, *t<sub>R</sub>* 31 min). Fr. 7-6 was further purified by semi-preparative HPLC (41% MeCN-H<sub>2</sub>O, 2.5 mL·min<sup>-1</sup>) to yield **2** (6 mg, *t<sub>R</sub>* 17 min), **1** (4.4 mg, *t<sub>R</sub>* 29 min), and **11** (700 mg, *t<sub>R</sub>* 40 min).

Aspergillamide C (**1**): colorless oil, [ $\alpha$ ]<sub>D</sub><sup>25</sup> -54 (*c* 0.20, MeOH); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 280 (2.83), 220 (3.30), 200 (3.63) nm; CD (0.25 mg·mL<sup>-1</sup>, MeOH)  $\lambda_{\max}$  ( $\Delta\epsilon$ ) 209 (+8.64), 227 (-4.65) nm; IR (film)  $\nu_{\max}$  3292, 2966, 2873, 1647, 1558,

1506, 1456, 1338 cm<sup>-1</sup>; HR-ESI-MS *m/z* 513.2482 [M + Na]<sup>+</sup> (Calcd. for C<sub>28</sub>H<sub>34</sub>N<sub>4</sub>NaO<sub>4</sub>, 513.2478); For <sup>1</sup>H and <sup>13</sup>C NMR data, see Table 1.

Aspergillamide D (**2**): colorless oil, [ $\alpha$ ]<sub>D</sub><sup>25</sup> -53 (*c* 0.21, MeOH); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 280 (2.64), 220 (3.36), 200 (3.65) nm; ECD (0.20 mg·mL<sup>-1</sup>, MeOH)  $\lambda_{\max}$  ( $\Delta\epsilon$ ) 201 (+10.65), 227 (-6.51) nm; IR (film)  $\nu_{\max}$  3305, 2968, 2876, 1616, 1539, 1516, 1458, 1089 cm<sup>-1</sup>; HR-ESI-MS *m/z* 513.2488 [M + Na]<sup>+</sup> (Calcd. for C<sub>28</sub>H<sub>34</sub>N<sub>4</sub>NaO<sub>4</sub>, 513.2478); For <sup>1</sup>H and <sup>13</sup>C 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 (Invitrogen, Carlsbad, USA) by monitoring the hydrolysis of the fluorogenic phosphatase substrate *p*-nitrophenyl phosphate (*p*NPP) (Aladdin) according to the manufacturer's instruction. IC<sub>50</sub> of compounds with more than 60% of inhibitory activity against MptpB was determined at different concentrations using two-fold serial dilution (1.5625–100  $\mu\text{mol}\cdot\text{L}^{-1}$ ). Oleonic acid was used as positive control (IC<sub>50</sub> 22.1  $\pm$  2.4  $\mu\text{mol}\cdot\text{L}^{-1}$ ). To determine the type of inhibition, different inhibitor concentrations (0, 5, and 10  $\mu\text{mol}\cdot\text{L}^{-1}$ ) and different concentrations of *p*NPP (0.05, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 mmol·L<sup>-1</sup>) 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 in 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<sup>-1</sup> of penicillin and 100  $\mu\text{g}\cdot\text{mL}^{-1}$  of streptomycin, at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. 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  $\times$  10<sup>3</sup> 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  $\mu\text{L}$  of MTT solution (5 mg·mL<sup>-1</sup> stock) was added to the cells, and then incubated for 1 h at 37 °C. The medium was removed carefully and 150  $\mu\text{L}$  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  $\mu\text{mol}\cdot\text{L}^{-1}$  on the preliminary screen-

ing process.

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