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ORIGINAL ARTICLE

A new cytotoxic indole-3-ethenamide from the halotolerant fungus *Aspergillus sclerotiorum* PT06-1

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A new cytotoxic indole-3-ethenamide (1) and two known compounds, 7-(3-methylbut-2-enyl)-1*H*-indole-3-carbaldehyde (2) and emodin (3) were isolated and identified from the ethyl acetate extract of *Aspergillus sclerotiorum* PT06-1 in a hypersaline nutrient-rich medium. On the basis of spectroscopic analysis and amino-acid analysis, the new structure of 1 was determined to be (*S*,*E*)-3-methyl-2-(*N*- methylacetamido)-*N*-(2-(7-(3-methylbut-2-enyl)-1H-indol-3-yl)vinyl)butanamide within 3:1 ratio of rotamers along the acetamido single bond in DMSO-d_6 at room temperature. Compound 1 showed moderate cytotoxicity against A-549 cells and weak cytotoxicity against HL-60 cells with the IC_{50} values of 3.0 and 27 μM, respectively. Compound 2 has been separated as natural product for the first time, and its NMR data were also reported for the first time in this study.
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Keywords: *Aspergillus sclerotiorum*; cytotoxicity; halotolerant fungus; indole-3-ethenamide

INTRODUCTION

Natural products produced by halotolerant microorganisms have been considered as an important source of bioactive novel compounds,[1–4] as hypersaline environment might activate some silent genes[5] and induce unique biosynthesis pathways.[6] Meanwhile, because of many of small peptides possessing a potential or an established use in cancer therapy, these compounds have intrigued us.[7] Our previous work revealed two novel cyclic hexapeptides,[8] and 11 new aspochracin-type cyclic tripeptides[9] from the metabolites of marine-derived halotolerant fungus *Aspergillus sclerotiorum* PT06-1 in hypersaline nutrient-limited and nutrient-rich media, respectively. Further chemical study led to the identification of a new indole-3-ethenamide (**1**), along with two known compounds, 7-(3-methylbut-2-enyl)-1*H*-indole-3-carbaldehyde (**2**)[10] and emodin (**3**)[11] from the ethyl acetate extract of the fermentation broth of *A. sclerotiorum* PT06-1 in a hypersaline nutrient-rich medium. New compound **1** displayed cytotoxicity against A-549 and HL-60 cell lines with IC_{50} values of 3.0 and 27 μM, respectively. NMR Data of **2**, isolated from the natural source for the first time, was also reported in this study for lack of the corresponding data in literature.

RESULTS

Physico-chemical properties

Compound **1**: yellow amorphous powder; $[\alpha]_D^{25}$ –67.5 (*c* 0.3, CH_3OH); HR-ESI-MS *m/z* 382.2490$[M+H]^+$ (calcd for $C_{23}H_{32}N_3O_2$, 382.2495); UV (CH_3OH) λ_{max} (log ε) nm 202 (4.67), 224 (4.56), 292 (4.43); IR ν_{max} cm^{-1} (KBr) 3388, 3279, 2969, 2930, 1689, 1633, 1532, 1447, 1324, 1226, 1060, 961, 779 cm^{-1}. ^1H and ^{13}C NMR (see Table 1).

Compound **2**: yellow amorphous powder; ^1H NMR ($CDCl_3$, 600 MHz) δ_H 12.16 (1H, br s, H-1), 9.94 (1H, s, 3-CHO), 8.29 (1H, d, *J*=2.2 Hz, H-2), 7.93 (1H, d, *J*=7.3 Hz, H-4), 7.15 (1H, 't' like, *J*=7.3, 7.0 Hz, H-5), 7.03 (1H, d, *J*=7.0 Hz, H-6), 5.41 (1H, tt, *J*=7.3, 1.5 Hz, H-2′), 3.58 (2H, d, *J*=7.3 Hz, H-1′), 1.73 (6H, s, H-4′/5′); ^{13}C NMR ($CDCl_3$, 150 MHz) δ_C 185.0 (d, 3-CHO), 138.3 (d, C-2), 135.8 (s, C-7a), 132.6 (s, C-3′), 125.6 (s, C-3a), 124.2 (s, C-7), 122.6 (d, C-5), 122.4 (d, C-6), 121.8 (d, C-2′), 118.6 (s, C-3), 118.5 (d, C-4), 28.9 (t, C-1′), 25.5 (q, C-4′), 17.8 (q, C-5′).

Cytotoxicity

The cytotoxicity of **1** was evaluated against A-549 cell line by the SRB method and HL-60 cell line by the MTT method, with the IC_{50} values of 3.0 μM and 27 μM, respectively.

Structure determination

Compound **1**, obtained as single peak during HPLC separation, was isolated as yellow amorphous powder. The molecular formula of **1** was determined to be $C_{23}H_{31}N_3O_2$ from the positive HRESIMS peak at *m/z* 382.2490 $[M+H]^+$ (cacld for $C_{23}H_{32}N_3O_2$, 382.2495). UV absorptions at λ_{max} at 202, 224 and 292 nm indicated an aromatic chromophore or extended conjugation. Broad IR absorptions at 3279 and 1633 cm^{-1} indicated the presence of amide NH and an amide

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carbonyl, respectively. The NMR data for **1**, however, were much more complex than expected. Interestingly, two sets of NMR signals appeared, with the ratio of 3:1 in DMSO-d_6 at room temperature. The ^{1}H and ^{13}C NMR spectra (Supplementary Figures S2 and S3) of the major one (**1a**) displayed characteristic signals for one indole and one isopentenyl moiety. ^{1}H-^{1}H COSY (Supplementary Figure S6) of HN-1/H-2, H-4/H-5/H-6 and H-8/H-9/HN-10, and the key HMBC (Supplementary Figure S7) correlations from H-1′ (δ_H 3.52) to C-6 (δ_C 120.7), C-7 (δ_C 125.0), C-7a (δ_C 135.7) and C-3′ (δ_C 132.3), from H-8 (δ_H 6.44) to C-2 (δ_C 123.5) and C-3a (δ_C 125.0) and from H-4′/H-5′ (δ_H 1.71) to C-2′ (δ_C 122.3), and C-3′ further supported that a 7-isopentenylindol-3-(2-aminoethenyl) indole moiety was presented in **1a**. The large coupling constant, $J_{8,9}$ (15.1 Hz) indicated *E*- configuration of the C_8=C_9 double bond. In addition, ^{1}H-^{1}H COSY of H-12/H-16/H-17 and H-16/H-18 and HMBC connections from H-12 (δ_H 4.71) to C-11 (δ_C 167.4) and C-14 (δ_C 170.9), from H-19 (δ_H 3.00) to C-12 (δ_C 60.9) and C-14, and from H-15 (δ_H 2.06) to C-14 suggested that a *N*-acetyl-*N*-methylvaline unit was also presented in **1a** (Figure 1). The key HMBC correlation between HN-10 (δ_H 10.16) and C-11 revealed that the two moieties were further connected into amide. The minor one (**1b**) shared the same 2D NMR correlations as those of **1a** (Supplementary Figure S5–S8). The obvious differences of ^{1}H and ^{13}C NMR between **1a** and **1b** main were δ_{CH-12}, δ_{CH-19} and δ_{H-15} (Table 1), indicating that **1a** and **1b** was a pair of conformers resulted from the rotation of acetamido single bond, $N_{13}C_{14}$. The synperiplanar (*sp*)/antiperiplanar (*ap*) interconversion of amide rotamers is sufficiently slow on the NMR spectroscopy that display steric and electronic differences.[12] The *sp* and *ap* configurations of **1a** and **1b,** respectively, were deduced from the upfield shift for CH-12 in **1a** and CH_3-19 in **1b**, respectively, due to the shielding effect of 14-CO.[13] This deduction was further supported by the NOE effects between H-15 and H-19 in **1a**, and between H-15 and H-12 in **1b**, respectively, in NOESY experiment (Figure 1 and Supplementary Figure S8). The absolute configuration of *N*-methylvaline unit was determined as L- by Marfey's method.[14] The FDAA derivatives of the acid hydrolysates of **1** gave the same retention time as that prepared from authentic *N*-Me-L-Val in HPLC analysis (Supplementary Figure S1). Thus, structure of **1** was determined to be (*S*,*E*)-3-methyl-2-(*N*-methylacetamido)-*N*-(2-(7-(3-methylbut-2-enyl)-1H-indol-3-yl)vinyl) butanamide. To the best of our knowledge, dipeptides possessing dehydrotryptamine functionality were scarcely found in nature.

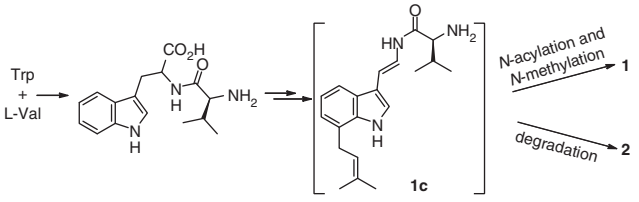
Table 1 ^{1}H and ^{13}C NMR (600 and 150 MHz) data for compound 1 (1a and 1b)[a]

	1a		*1b*	
Position	δ_H *(J in Hz)*	δ_C	δ_H *(J in Hz)*	δ_C
1-NH	11.08 (1H, br s)	—	11.12 (1H, br s)	—
2	7.41 (1H, d, 2.8)	123.5 d	7.46 (1H, d, 2.3)	123.7 d
3	—	112.2 s	—	112.2 s
3a	—	125.0 s	—	125.0 s
4	7.45 (1H, d, 7.8)	116.8 d	7.47 (1H, d, 7.8)	116.8 d
5	6.99 (1H, t, 7.8)	119.7 d	6.99 (1H, t, 7.8)	119.8 d
6	6.89 (1H, d, 7.3)	120.7 d	6.90 (overlay)	120.7 d
7	—	125.0 s	—	125.0 s
7a	—	135.7 s	—	135.7 s
8	6.44 (1H, d, 15.1)	107.4 d	6.48 (1H, d, 15.1)	107.8 d
9	7.27 (1H, dd, 15.1, 10.1)	119.4 d	7.30 (1H, dd, 15.1, 10.1)	119.2 d
10-NH	10.16 (1H, d, 10.1)	—	10.26 (1H, d, 10.1)	—
11	—	167.4 s	—	166.7 s
12	4.71 (1H, d, 11.0)	60.9 d	3.88 (1H, d, 10.6)	66.3 d
14	—	170.9 s	—	170.3 s
15	2.06 (3H, s)	22.2 q	2.16 (3H, s)	22.3 q
16	2.16 (1H, m)	26.9 d	2.25 (1H, m)	27.4 d
17	0.90 (3H, d, 6.4)	19.5 q	0.92 (3H, d, 6.4)	19.4 q
18	0.79 (3H, d, 6.9)	19.0 q	0.84 (3H, d, 6.4)	18.7 q
19	3.00 (3H, s)	31.4 q	2.82 (3H, s)	28.4 q
1′	3.52 (2H, d, 6.9)	29.1 t	3.52 (2H, d, 6.9)	29.1 t
2′	5.42 (1H, t, 6.6)	122.3 d	5.42 (1H, t, 6.6)	122.3 d
3′	—	132.3 s	—	132.3 s
4′	1.71 (3H, s)	25.7 q	1.71 (3H, s)	25.7 q
5′	1.71 (3H, s)	17.9 q	1.71 (3H, s)	17.9 q

[a]The assignments were based on DEPT, HMQC, ^{1}H-^{1}H COSY and HMBC experiments, and recorded in DMSO-d_6 using TMS as internal standard.

Biosynthesis

Compound **1** was probably biosynthesized mainly via a mixed amino-acid mevalonic-acid pathway. Tryptophan and valine condensed to a dipeptide that then underwent decarboxylation, dehydrogenation and isoprenylation with mevalonic acid to form an intermediate **1c**. The intermediate **1c** was postulated to further undergo *N*-acylation



Scheme 1 The postulated biosynthesis of **1** and **2**.

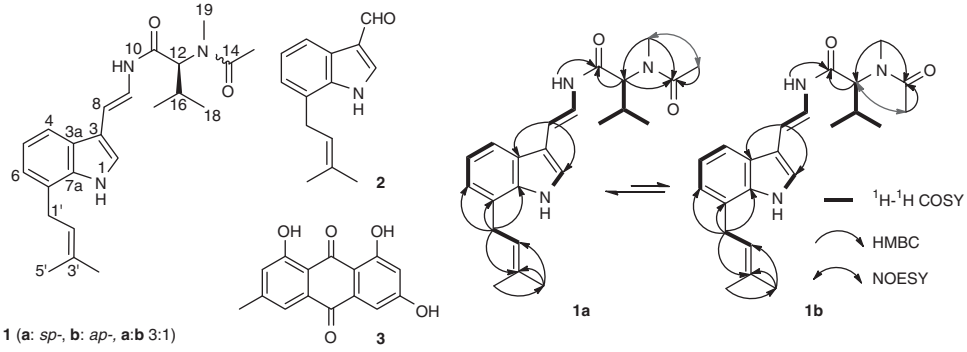


Figure 1 Structures of **1**–**3** and the key ^{1}H-^{1}H COSY, HMBC and NOESY correlations of **1** (**a** and **b**). A full color version of this figure is available at *The Journal of Antibiotics* journal online.

and *N*-methylation to produce the bioactive **1**. Compound **2** might be resulted from the degradation of intermediate **1c** (Scheme 1).

METHODS

General experimental procedures

Optical rotations were obtained on a JASCO P-1020 digital polarimeter (JASCO Corporation, Tokyo, Japan). IR spectra were recorded using a Bruker model. UV spectra were recorded on Beckman DU 640 spectrophotometer (Beckman, Brea, CA, USA). ^{1}H, ^{13}C NMR and DEPT spectra and 2D-NMR were recorded on a JEOL JNM-ECP 600 spectrometer (JEOL Ltd., Tokyo, Japan) using TMS as internal standard and chemical shifts were recorded as δ values. ESI-MS was measured on a Q-TOF Ultima Global GAA076 LC mass spectrometer (Waters, Milford, MA, USA). Semipreparative HPLC was performed using an ODS column (YMC-pack ODS-A, 10×250 mm, 5 μM, 4 ml min^{-1}).

Strain

The halotolerant fungus *A. sclerotiorum* PT06-1 was isolated from sediments collected in the Putian salt field, Fujian Province of China. It was identified according to its morphological characteristics and 18S rRNA sequences.[8,9] The voucher specimen is deposited in our laboratory at −80 °C. The working strain was prepared on potato dextrose agar slants containing 10% NaCl and stored at 4 °C.

Fermentation

The fungus *A. sclerotiorum* PT06-1 was incubated on a rotary shaker (160 rpm) at 28 °C for 16 days in 500 ml×200 conical flasks containing the liquid medium (150 ml per flask) composed of glucose (1.5 g), maltose (3 g), mannitol (3 g), yeast extract (0.45 g), monosodium glutamate (1.5 g), corn steep liquor (0.15 g), NaCl (12 g), $MgSO_4$ (0.75 g), KH_2PO_4 (0.75 g), NH_4Cl (0.75 g) and KCl (0.75 g), adjusting its pH to 7.0.

Extraction and isolation

The fermented whole broth (30 l) was filtered through cheesecloth to separate into supernatant and mycelia. The former was concentrated *in vacuo* to about a quarter of original volume and then extracted three times with the same volume of ethyl acetate to give an ethyl acetate solution, while the latter was extracted three times with 5 l acetone-H_2O (4:1). The acetone solution was evaporated under reduced pressure to afford an aqueous solution. The aqueous solution was extracted three times with the same volume of ethyl acetate to give another ethyl acetate solution. Both the ethyl acetate solutions were combined and concentrated *in vacuo* to give a crude extract (50.2 g). The crude extract was then subjected to vacuum liquid chromatography using step gradient elution with $CHCl_3$–MeOH (100:0, 100:1, 50:1, 30:1, 20:1, 10:1) to give six fractions (fractions 16) based on TLC properties. Fraction 3 (7 g) eluted with $CHCl_3$–MeOH (50:1) was further applied to ODS column chromatography using step gradient elution with H_2O-MeOH into five subfractions (fractions 3.13.5). The subfraction 3.4 (480 mg), eluted with MeOH–H_2O (4:1), was then separated by semipreparative HPLC (80% MeOH-H_2O) to yield **3** (10 mg, t_R 5 min), **2** (3 mg, t_R 10 min) and **1** (8 mg, t_R 16 min).

Absolute configuration of amino acid in 1 by Marfey's method

Compound **1** (1 mg) was hydrolyzed in HCl (6 M; 1 ml) for 20 h at 110 °C.[14] The solution was then evaporated to dryness and redissolved in H_2O (250 μl). A 100 μl of 1% (w/v) solution of L-FDAA (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) in acetone was then added to an aliquot (50 μl) of the acid hydrolysate solution. After addition of $NaHCO_3$ solution (1M; 20 μl), the mixture was incubated at 45 °C for 1 h. The reaction was quenched by addition of HCl (2 M, 10 μl). Analyses of the FDAA derivatized hydrolysate of **1** and standard FDAA-derivatized *N*-Me-Val were carried out by HPLC (solvents: A water+0.2%TFA, B MeCN; linear gradient: 0 min 25% B, 40 min 60% B, 45 min 100% B; 30 °C 1 ml min^{-1}; UV detection at λ 340 nm). Retention times for the *N*-Me-valine (*N*-Me-Val) derivatives of hydrolysates of **1** and the authentic *N*-Me-L-Val and *N*-Me-D-Val were t_R 28.3 min, 28.4 min and 30.8 min, respectively. Co-injection of the authentic sample with the hydrolysate confirmed that the *N*-Me-Val residue was L-configuration (Supplementary Figure S1).

Biological assay

Compounds **1** was evaluated for cytotoxic effects on HL-60 cell line using the MTT method[15] and on A-549 cell line using the SRB method.[16] In the MTT assay, cell lines were grown in RPMI-1640 supplemented with 10% FBS under a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cell suspensions, 200 μl, at a density of 5×10^4 cell per ml were plated in 96-well microtiter plates and incubated for 24 h. Thereafter, 2 μl of the test solutions (in MeOH) were added to each well and further incubated for 72 h. The MTT solution (20 μl, 5 mg ml^{-1} in IPMI-1640 medium) was then added to each well and incubated for 4 h. Old medium containing MTT (150 μl) was then gently replaced by DMSO and pipetted to dissolve any formazan crystals formed. Absorbance was then determined on a Spectra Max Plus plate reader (Molecular Devices, Sunnyvale, CA, USA) at 540 nm. In the SRB assay, 200 μl of the cell suspensions were plated in 96-well plates at a density of 2×10^5 cell per ml. Thereafter, 2 μl of the test solutions (in MeOH) was added to each well and the culture was further incubated for 24 h. The cells were fixed with 12% trichloroacetic acid and the cell layer stained with 0.4% SRB. The absorbance of the SRB solution was measured at 515 nm. The IC_{50} values were obtained using the Bliss method.

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