A new cyclopeptide metabolite of marine gut fungus from *Ligia oceanica*

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A new cyclopeptide, together with three known amino acid derivatives, was isolated from marine-derived fungus *Aspergillus flavipes*, which was found in the gut of isopod *Ligia oceanica*. The novel peptide contains four amino acid units, proline, 5-methoxyanthranilic acid, isoleucine and 3-aminoacrylic acid. Its structure was determined on the basis of NMR, HR-MS and MS\(^n\) spectral data analysis. The two unusual amino acid residues, 5-methoxyanthranilic acid and 3-aminoacrylic acid, were first found in natural product. The known compound \(N\)-benzoyl-phenylalanine methyl ester was first found as fungal metabolite. This is the first report of natural products isolated from marine gut fungi.

**Keywords**: gut fungus; *Aspergillus flavipes*; cyclopeptide; 5-methoxyanthranilic acid; 3-aminoacrylic acid

1. Introduction

In recent years marine-derived fungi have become an important resource of natural products. Hundreds of fungal metabolites were reported one year and these compounds usually showed cytotoxicity, anti-fouling, anti-infective and anti-parasitic activities (Rateb & Ebel 2011). So far the major sources of marine fungi are alga and sponges. Actually various microorganisms including fungi were found in the gut of marine isopods (Cafaro 1999, 2000; White 1999; Zimmer et al. 2001; Roa et al. 2009). These gut symbiote showed some ecological roles such as chemical defence (Lindquist et al. 2005). Therefore, gut fungi may be an interesting source of bioactive marine natural products. In the course of our chemical study of marine fungal metabolites (Xu et al. 2006, 2008), a new cyclopeptide, together with three known ones (Figure 1), was isolated from the broth of *Aspergillus flavipes* (Z-4), which were found in the gut of marine isopod *Ligia oceanica*. *A. flavipes* is an active producer of natural products including cytochalasans (Zhou et al. 2004; Rochfort et al. 2005; Lin et al. 2009), phenols (Kwon et al. 2010, 2012), cerebrosides (Jiang et al. 2004), alkaloids (Barrow & Sun 1994), peptide (Barrow et al. 1994) and so on. In this work, the structural identification of one new cyclopeptide was described by NMR and mass spectral data analysis.

2. Results and discussion

HR-ESI-MS data of 1 showed quasi-molecular ion peaks at \(m/z\) 429.2136 \([M + H]^+\) and \(m/z\) 879.4009 \([2M + Na]^+\) and indicated that the molecular formula is \(C_{22}H_{28}N_4O_5\) (calcd for \(C_{22}H_{29}N_4O_5\), 429.2132). The result was supported by 1D NMR data. \(^{13}\)C NMR gave four
carboxyl signals (170.2, 168.6, 168.4 and 167.7) and indicated that 1 was a tetrapeptide and the interpretation of 1D (see Section 3.3) and 2D NMR (Figure 2S) data also revealed four amino acid residues (A–D). In the proton NMR spectrum, three signals [(δ 8.28, d, J = 8.8 Hz), (δ 7.00, dd, J = 8.8, 2.8 Hz), (δ 6.95, d, J = 2.8 Hz)] indicated a 1,2,4-trisubstituted benzene group. These substitutes were determined as carbonyl, methoxyl and NH through HMBC (heteronuclear multiple-bond correlation) signals between signals of both benzene cycle and substitutes, from δH 3.82 (CH3O) to δC 155.7, δH 8.28 to δC 170.2 (carbonyl), and δH 9.72 (NH) to δC 125.8, respectively. Thus substructure A was identified as 5-methoxyanthranilic acid residue. The 1H–1H COSY correlations among proton signals of (δ 9.44, d, J = 11.6 Hz), (δ 5.94, dd, J = 11.6, 9.0 Hz) and (δ 5.30, d, J = 9.0 Hz) indicated a −NH−CH−CH− group with Z configuration. Additional HMBC signals from δH 5.30 to δC 167.7 confirmed a 3-aminoacrylic acid residue (C). Three aliphatic methine groups (δC 58.8, 58.3, 32.3), four aliphatic methylene groups (δC 39.6, 26.6, 24.9, 21.0) and two methyl groups (δC 16.0, 10.3) (DEPT135 and DEPT90, see Supplementary file) were assigned to two independent spin systems of the types CH2−CH2−CH2−CH and CH3−CH2−CH(CH)−CH3 by heteronuclear multiple quantum coherence and 1H−1H COSY spectra (Figure S2), suggesting the existence of proline (B) and isoleucine (D) residues. HMBC signals from δH 9.72 to δC 168.4 and δH 9.44 to δC 168.6 indicated the linkages between A and B, C and D, respectively. The result was supported by the major ESI-MSn fragment ion peaks at m/z 181 [M + H − A − B]+ and m/z 153 [M + H − A − B − CO]+. Thus compound 1 was determined as shown in Figure 1. The tetrapeptide includes two unusual amino acid residues, 5-methoxyanthranilic acid and 3-aminoacrylic acid. To the best of our knowledge, this is the first report of cyclopeptide containing these two amino acid residues. Known compounds (2–4) were also identified as asperphenamate, N-benzoyl-phenylalanine methyl ester and N-benzoylphenylalaninol, respectively, by the 1D and 2D NMR data analysis and the comparison with reported data (Clark & Hufford 1977; Balunas et al. 2009).

The fungus A. flavipes, terrestrial or marine, was reported to produce various secondary metabolites. This is the first report about this strain isolated from the gut environment of marine isopod. Its products included one novel cyclic tetrapeptide containing two unusual amino acid residues, 5-methoxyanthranilic acid and 3-aminoacrylic acid residues. One of the known
products, N-benzoyl-phenylalanine methyl ester, was isolated as a natural product from a plant Brassaiopsis glomerulata. This is the first time reported as a fungal metabolite. It is wondered that these rare metabolites were related with gut environment.

3. Experimental

3.1. General experimental procedures
NMR spectra were recorded on a Bruker DRX 400 with trimethylsilyl as internal standard. HR-ESI-MS spectra were measured on an Agilent 6224 TOF LC/MS. MS/MS spectra were obtained by Finnigan LTQ linear ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). Medium 2216E (peptone 5.0 g, yeast extract 1.0 g, FePO₄ 0.01 g dissolved in 1000 mL seawater, pH adjusted to 7.6–7.8, added 16 g agar for solid medium).

3.2. Fungal isolation and fermentation
Marine isopod L. oceanica was collected in Zhoushan, Zhejiang province of China, in December 2011. Gut fungus (Z-4) was grown on solid 2216E medium (Qingdao Hope Biol-Technology Co., Ltd, Qingdao, China) for a period of 4 weeks at room temperature. Z-4 was identified as A. flavipes by 18S rDNA analysis. The strain Z-4 was cultured in 500-mL Erlenmeyer flasks containing 2216E liquid media (peptone 5.0 g, yeast extract 1.0 g, FePO₄ 0.01 g dissolved in 1000 mL seawater, pH adjusted to 7.6–7.8, 200 mL £ 100) for 4 weeks at room temperature.

3.3. Extraction and purification
The culture broth was filtered and extracted with ethyl acetate (EtOAc). The extract (10 g) was subjected to silica gel column chromatography eluted in gradient with hexane–EtOAc (98:2, 95:5, 90:10, 80:20, 50:50) and CH₂Cl₂–MeOH (95:5, 90:10, 80:20 and 50:50), successively. Subfraction (hexane–EtOAc 70:30) was further separated by silica gel column chromatography eluted with hexane–EtOAc (80:20) and its fractions were purified by semi-preparative HPLC (COSMOSIL PACKED COLUMN, 5C18-MS-II column, 10ID £ 250 mm) to obtain compound 1 (3 mg, 58% ACN–H₂O), Asperphenamate (18 mg, 76% MeOH–H₂O), N-benzoyl-phenylalaninol (3 mg, 43% ACN–H₂O) and N-benzoyl-phenylalanine methyl ester (17 mg, 43% ACN–H₂O). The elution of these compounds was monitored at 254 nm.

3.3.1. Compound 1
C₂₂H₂₈N₄O₅, HR-ESI-MS m/z 429.2136 [M + H]+ (calcld for C₂₂H₂₉N₄O₅, 429.2132) and m/z 879.4009 [2M + Na]+.¹H (400 MHz, CDCl₃): δ 8.28 (d, J = 8.8 Hz, A-2), 7.00 (dd, J = 8.8, 2.8 Hz, A-3), 6.95 (d, J = 2.8 Hz, A-5), 9.72 (s, A-NH), 3.82 (s, A-MeO), 4.55 (d, J = 4.4 Hz, B-1), 2.62 (d, J = 13.2 Hz, B-2α), 1.68 (m, B-2β), 1.78 (m, B-3α), 1.45 (m, B-3β), 4.72 (d, J = 12.2 Hz, B-4α), 2.81 (m, B-4β), 5.30 (d, J = 9.0 Hz, C-1), 5.94 (dd, J = 11.6, 9.0 Hz, C-2), 9.44 (d, J = 11.6 Hz, C-NH), 4.19 (d, J = 9.6 Hz, D-1), 2.13 (m, D-2), 1.45 (m, D-3), 0.98 (d, J = 6.8 Hz, D-4), 0.96 (t, J = 6.8 Hz, D-5), 7.12 (m, D-NH) and ¹³C NMR (100 MHz, CDCl₃): δ 170.2 (A–C–O), 129.4 (A-1), 123.4 (A-2), 116.0 (A-3), 155.7 (A-4), 113.2 (A-5), 125.8 (A-6), 55.7 (A-MeO), 168.4 (B–C–O), 58.8 (B-1), 26.6 (B-2), 21.0 (B-3), 39.6 (B-4), 167.7 (C–C–O), 104.8 (C-1), 129.5 (C-2), 168.6 (D–C–O), 58.3 (D-1), 32.2 (D-2), 24.9 (D-3), 16.0 (D-4), 10.3 (D-5).

4. Conclusion
This work reported that four amino acid derivatives, one novel peptide and three known peptides, were isolated from a marine gut fungus. The new peptide concluded two unusual amino
acid residues and known compound N-benzyol-phenylalanine methyl ester was isolated as fungal metabolite for the first time. This report demonstrates that gut fungi are the possible producers of marine natural products.

Supplementary material
Supplementary material relating to this article is available online.

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References
Balunas MJ, Su B, Riswan S, Fong HHS, Brueggemeier RW, Pezzuto JM, Kinghorn AD. 2009. Isolation and characterization of aromatase inhibitors from Brassaiopsis glomerulata (Araliaceae). Phytochem Lett. 2:29–33.
Barrow CJ, Doleman MS, Bobko MA, Cooper R. 1994. Structure determination, pharmacological evaluation, and structure-activity studies of a new cyclic peptide substance P antagonist containing the new amino acid 3-prenyl-beta-hydroxytyrosine, isolated from Aspergillus flavipes. J Med Chem. 37:356–363.
Barrow CJ, Sun HH. 1994. Spiroquinazoline, a novel substance P inhibitor with a new carbon skeleton, isolated from Aspergillus flavipes. J Nat Prod. 57:471–476.
Cafaro MJ. 1999. Baltomyces, a new genus of gut-inhabiting fungi in an isopod. Mycologia. 91:517–519.
Cafaro MJ. 2000. Gut fungi of isopods: the genus Palavascia. Mycologia. 92:361–369.
Clark AM, Hufford CD. 1977. Synthesis of asperphenamate, a novel fungal metabolite. Phytochemistry. 17:552–553.
Jiang T, Li T, Li J, Fu HZ, Pei YH, Lin WH. 2004. Cerebroside analogues from marine-derived fungus Aspergillus flavipes. J Asian Nat Prod Res. 6:249–257.
Kwon YJ, Sohn MJ, Kim CJ, Koshino H, Kim WG. 2012. Flavimycins A and B, dimeric 1,3-dihydroisobenzofurans with peptide deformylase inhibitory activity from Aspergillus flavipes. J Nat Prod. 75:271–274.
Kwon YJ, Zheng CJ, Kim WG. 2010. Isolation and identification of FR198248, a Hydroxylated 1,3-dihydroisobenzofuran, from Aspergillus flavipes as an inhibitor of peptide deformylase. Biosci Biotechnol Biochem. 74:390–393.
Lin ZJ, Zhang GJ, Zhu TJ, Liu R, Wei HJ, Gu QQ. 2009. Bioactive cytochalasins from Aspergillus flavipes, an endophytic fungus associated with the mangrove plant acanthus ilicifolius. Helv Chim Acta. 92:1538–1544.
Lindquist N, Barber PH, Weisz JB. 2005. Episymbiotic microbes as food and defence for marine isopods: unique symbioses in a hostile environment. Proc R Soc Lond Ser B-Biol Sci. 272:1209–1216.
Rateb ME, Ebel R. 2011. Secondary metabolites of fungi from marine habitats. Nat Prod Rep. 28:290–344.
Roa JJH, Virella CR, Cafaro MJ. 2009. First survey of arthropod gut fungi and associates from Vieques, Puerto Rico. Mycologia. 101:896–903.
Rochfort S, Ford J, Owenen S, Wan SS, George S, Wildman H, Tait RM, Meurer-Grimes B, Cox S, Coates J, Rhodes D. 2005. A novel aspochalasin with HIV-1 integrase inhibitory activity from Aspergillus flavipes. J Antibiot. 58:279–283.
White MM. 1999. Legerioides, a new genus of Harpellales in isopods and other Trichomycetes from New England, USA. Mycologia. 91:1021–1030.
Xu JZ, Nakazawa T, Ukai K, Kobayashi H, Mangindaan REP. Wewengkang DS, Rotinsulu H, Namikoshi M. 2008. Tetrahydrodrostroycin and 1-deoxysterterahydrostroycin, two new hexahydroanthrone derivatives, from a marine-derived fungus Aspergillus sp. J Antibiot. 61:415–419.
Xu JZ, Takasaki A, Kobayashi H, Oda T, Yamada J, Mangindaan REP, Ukai K, Nagai H, Namikoshi M. 2006. Four new macrocyclic trichothecenes from two strains of marine-derived fungi of the genus Myrothecium. J Antibi. 59:451–455.
Zhou GX, Wieratine EMK, Bigelow D, Pierson LS, VanEten HD, Gunatilaka AAL. 2004. Aspochalasins I, J, and K: three new cytotoxic cytochalasans of Aspergillus flavipes from the rhizosphere of Ericameria laricifolia of the Sonoran Desert. J Nat Prod. 67:328–332.
Zimmer M, Danko JP, Pennings SC, Danford AR, Ziegler A, Uglow RF, Carefoot TH. 2001. Hepatopancreatic endosymbionts in coastal isopods (Crustacea: Isopoda), and their contribution to digestion. Mar Biol. 138:955–963.