An Antimalarial Tetrapeptide from the Entomopathogenic Fungus *Hirsutella* sp. BCC 1528

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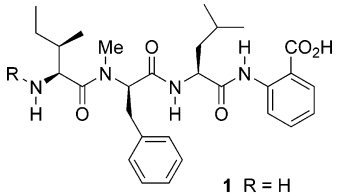
Hirsutellic acid A (**1**), a new linear tetrapeptide possessing an anthranilic acid residue at the C-terminus, was isolated from a fermentation broth of the entomopathogenic fungus *Hirsutella* sp. BCC 1528. The structure of this compound was elucidated by NMR and MS analyses, and its absolute configuration was deduced by HPLC analysis of the acid hydrolysate using a chiral column. Hirsutellic acid A exhibits activity against the malarial parasite *Plasmodium falciparum* K1 with an IC_{50} value of 8.0 μM, while it was noncytotoxic to Vero cells at a concentration of 95 μM.

As part of our research program on bioactive fungal metabolites, we have been investigating the constituents from fermentation broths of insect pathogenic fungi that were collected from various places in Thailand.[1] We report herein the isolation, structure elucidation, and biological activities of a new tetrapeptide, hirsutellic acid A (**1**), from *Hirsutella* sp. BCC 1528 (collected on an Orthoptera-cricket nymph/leaf litter). Recently there have been several reports, including our own work, on the low molecular weight secondary metabolites from the genus *Hirsutella*: hirsutellones (antitubercular alkaloids)[2] and hirsutatins (cyclohexadepsipeptides)[3] from *H. nivea* BCC 2594, hirsutellide A (a cyclohexadepsipeptide) from *H. kobayasii* BCC 1660,[4] and hirsutide (a cyclotetrapeptide) from a New Zealand *Hirsutella* sp. (CANU-E1101).[5]



1 R = H
2 R = Ac

Hirsutellic acid A (**1**) was isolated as a colorless amorphous solid. The molecular formula of **1** was determined by HRMS (ESI-TOF) and ^{13}C NMR as $C_{29}H_{40}N_4O_5$. The IR spectrum shows broad and intense absorption bands at ν_{max} 3447 and 1653 cm^{-1}. A tetrapeptide structure of **1** was suggested by ^{1}H and ^{13}C NMR spectra, exhibiting three amide carbonyl carbons and a carboxylic acid (δ_C 168.9, 171.1, 171.3, and 173.9). Analysis of NMR data (^{1}H, ^{13}C, DEPT, COSY, HMQC, and HMBC) revealed the structures of three amino acid residues: an isoleucine (Ile), a leucine (Leu), and an *N*-methylphenylalanine (*N*-Me-Phe). The structure of the remaining residue was elucidated by NMR analysis as an anthranilic acid (Abz) derivative. A 1,2-disubstituted benzene moiety with four protons was evident from δ_H 8.70 (H-4, d, J = 8.4 Hz), 7.40 (H-5, br t, J = 7.6 Hz), 6.92 (H-6, br t, J = 7.5 Hz), and 7.87 (H-7, br d, J = 7.5 Hz). HMBC correlations from H-7 (δ_H 7.87) to a carboxyl carbon at δ_C 173.9 (C-1) and a quaternary carbon at δ_C 140.7 (C-3), and from H-5 (δ_H 7.40) to C-3, indicated the proposed partial structure (Figure 1). The ^{1}H and ^{13}C chemical shifts assigned to this aromatic moiety were similar to those of *N*-acetylanthranilic acid. The sequence of the four amino acid residues was established by analysis of HMBC and NOESY data as shown in Figure 1. Thus, the HMBC correlations from amide N-C*H*$_3$ of *N*-Me-Phe to the carbonyl (C-1) of Ile and C-2 of *N*-Me-Phe indicated the connection of Ile and *N*-Me-Phe. NOESY correlations between H-2 of *N*-Me-Phe and amide N*H* of Leu, and between H-2 of Leu and amide N*H* of Abz, revealed the connectivity of *N*-Me-Phe, Leu, and Abz. The presence of free amine at the N-terminus (Ile) was confirmed by acetylation of **1** (Ac_2O, pyridine, rt), giving a monoacetate derivative **2**. The downfield shift of H-2 of Ile (δ_H 5.25, dd, J = 9.4, 2.0 Hz) in **2** was observed (δ_H 4.10 for **1**), and this proton was coupled with acetamide N*H* (δ_H 6.68, d, J = 9.4 Hz).

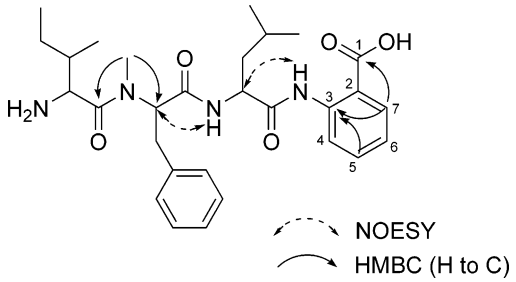


Figure 1. Selected HMBC and NOESY correlations for **1**.

The absolute configuration of the amino acid residues in **1** was addressed by HPLC analysis of its acid hydrolysate using a ligand-exchange-type chiral column (Sumichiral OA-5000). Co-injection using all possible D- and L-amino acids revealed that the hydrolysate of **1** was composed of an L-*allo*-isoleucine, an *N*-methyl-D-phenylalanine, an L-leucine, and an anthranilic acid. It should be noted that naturally occurring peptides possessing an anthranilic acid residue are not common; reported examples are viridic acid (from *Penicillium viridicatum*),[6] cycloaspeptides (from *Aspergillus* sp. and *Penicillium ribeum*),[7,8] and dictinamides (from an unidentified marine fungus K063).[9]

Hirsutellic acid A (**1**) exhibited activity against *Plasmodium falciparum* K1 with an IC_{50} value of 8.0 μM, while it was noncytotoxic to Vero cells (African green monkey kidney fibroblasts) at a concentration of 95 μM. It was also inactive against three cancer cell lines, KB, BC, and NCI-H187, at 38 μM. Although the in vitro antimalarial activity of **1** was much weaker when compared with those of standard drugs, the noncytotoxicity deserves further studies.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO P-1030 digital polarimeter. UV spectra were recorded on a Varian CARY 1E UV−visible spectrophotometer. FT-IR spectra were recorded on a Bruker Vector 22 spectrometer. NMR spectra were recorded on a Bruker AV500D spectrometer. ESI-TOF mass spectra were measured with a Micromass LCT mass spectrometer.

Fungal Material. *Hirsutella* sp. was collected on an Orthoptera-cricket nymph/leaf litter and identified by Dr. Nigel L. Hywel-Jones,

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Table 1. NMR Data for Compound **1** (in $CDCl_3$)

unit/position	δ_C (mult.)	δ_H (mult., *J* in Hz)
Abz		
1	173.9 (s)	
2	119.7 (s)	
3	131.1 (d)	7.87 (d, 7.5)
4	122.4 (d)	6.92 (dd, 7.5, 7.4)
5	133.1 (d)	7.40 (dd, 7.6, 7.6)
6	120.2 (d)	8.70 (d, 8.4)
7	140.7 (s)	
N*H*		12.17 (br s)
L-Leu		
1	171.3 (s)[a]	
2	54.3 (d)[b]	4.64 (m)
3	41.2 (t)	1.84 (m)
		1.63 (m)
4	25.1 (d)	1.62 (m)
5	21.5 (q)	0.91 (d, 6.3)
5′	23.2 (q)	0.95 (d, 6.4)
N*H*		7.19 (s)
N-Me-D-Phe		
1	168.9 (s)	
2	56.9 (d)	5.59 (dd, 10.1, 6.4)
3	34.1 (t)	3.07 (dd, 14.6, 10.5)
		3.21 (dd, 14.7, 6.2)
4	136.2 (s)	
5, 9	129.0 (d)	7.24 (m)
6, 8	128.7 (d)	7.23 (m)
7	127.0 (d)	7.21 (m)
N-*CH*$_3$	30.6 (q)	2.92 (s)
L-*allo*-Ile		
1	171.1 (s)[a]	
2	54.4 (d)[b]	4.10 (d, 3.2)
3	36.1 (d)	1.28 (m)
4	25.8 (t)	1.13 (m)
		1.16 (m)
5	11.7 (q)	0.71 (t, 7.4)
4′	13.4 (q)	0.55 (d, 6.7)

[a,b] Assignment of carbons can be interchanged.

BIOTEC. This fungus was deposited at the BIOTEC Culture Collection (BCC) as BCC 1528 on December 4, 1997.

Fermentation and Isolation. *Hirsutella* sp. BCC 1528 was maintained on potato dextrose agar at 25 °C, cut into pieces (1 × 1 cm) and inoculated into 2 × 250 mL Erlenmeyer flasks containing 25 mL of potato dextrose broth (PDB; composition: potato starch 4.0 g, dextrose 20.0 g, per liter) (10 pieces for each flask). After incubation at 25 °C for 7 days on a rotary shaker (200 rpm), each primary culture was transferred into a 1 L Erlenmeyer flask containing 250 mL of the same liquid medium (PDB) and incubated at 25 °C for 7 days on a rotary shaker (200 rpm). Each 25 mL portion of the secondary cultures (in 2 flasks) was transferred into 20 × 1 L Erlenmeyer flasks each containing 250 mL of PDB, and static fermentation was carried out at 25 °C for 7 days. The cultures were harvested by filtration. The residues (mycelia) were macerated in MeOH (1 L, rt, 3 days) and filtered. The MeOH solution was washed with hexane (700 mL) and concentrated under reduced pressure. The residue was extracted with EtOAc (500 mL) and concentrated to leave a brown gum (390 mg). This mycelial extract was passed through a Sephadex LH-20 column (2.5 × 70 cm) with MeOH as eluent, and the 300−450 mL elute contained the tetrapeptide. This process was repeated to obtain a fraction mainly containing **1** (27.3 mg). Fractionation by silica gel column chromatography ($MeOH/CH_2Cl_2$) gave compound **1** as a colorless amorphous solid (8.3 mg; R_f 0.35, 20% MeOH in CH_2Cl_2). Compound **1** was also isolated from the culture filtrate by EtOAc extraction and subsequent chromatography (10.7 mg).

Hirsutellic acid A (1): colorless solid; mp 131−132 °C; $[\alpha]^{24}_D$ +148 (*c* 0.305, MeOH); UV (MeOH) λ_{max} (log ϵ) 212 (4.77), 254 (4.38), 297 (3.76) nm; IR (KBr) ν_{max} 3447, 1653, 1501, 1372, 758 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 1; HRMS (ESI-TOF) *m*/*z* 547.2854 (calcd for $C_{29}H_{40}N_4O_5Na$, 547.2897) $[M + Na]^+$.

Acetylation of 1. Compound **1** (2.7 mg) was treated with Ac_2O (0.2 mL) in pyridine (0.3 mL) at rt for 24 h. After the usual aqueous workup, the crude product was purified by column chromatography on silica gel (5% MeOH in CH_2Cl_2) to obtain the acetate derivative **2** (3.0 mg).

Compound 2: colorless amorphous solid; ^{1}H NMR ($CDCl_3$, 500 MHz) δ *N*-acetyl-L-*allo*-isoleucine residue, 6.68 (1H, d, *J* = 9.4 Hz, N*H*), 5.25 (1H, dd, *J* = 9.4, 2.0 Hz, H-2), 2.12 (3H, s, acetyl), 1.23 (1H, m, H-4a), 1.03 (1H, m, H-4b), 0.93 (1H, m, H-3), 0.87 (3H, t, *J* = 7.3 Hz, H-5), 0.35 (3H, d, *J* = 6.7 Hz, H-4′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ *N*-acetyl-L-*allo*-isoleucine residue, 174.2 (s, C-1), 172.9 (s, CH_3CO), 52.7 (d, C-2), 37.9 (d, C-3), 27.0 (t, C-4), 24.0 (q, CH_3-CO), 13.4 (q, C-4′), 11.9 (q, C-5); HRMS (ESI-TOF) *m*/*z* 589.2997 (calcd for $C_{31}H_{42}N_4O_6Na$, 589.3002) $[M + Na]^+$.

Hydrolysis of 1 and HPLC Analysis of the Hydrolysate.[3,10,11] Compound **1** (1.0 mg) was hydrolyzed with 6 N HCl (0.6 mL) at 110−120 °C for 24 h. After concentration to dryness, the residue was dissolved in MeOH (100 μL) and subjected to HPLC analysis (column: Sumichiral OA-5000, 4.6 × 150 mm; mobile phase 20% MeOH in 2 mM aqueous $CuSO_4$; flow rate 1 mL/min; UV 235 nm). Co-injection using standard amino acids, L-Ile (t_R 10.5 min), D-Ile (t_R 13.5 min), L-*allo*-Ile (t_R 8.9 min), D-*allo*-Ile (t_R 11.0 min), L-Leu (t_R 11.0 min), D-Leu (t_R 14.4 min), *N*-Me-L-Phe (t_R 27.8 min), *N*-Me-D-Phe (t_R 30.0 min), and anthranilic acid (t_R 61.0 min), revealed that the hydrolysate of the tetrapeptide **1** consisted of L-*allo*-Ile, *N*-Me-D-Phe, L-Leu, and anthranilic acid.

Biological Assays. The assay for activity against *P. falciparum* (K1, multi-drug-resistant strain) was performed using the microculture radioisotope technique described by Desjardins.[12] IC_{50} represents the concentration that causes 50% reduction of parasite growth as indicated by the in vitro uptake of [^{3}H]-hypoxanthine by *P. falciparum*. Compound **1** showed an IC_{50} value of 8.0 μM. An IC_{50} value of a standard antimalarial compound, dihydroartemisinin, was 4.2 nM in the same assay system. Cytotoxicity of the purified compounds against African green monkey kidney fibroblasts (Vero), human epidermoid carcinoma cells (KB), human breast cancer cells (BC), and human lung cancer cells (NCI-H187) was evaluated using the colorimetric method.[13]

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