Cyclohexadepsipeptides from the Insect Pathogenic Fungus *Hirsutella nivea* BCC 2594

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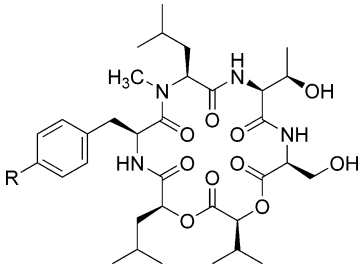
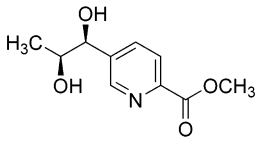
Two new cyclohexadepsipeptides, hirsutatins A (**1**) and B (**2**), were isolated from a culture filtrate of the insect pathogenic fungus *Hirsutella nivea* BCC 2594. Structures of these compounds were elucidated primarily by NMR and mass spectroscopic analyses. The α-carbon stereochemistry of **1** was established by HPLC analysis of its acid hydrolysate using a chiral column. Hirsutatin B (**2**) exhibited activity against the malarial parasite *Plasmodium falciparum* K1 with an IC_{50} value of 5.8 μg/mL, while hirsutatin A (**1**) was inactive at a concentration of 20 μg/mL.

As part of an ongoing research program on novel bioactive fungal metabolites, we have been conducting a chemical investigation of insect pathogenic fungi that were collected at various places in Thailand. Recently we reported the isolation and structure elucidation of five new antimycobacterial alkaloids, hirsutellones A−E, from the mycelial MeOH extract of *Hirsutella nivea* BCC 2594.[1] Subsequent investigation of the EtOAc extract from culture filtrate led to the isolation of two new cyclohexadepsipeptides, hirsutatins A (**1**) and B (**2**), as minor constituents together with a known picolinic acid derivative, CJ-14,877 (**3**).[2] In this paper, we describe the isolation, structural elucidation, and biological activities of these depsipeptides.



1 : R = H
2 : R = OCH_3



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Hirsutatin A (**1**) was obtained as a colorless amorphous solid. The molecular formula of **1** was determined by HRMS (ESI-TOF) and ^{13}C NMR as $C_{34}H_{52}N_4O_{10}$. The IR spectrum of **1** showed intense broad absorption bands of esters and amides at ν_{max} 1749, 1654, and 1640 cm^{-1}. A hexadepsipeptide structure of **1** was suggested by ^1H and ^{13}C NMR spectroscopic data ($CDCl_3$), showing six carbonyl carbon signals at δ_C 172.3, 171.9, 171.2, 170.1, 169.7, and 167.7 and six α-protons at δ_H 5.50 (dd, J = 7.5, 4.8 Hz), 5.34 (m), 4.72 (br d, J = 7.1 Hz), 4.67 (d, J = 5.0 Hz), 4.41 (br d, J = 8.5 Hz), and 3.49 (m). Three amide protons (N*H*) at δ_H 8.16 (d, J = 7.1 Hz), 7.54 (d, J = 8.7 Hz), and 6.69 (d, J = 8.7 Hz) and an *N*-methyl-amide proton signal at δ_H 3.08 (3H, s) were also observed. Analysis of 2D NMR data (COSY, HMQC, and HMBC) revealed the structures of two 2-hydroxycarboxylic acid and four amino acid residues. Two oxymethines, δ_H 4.67 (δ_C 79.3) and 5.50 (δ_C 73.6), attached respectively with an isopropyl and an isobutyl group, indicated the presence of 2-hydroxyisovaleric acid and 2-hydroxyisocaproic acid residues. An additional oxymethine, δ_H 4.66 (br q, J = 6.4 Hz; δ_C 65.3), and an oxymethylene, δ_H 4.19−4.20 (2H, m; δ_C 62.8), were assigned to the β-position of threonine (Thr) and serine (Ser), respectively. The presence of a phenylalanine (Phe) residue was evident from characteristic signals of a benzyl group attached to a methine at δ_C 49.9 (C-2; δ_H 5.34). The remaining *N*-methylamiono acid was assigned to *N*-methylleucine (*N*-Me-Leu), which was confirmed by the HMBC correlation from amide methyl protons (δ_H 3.08, 3H, s) to the α-carbon at δ_C 65.1 (δ_H 3.49, m). The connectivity of each residue was established by analysis of HMBC and NOESY data (Figure 1). Thus, α-protons of 2-hydroxyisovaleric acid, 2-hydroxyisocaproic acid, Phe, and *N*-Me-Leu were correlated respectively to carbonyl carbons of Ser, 2-hydroxyisovaleric acid, 2-hydroxyisocaproic acid, and Phe. The amide N*H* of Thr showed HMBC correlation to the carbonyl carbon of *N*-Me-Leu, as well as NOESY cross signals to the methylene protons (δ_H 1.68, 1.47) of *N*-Me-Leu. The linkage of Thr-Ser was indicated by a NOESY correlation between the amide N*H* of Ser and the α-proton of Thr.

The α-carbon stereochemistry of hirsutatin A (**1**) was established by HPLC analysis of its acid hydrolysate using a ligand-exchange-type chiral column (see Experimental Section). Co-injection with standard D- and L-amino acids and 2-hydroxycarboxylic acids revealed that all residues in cyclohexadepsipeptide **1** possess the 2*S*-configuration: a L-2-hydroxyisovaleric acid, a L-2-hydroxyisocaproic acid, a L-Phe, a *N*-Me-L-Leu, a L-Thr, and a L-Ser.

The molecular formula of hirsutatin B (**2**), $C_{35}H_{54}N_4O_{11}$, was determined on the basis of HRMS and ^{13}C NMR data. The ^1H and ^{13}C NMR spectra for compound **2** were similar to those of **1** except for replacement of the benzyl group in **1** by *p*-methoxybenzyl in **2**. The methoxyl protons at δ_H 3.78

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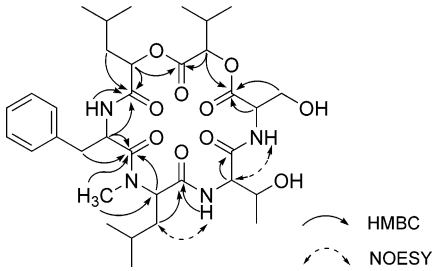

Figure 1. Selected HMBC and NOESY correlations for **1**.

(3H, s; attached to δ_C 55.2) in **2** showed HMBC correlation to the downfield quaternary aromatic carbon situated at δ_C 158.3 and also exhibited NOESY correlation with δ_H 6.81 aromatic protons (2H, d, J = 8.5 Hz, H-5 and H-9). Therefore, hirsutatin B (**2**) should possess an *O*-methyltyrosine (*O*-Me-Tyr) residue instead of phenylalanine in **1**. The similarity of the NMR data between compounds **1** and **2** strongly suggested that these cyclohexadepsipeptides possess identical stereochemistry and have similar conformation in $CDCl_3$.

The NMR, MS, IR, and UV spectra and optical rotation data ($[\alpha]^{30}_D$ +27°, *c* 0.50, MeOH) for **3** were consistent with those of CJ-14,877, which was previously isolated from the fermentation broth of a basidomycete, *Marasmiellus* sp. CL21624, as a cytokine production inhibitor.[2]

Hirsutatin B (**2**) exhibited activity against the malarial parasite *Plasmodium falciparum* K1 with an IC_{50} value of 5.8 μg/mL, while hirsutatin A (**1**) was inactive at a concentration of 20 μg/mL. Both **1** and **2** showed weak activity against *Mycobacterium tuberculosis* $H_{37}Ra$, both with the same MIC value of 50 μg/mL. However, these compounds were noncytotoxic to Vero cells at a concentration of 50 μg/mL.

Experimental Section

General Experimental Procedures. Melting points were measured with an Electrothermal IA9100 digital melting point apparatus and are uncorrected. 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 taken on a Bruker VECTOR 22 spectrometer. NMR spectra were taken on a Bruker AV500D spectrometer. ESI-TOF mass spectra were measured with a Micromass LCT mass spectrometer.

Fungal Material. *Hirsutella nivea* Hywel-Jones (Ascomycota, Mitosporic, Hypocreales, Clavicipitaceae)[3] was collected, identified, and isolated from a Homoptera leaf-hopper, Khao Yai National Park, Central Thailand, by Dr. Nigel L. Hywel-Jones. This fungus was deposited at the BIOTEC Culture Collection as BCC 2594.

Fermentation and Isolation. BCC 2594 was maintained on potato dextrose agar at 25 °C for 16 days, and the agar was cut into pieces (1 × 1 cm) and inoculated into 4 × 250 mL Erlenmeyer flasks containing 25 mL of potato dextrose broth (PDB; composition: potato starch 4.0 g, dextrose 20.0 g, per liter). After incubation at 25 °C for 8 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 8 days on a rotary shaker (200 rpm). Each 25 mL portion of the secondary cultures (in 4 flasks) was transferred into 32 × 1 L Erlenmeyer flasks each containing minimum salt medium (composition: glucose 20.0 g, NH_4NO_3 3.0 g, KH_2PO_4 0.5 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $CaCl_2$ 0.5 g, and yeast extract 1.0 g, per L), and static fermentation was carried out at 25 °C for 40 days. The cultures were filtered to separate mycelia and filtrate. Investigation of the CH_3OH extract from the mycelia was previously reported.[1] The filtrate (ca. 8 L) was extracted with an equal volume of EtOAc. The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure to obtain a dark brown gum (1.05 g). This extract was passed through a Sephadex LH-20 column (3 × 25 cm) using CH_3OH as eluent. Fractions of 150−250 mL elutes were combined and subjected to column chromatography on silica gel (CH_3OH/CH_2Cl_2, step gradient elution with 5:95 to 20:80). The CH_3OH/CH_2Cl_2 = 10:90 elute (34 mg) contained depsipeptides, while compound **3** (533 mg) was obtained from the CH_3OH /CH_2Cl_2 = 15:85 elute. The former was subjected to HPLC (peaks observed at 210 nm) using a reversed-phase column (LiChroCART RP-18, 10 μm, 10 × 250 mm) with CH_3CN/H_2O = 65:35 as eluent at a flow rate of 3 mL/min to obtain a mixture of **1** and **2** (8.0 mg). This mixture was separated using the same column eluted with CH_3CN/H_2O = 40:60 (flow rate 3 mL/min) to obtain pure compounds **2** (3.3 mg, t_R 5.0 min) and **1** (2.7 mg, t_R 6.5 min).

Hirsutatin A (1): colorless solid; $[\alpha]^{25}_D$ −66° (*c* 0.15, CH_3OH); UV (MeOH) λ_{max} (log ϵ) 204 (4.10), 275 sh (3.11) nm; IR ($CHCl_3$) ν_{max} 3328, 2925, 1749, 1654, 1630, 1253 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 2-hydroxyisovaleric acid 4.67 (1H, d, J = 5.0 Hz, H-2), 2.29 (1H, m, H-3), 1.06 (3H, d, J = 6.7 Hz, H-4), 1.05 (3H, d, J = 6.8 Hz, 3-CH_3); 2-hydroxyisocaproic acid 5.50 (1H, dd, J = 7.5, 4.8 Hz, H-2), 1.70 (1H, m, H-3a), 1.57 (1H, m, H-3b), 1.63 (1H, m, H-4), 0.94 (3H, d, J = 6.2 Hz, H-5), 0.93 (3H, d, J = 6.1 Hz, 4-CH_3); Phe 7.54 (1H, d, J = 8.7 Hz, N-*H*), 7.27 (2H, m, H-6 and H-8), 7.23 (3H, m, H-5, H-7, and H-9), 5.34 (1H, m, H-2), 3.01 (1H, dd, J = 12.9, 4.7 Hz, H-3a), 2.89 (1H, dd, J = 12.9, 10.3 Hz, H-3b); *N*-Me-Leu 3.49 (1H, m, H-2), 3.08 (3H, s, N-CH_3), 1.68 (1H, m, H-3a), 1.47 (1H, m, H-3b), 1.30 (1H, m, H-4), 0.86 (3H, d, J = 6.4 Hz, H-5), 0.81 (3H, d, J = 6.4 Hz, 4-CH_3); Thr 6.69 (1H, d, J = 8.7 Hz, N-*H*), 4.66 (1H, br q, J = 6.4 Hz, H-3), 4.41 (1H, br d, J = 8.5 Hz, H-2), 1.18 (3H, d, J = 6.5 Hz, H-4); Ser 8.16 (1H, d, J = 7.1 Hz, N-*H*), 4.72 (1H, br d, J = 7.1 Hz, H-2), 4.19−4.20 (2H, m, H-3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 2-hydroxyisovaleric acid 167.7 (s, C-1), 79.3 (d, C-2), 30.4 (d, C-3), 18.8 (q, 3-*C*H_3), 17.6 (q, C-4); 2-hydroxyisocaproic acid 169.7 (s, C-1), 73.6 (d, C-2), 42.2 (t, C-3), 24.6 (d, C-4), 23.5 (q, C-5), 22.1 (q, 4-*C*H_3); Phe 172.3 (s, C-1), 135.8 (s, C-4), 129.4 × 2 (d, C-5 and C-9), 128.6 × 2 (d, C-6 and C-8), 127.1 (d, C-7), 49.9 (d, C-2), 39.6 (t, C-3); *N*-Me-Leu 171.9 (s, C-1), 65.1 (d, C-2), 40.6 (q, N-*C*H_3), 36.8 (t, C-3), 24.5 (d, C-4), 23.0 (q, 4-*C*H_3), 21.7 (q, C-5); Thr 171.2 (s, C-1), 65.3 (d, C-3), 57.3 (d, C-2), 19.1 (q, C-4); Ser 170.1 (s, C-1), 62.8 (t, C-3), 55.6 (d, C-2); HRMS (ESI-TOF) *m/z* 699.3586 (calcd for $C_{34}H_{52}N_4O_{10}Na$, 699.3581) $[M + Na]^+$.

Hirsutatin B (2): colorless solid; $[\alpha]^{25}_D$ −65° (*c* 0.185, MeOH); UV (MeOH) λ_{max} (log ϵ) 202 (3.90), 226 (3.77), 277 (2.99) nm; IR ($CHCl_3$) ν_{max} 3328, 2924, 1742, 1654, 1630, 1510, 1248, 753 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 2-hydroxyisovaleric acid 4.67 (1H, d, J = 6.0 Hz, H-2), 2.29 (1H, m, H-3), 1.06 (3H, d, J = 6.7 Hz, H-4), 1.04 (3H, d, J = 6.8 Hz, 3-CH_3); 2-hydroxyisocaproic acid 5.49 (1H, dd, J = 7.5, 4.8 Hz, H-2), 1.69 (1H, m, H-3a), 1.56 (1H, m, H-3b), 1.63 (1H, m, H-4), 0.94 (3H, d, J = 6.1 Hz, H-5), 0.93 (3H, d, J = 6.2 Hz, 4-CH_3); *O*-Me-Tyr 7.52 (1H, d, J = 8.6 Hz, N-*H*), 7.14 (2H, d, J = 8.5 Hz, H-5 and H-9), 6.81 (2H, d, J = 8.5 Hz, H-6 and H-8), 5.30 (1H, m, H-2), 3.78 (3H, s, O-CH_3), 2.93 (1H, dd, J = 13.1, 4.9 Hz, H-3a), 2.82 (1H, dd, J = 13.1, 10.2 Hz, H-3b); *N*-Me-Leu 3.49 (1H, m, H-2), 3.09 (3H, s, N-CH_3), 1.70 (1H, m, H-3a), 1.48 (1H, m, H-3b), 1.30 (1H, m, H-4), 0.87 (3H, d, J = 6.4 Hz, H-5), 0.83 (3H, d, J = 6.4 Hz, 4-CH_3); Thr 6.69 (1H, d, J = 8.7 Hz, N-*H*), 4.66 (1H, br q, J = 6.6 Hz, H-3), 4.41 (1H, br d, J = 8.7 Hz, H-2), 1.19 (3H, d, J = 6.5 Hz, H-4); Ser 8.16 (1H, d, J = 7.0 Hz, N-*H*), 4.72 (1H, br d, J = 7.1 Hz, H-2), 4.19 (2H, br s, H-3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 2-hydroxyisovaleric acid 167.7 (s, C-1), 79.3 (d, C-2), 30.4 (d, C-3), 18.8 (q, 3-*C*H_3), 17.6 (q, C-4); 2-hydroxyisocaproic acid 169.6 (s, C-1), 73.6 (d, C-2), 42.2 (t, C-3), 24.6 (d, C-4), 23.4 (q, C-5), 22.1 (q, 4-*C*H_3); *O*-Me-Tyr 172.4 (s, C-1), 158.3 (s, C-7), 130.4 × 2 (d, C-5 and C-9), 127.7 (s, C-4), 113.0 × 2 (d, C-6 and C-8), 50.0 (d, C-2), 55.2 (q, O-*C*H_3), 38.7 (t, C-3): *N*-Me-Leu 171.9 (s, C-1), 65.1 (d, C-2), 40.7 (q, N-*C*H_3), 36.8 (t, C-3), 24.4 (d, C-4), 23.0 (q, 4-*C*H_3), 21.7 (q, C-5); Thr 171.2 (s, C-1), 65.3 (d, C-3), 57.3 (d, C-2), 19.0 (q, C-4); Ser 170.1 (s, C-1), 62.8 (t, C-3), 55.6 (d, C-2); HRMS (ESI-TOF) *m/z* 729.3687 (calcd for $C_{35}H_{54}N_4O_{11}Na$, 729.3687) $[M + Na]^+$.

Acid Hydrolysis of 1. Compound **1** (0.6 mg) was hydrolyzed with 6 N HCl (0.8 mL) at 110−120 °C for 15 h. After concentration to dryness, the residue was dissolved in MeOH (100 μL) and subjected to HPLC analysis.

HPLC Analysis of the Hydrolysate.[4,5] HPLC analysis of the depsipeptide hydrolysate was performed using a ligand-exchange-type chiral column: SUMICHIRAL OA-5000, 5 μm, 4.6 × 150 mm (Sumika Chemical Analysis Service, Ltd); flow rate 1 mL/min, UV 235 nm. Standard D- and L-amino acids and 2-hydroxycarboxylic acids were used for co-injection experiments. Three mobile phase conditions were employed for polarity reasons: (1) 5% CH_3OH in 2 mM $CuSO_4$, L-Ser (t_R 3.7 min), D-Ser (t_R 3.9 min), L-Thr (t_R 4.0 min), D-Thr (t_R 4.5 min); (2) 5% 2-propanol in 2 mM $CuSO_4$, *N*-Me-L-Leu (t_R 12.8 min), *N*-Me-D-Leu (t_R 16.7 min), L-Phe (t_R 29.0 min), D-Phe (t_R 40.5 min); (3) 15% 2-propanol in 2 mM $CuSO_4$, L-2-hydroxyisovaleric acid (t_R 14.6 min), D-2-hydroxyisovaleric acid (t_R 24.1 min), L-2-hydroxyisocaproic acid (t_R 45.0 min), D-2-hydroxyisocaproic acid (t_R 61.2 min). These analyses revealed that the hydrolysate of **1** consisted of L-Ser, L-Tyr, *N*-Me-L-Leu, L-Phe, L-2-hydroxyisovaleric acid, and L-2-hydroxyisocaproic acid.

Biological Assays. An assay for activity against *Plasmodium falciparum* (K1, multi-drug-resistant strain) was performed using the microculture radioisotope technique described by Desjardins.[6] A standard antimalarial compound, dihydroartemisinin, showed an IC_{50} value of 1.8 ng/mL in the same assay system. Growth inhibitory activity against *Mycobacterium tuberculosis* $H_{37}Ra$ was determined using the Microplate Alamar Blue Assay (MABA) described by Collins and Franzblau.[7] Isoniazid, a standard antitubercular drug, showed a MIC value of 0.050 μg/mL. Cytotoxicity of the purified compounds against Vero cells (African green monkey kidney fibroblast) was evaluated using the colorimetric method.[8] Ellipticine (IC_{50} 0.60 ± 0.20 μg/mL) was used as a positive control.

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