Hirsutellide A, a New Antimycobacterial Cyclohexadepsipeptide from the Entomopathogenic Fungus *Hirsutella kobayasii*

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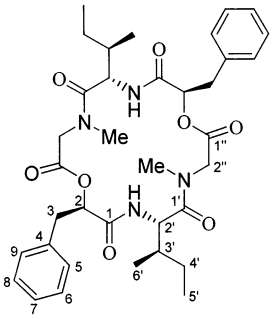
A new cyclohexadepsipeptide, named hirsutellide A (**1**), was isolated from a cell extract of the entomopathogenic fungus *Hirsutella kobayasii* BCC 1660. The structure of **1** was elucidated by analyses of spectroscopic data, and its absolute stereochemistry was addressed by the use of Marfey's method. Hirsutellide A (**1**) exhibited antimycobacterial and antimalarial activities, but was inactive toward the Vero cell line (at 50 μg/mL).

The incidence of tuberculosis has rapidly increased worldwide, particularly among those associated with HIV infection. It is estimated that approximately one-third of the global population is infected with *Mycobacterium tuberculosis* and that seven to eight million new cases of tuberculosis occur each year.[1] Development of new drugs for use against the emerging drug-resistant strains of *M. tuberculosis* is therefore urgently needed. As part of our continuing search for biologically active compounds from plants and microorganisms,[2] we report herein a new antimycobacterial cyclohexadepsipeptide, named hirsutellide A (**1**), from a cell extract of *Hirsutella kobayasii* BCC 1660. It should be noted that entomopathogenic fungi of the genus *Hirsutella* have rarely been chemically explored; only a toxic polypeptide, hirsutellin A, has been reported to date.[3]



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Hirsutellide A (**1**) was obtained as an off-white solid. Its IR exhibited characteristic stretchs of an amide NH (3292 cm^{-1}), an ester carbonyl (1752 cm^{-1}), and amide carbonyls (1663 and 1634 cm^{-1}). The ^1H NMR spectrum ($CDCl_3$) showed signals of protons of three methyl groups (singlet at δ_H 3.27, doublet at δ_H 0.87, and triplet at δ_H 0.91), three nonequivalent methylenes at δ_H 1.19−4.46, two downfield methines at δ_H 4.93 and 5.63, aromatic protons at δ_H 7.16−7.28, and an amide NH at δ_H 7.57. Analyses of ^{13}C NMR, DEPT, and HMQC spectral data revealed that hirsutellide A (**1**) possessed 18 carbons (two equivalent carbon signals

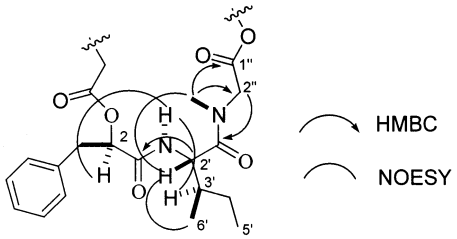

Figure 1. Selected HMBC and NOESY correlations of hirsutellide A (**1**).

at δ_C 129.1 (C-5 and C-9) and δ_C 128.6 (C-6 and C-8) of a substituted benzene ring). However, a molecular formula, $C_{36}H_{48}O_8N_4$, was inferred by the ESITOF mass spectrum [accurate mass observed at *m/z* 665.3579 $(M + H)^+$, Δ +2.9 mmu]; hirsutellide A (**1**) therefore possessed a C_2 symmetry. Analyses of $^1H-^1H$ COSY, NOESY, HMQC, and HMBC spectral data unambiguously revealed the presence of isoleucine, sarcosine, and 2-hydroxy-3-phenylpropanoic acid in hirsutellide A (**1**) (Figure 1). The $^1H-^1H$ COSY spectrum of **1** established the partial structure from H-2′ to H-6′ of the isoleucine residue and also showed correlations between H-2′ of isoleucine to an adjacent amide proton (NH) as well as between H-2 and H-3 of 2-hydroxy-3-phenylpropanoic acid. The HMBC spectrum of hirsutellide A (**1**) assisted in the assignment of the amino acid sequence in **1** (H-2′ of isoleucine to C-1 of 2-hydroxy-3-phenylpropanoic acid and H-2″ of sarcosine to C-1′ of isoleucine), as depicted in Figure 1. The position of the *N*-methyl at the sarcosine residue was also assigned by HMBC, from which correlations of the singlet methyl protons (δ_H 3.27) to C-1″ (δ_C 166.8) and C-2″ (δ_C 51.7) of sarcosine were observed (Figure 1). On the basis of these spectral data, the chemical structure of hirsutellide A (**1**) was secured. Complete assignment of protons and carbons in **1** is shown in Table 1.

The relative stereochemistry of hirsutellide A (**1**) was successfully assigned by analyses of the NOESY spectrum. Correlations from the amide proton (NH) to H-3′ of isoleucine and to H-2 of 2-hydroxy-3-phenylpropanoic acid were observed, suggesting that these protons were coplanar (Figure 1). The NOESY spectral data of **1** also revealed correlations between H-2′ and the methyl protons (H-6′) of isoleucine and between H-2′ of isoleucine and the *N*-methyl of sarcosine. The absolute stereochemistry in

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Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Spectral Data ($CDCl_3$) of Hirsutellide A (**1**)

unit	δ_C, multiplicity	δ_H, multiplicity (J in Hz)
2-hydroxy-3-phenyl-propanoic acid		
1	168.8, s	
2	74.1, d	5.63, dd (11.8, 2.9)
3	38.7, t	2.74, dd (14.0, 11.9)
		3.68, dd (14.0, 2.8)
4	136.1, s	
5, 9	129.1, d	7.16, br d (7.0)
6, 8	128.6, d	7.28, dd (7.0, 7.0)
7	127.1, d	7.23, m
L-*allo*-isoleucine		
1′	174.1, s	
2′	52.3, d	4.93, dd (10.1, 9.7)
3′	35.8, d	2.24, m
4′	24.2, t	1.19, m
		1.55, m
5′	10.2, q	0.91, t (7.4)
6′	15.4, q	0.87, d (6.7)
NH		7.57, d (9.7)
sarcosine		
1″	166.8, s	
2″	51.7, t	3.20, d (17.1)
		4.46, d (17.2)
*N*Me	37.9, q	3.27, s

hirsutellide A (**1**) was addressed by the use of Marfey's method.[4] Hirsutellide A (**1**) was hydrolyzed and subsequently derivatized with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alaninamide, FDAA). HPLC analysis established the L stereochemistry of the isoleucine unit in **1**; however, the reversed-phase column failed to distinguish between L-isoleucine and L-*allo*-isoleucine. The problem was solved using a chiral column, from which the presence of L-*allo*-isoleucine in hirsutellide A (**1**) was firmly established. The absolute configuration at C-2 of 2-hydroxy-3-phenylpropanoic acid was assigned as *R* according to the NOESY spectrum of **1**.

Hirsutellide A (**1**) exhibited antimycobacterial activity with a MIC (minimum inhibitory concentration) of 6−12 μg/mL, but showed no cytotoxic effect toward Vero cells at 50 μg/mL. Additionally, hirsutellide A (**1**) also possessed weak in vitro antimalarial activity, with an IC_{50} value of 2.8 μg/mL.

Experimental Section

General Experimental Procedures. The IR spectra and optical rotations were measured on a Perkin-Elmer 2000 spectrometer and Jasco DIP370 polarimeter, respectively. The UV spectra were recorded on a Cary 1E UV−vis spectrophotometer. The ^{1}H, ^{13}C, DEPT, ^{1}H−^{1}H COSY, NOESY, HMQC, and HMBC experiments were carried out on a Bruker DRX 400 NMR spectrometer, operating at 400 MHz for proton and 100 MHz for carbon. The ESI-TOF mass spectra were obtained from a Micromass LCT mass spectrometer, and the lock mass calibration was applied for the determination of accurate mass.

Fungal Material, Extraction, and Isolation. The fungus *H. kobayasii* BCC 1660 was collected from Kaeng Krachan National Park, Phetchburi, Thailand, and identified by Dr. Nigel Leslie Hywel-Jones of the Mycology Research Unit, National Center for Genetic Engineering and Biotechnology (BIOTEC). The specimen has been deposited (registration no. BCC 1660) at the BIOTEC Culture Collection. *H. kobayasii* BCC 1660 was cultured in potato dextrose broth (5 L culture); cells were separated from the broth by filtration and subsequently extracted twice with CH_2Cl_2 to yield 1.3 g of a crude extract. The extract was subjected to a Sephadex LH-20 column (eluted with MeOH), and the fraction containing hirsutellide A (**1**) was further purified by silica gel column chromatography (eluted with acetone/hexane, gradient elution from 5:95 to 30:70) to afford **1** (18 mg).

Hirsutellide A (1): off-white solid; $[\alpha]^{28}_D$ −13.6° (*c* 0.25, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 212 (4.32) and 257 (sh) nm; IR (KBr) ν_{max} 3292, 3030, 3012, 2967, 2933, 1752, 1663, 1634, 1527, 1464, 1262, 1132, 1095, 1061 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; ESITOF MS *m*/*z* 665.3579 $(M + H)^+$, calcd for $(C_{36}H_{48}O_8N_4 + H)^+$, 665.3550.

Determination of Amino Acid Configuration by Marfey's Method. A mixture of hirsutellide A (**1**) (1 mg) and 2 M HCl (2 mL) was heated to reflux at 110 °C for 14 h, after which it was evaporated to dryness. The residue was dissolved in 0.5 mL of H_2O, then 2 mL of 1 M $NaHCO_3$ and 1 mL of 1% Marfey's reagent (FDAA)[4] in acetone were added. The reaction mixture was incubated at 37 °C for 1 h, quenched with 0.2 mL of 2 N HCl, and subjected to HPLC analysis (C_{18} reversed-phase column, eluted with MeCN/H_2O (30:70), flow rate 1.0 mL/min, and UV detector set at 340 nm). D and L forms of isoleucine and *allo*-isoleucine were separately derivatized with FDAA in the same manner as that described for **1**. Under the HPLC conditions employed, D- and L-isoleucine (and *allo*-isoleucine) had retention times of 41.26 and 19.62 min, respectively. The residue in **1** was found to be the L form; however, the reversed-phase column failed to distinguish between L-isoleucine and L-*allo*-isoleucine. This problem was solved using a chiral column (ChiraDex, Merck), eluted with MeOH/H_2O (30:70), at a flow rate of 0.7 mL/min. Under these HPLC conditions, L-isoleucine and L-*allo*-isoleucine exhibited retention times of 7.08 and 7.88 min, respectively. The sample was co-injected with standard compounds to finally establish the amino acid from hirsutellide A (**1**) as L-*allo*-isoleucine.

Bioassays. The antimycobacterial activity was assessed against *Mycobacterium tuberculosis* H37Ra using the Microplate Alamar Blue Assay (MABA).[5] Standard drugs, isoniazid and kanamycin sulfate, the reference compounds for the antimycobacterial assay, showed minimum inhibitory concentrations (MICs) of 0.040−0.090 and 2.0−5.0 μg/mL, respectively. The antimalarial activity was evaluated against the parasite *Plasmodium falciparum* (K1, multidrug-resistant strain), which was cultured continuously according to the method of Trager and Jensen.[6] Quantitative assessment of antimalarial activity in vitro was determined by means of the microculture radioisotope technique based upon the method described by Desjardins et al.[7] The inhibitory concentration (IC_{50}) represents the concentration that causes 50% reduction in parasite growth as indicated by the in vitro uptake of [^{3}H]-hypoxanthine by *P. falciparum*. An IC_{50} value of 1 ng/mL was observed for the standard compound, artemisinin, in the same test system. Cytotoxicity was determined by employing the colorimetric method described by Skehan and co-workers.[8] The reference compound, ellipticine, exhibited activity toward the BC-1 and KB cell lines, both with an IC_{50} of 0.3 μg/mL.

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Supporting Information Available: ^{1}H, ^{13}C, ^{1}H−^{1}H COSY, NOESY, HMQC, HMBC, and ESI-TOF MS spectra of hirsutellide A (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

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