Hirsutide, a Cyclic Tetrapeptide from a Spider-Derived Entomopathogenic Fungus, *Hirsutella* sp.

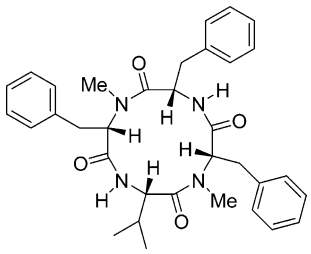
Gerhard Lang,[†] John W. Blunt,[†] Nicholas J. Cummings,[‡] Anthony L. J. Cole,[‡] and Murray H. G. Munro*,[†]

Department of Chemistry and School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand

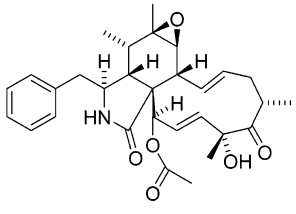
Received May 2, 2005

The entomopathogenic fungus *Hirsutella* sp., isolated from an infected spider, was found to produce the new cyclotetrapeptide hirsutide (**1**), cyclo-(L-*N*Me-Phe-L-Phe-L-*N*Me-Phe-L-Val), along with the known cytochalasin Q (**2**), using a cytotoxicity-guided isolation procedure. The structure of **1** was elucidated using one- and two-dimensional NMR experiments, mass spectrometry, and Marfey's method for analyzing the configuration of the amino acids.

While entomopathogenic fungi infecting insects have been the source of many new and biologically active metabolites,[1,2] the metabolic potential of fungal species from other arthropods, e.g., spiders and mites, remains largely unexplored. Within a program aimed at isolating novel bioactive natural products from New Zealand fungi, a *Hirsutella* strain isolated from an infected spider was investigated. The only low molecular weight metabolites hitherto described from *Hirsutella* species are the cyclodepsipeptide hirsutellide A from *H. kobayasii*,[3] the antimycobacterial hirsutellones from *H. nivea*,[4] and phomalactone, which is also produced by fungi of other genera, from *H. thompsonii*.[5] *H. thompsonii* also produces the protein hirsutellin A, a compound that shows strong and selective cytotoxicity against insect cells.[6,7] In this current work a further *Hirsutella* metabolite, the cyclotetrapeptide **1**, was isolated and characterized. Additionally, we report for the first time the production of a cytochalasin, i.e., cytochalasin Q (**2**),[8] by a *Hirsutella* species.



1



2

For chemical investigation, the fungus was grown on half-strength SDY agar, which was then extracted with EtOAc. The extract displayed moderate cytotoxic activity (IC_{50} = 11 μg/mL) against P388 murine leukemia cells. HPLC-UV and -MS analysis showed two major compounds, **1** and **2**, with the molecular masses 568 and 507, respectively, which were subsequently isolated for structural elucidation and characterization of biological activities.

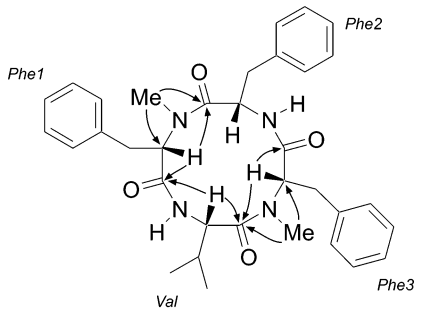
Four signals between 4 and 5 ppm in the ^{1}H NMR spectrum of **1** suggested a peptidic structure for this compound, with these signals being attributable to the α-protons of four amino acid units. A TOCSY experiment revealed the identity of the amino acids, namely, three phenylalanine residues, for which the diastereotopic β-protons were observed between 2.9 and 3.8 ppm, and one valine unit with the β-proton at 2.1 ppm and the two methyl doublets at 0.88 and 0.72 ppm.

The α-proton of the valine unit (4.27 ppm) coupled to an NH proton, whose signal was overlapping with those of the three phenyl groups (7.05−7.35 ppm). The α-proton of one of the phenylalanines (4.90 ppm) was broadened, possibly due to dynamic effects. Changing the solvent from $CDCl_3$ to C_6D_6 did not improve the spectrum, but heating to 50 °C caused the signal at 4.90 ppm to become sharp, while the previously sharp signal of the valine α-proton became broader. A COSY spectrum acquired under this set of conditions revealed that the phenylalanine α-proton, which before had shown a broad signal, was also coupled to an NH proton. The proton spectrum also showed signals for two *N*-methyl groups, which were attached to the remaining two phenylalanine units. The presence of two NH-protons and two *N*-methyl singlets in the spectrum of a tetrapeptide suggested a cyclic structure, which also is in accordance with the molecular formula of $C_{34}H_{40}N_4O_4$, derived from the HRESIMS.

By acid hydrolysis of the peptide and analysis using Marfey's method,[9] all four amino acid units were shown to have an *S*- (or L-) configuration. Thus, the task of determining the sequence of the amino acid units in the peptide was reduced to identifying which of the phenylalanines was not *N*-methylated. This was achieved by observing long-range H,C-couplings of the α-protons and *N*-methyl groups to the carbonyl carbons. Since the carbonyl chemical shifts were not resolvable in the HMBC experiment, the IMPRESS technique with its higher resolution in the F1 dimension was applied. The interpretation of the IMPRESS data was further complicated by the identical chemical shifts of the carbonyl carbons of the valine and the nonmethylated phenylalanine unit, but the only structure consistent with all observed H,C-couplings (see Figure 1)

* To whom correspondence should be addressed. Tel: +64-3-3642434. Fax: +64-3-3642429. E-mail: murray.munro@canterbury.ac.nz.
[†] Department of Chemistry.
[‡] School of Biological Sciences.

10.1021/np0501536 CCC: $30.25
Published on Web 07/26/2005



1

Figure 1. Long-range H,C-couplings indicative of the amino acid sequence of **1**.

was the structure *N*-methylated at the two phenylalanines directly bound to the valine unit (i.e., *Phe1* and *Phe3*) and a free amide proton at the "middle" phenylalanine residue (*Phe2*). Compound **1** was named hirsutide after the producing organism.

A second compound isolated from the extract was found to be cytochalasin Q (**2**) after extensive evaluation of the NMR data.[8] This compound was also found to be responsible for the cytotoxic activity of the extract, with an IC_{50} against P388 cells[10] of 2.4 μM. Hirsutide displayed no cytotoxicity against P388 cells and was also inactive in an agar diffusion assay against *Escherichia coli*, *Bacillus subtilis*, *Candida* albicans, *Cladosporium resinae*, and *Trichophyton mentagrophytes*.[10]

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Perkin-Elmer 341 polarimeter. NMR spectra were recorded on a Varian INOVA AS-500 spectrometer (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively), using the signals of the residual solvent protons and the solvent carbons as internal references (δ_H 7.25 and δ_C 77.01 ppm for $CDCl_3$). HRESIMS were acquired using a Micromass LCT TOF mass spectrometer. Solvents used for extraction and isolation were distilled prior to use. Cytotoxicity against P388 cells and antimicrobial activities were measured using a standard protocol.[10]

Fungus. A dead spider was collected from the base of a tree stump in indigenous rainforest in June 2004 near Lake Kaniere, Westland, New Zealand. The host was completely covered with fungal hyphae bearing scattered phialides with swollen bases abruptly tapering to thin necks, producing one-celled ellipsoidal conidia encased in a mucous sheath. The fungus was identified as a species of *Hirsutella*.[11] Perithecial ascomata containing filiform, multiseptate ascospores were also immersed in the fungal stroma and probably represent a *Torrubiella* teleomorph of the fungus as reported for other *Hirsutella* species.[12] Conidia were inoculated onto plates of half-strength Sabouraud dextrose yeast agar and incubated at 20 °C. Cultures produced conidiogenous cells and conidia similar to those observed on the host. Ascomata were not produced in cultures. A sample of this *Hirsutella* sp. has been deposited in the culture collection of the School of Biological Sciences, University of Canterbury (CANU-E1101). For chemical investigation the *Hirsutella* sp. was cultured for 35 days on 24 plates of half-strength Sabouraud dextrose yeast agar at 26 °C.

Extraction and Isolation. The fungal agar cultures were macerated and exhaustively extracted with EtOAc. The EtOAc extract was partitioned against H_2O and dried to give a crude extract, which was subsequently partitioned between petroleum ether and MeOH−H_2O (9:1). The resulting MeOH phase was concentrated (49 mg) and subjected to semipreparative HPLC under isocratic conditions (Phenomenex Luna C18, 10 × 250 mm, 5 μm; 62% MeCN + 38% H_2O with 0.05% TFA).

Table 1. ^{1}H and ^{13}C NMR Data ($CDCl_3$ at 296 K) of Hirsutide (**1**)

position	^{13}C [ppm]	^{1}H [ppm][a]	position	^{13}C [ppm]	^{1}H [ppm]
Phe1			Phe3		
CO	170.2		CO	169.4	
α	63.0	4.61 *d*	α	62.7	4.56 d
β	34.1	a 3.66 *bd*	*β*	34.6	a 3.77 bd
		b 2.78			b 2.89
γ	137.2[b]		*γ*	137.0[b]	
δ	129.2[c]	7.05−7.35[d]	*δ*	128.2[c]	7.05−7.35[d]
ϵ	128.9[c]	7.05−7.35[d]	*ϵ*	128.2[c]	7.05−7.35[d]
ζ	128.9[c]	7.05−7.35[d]	*ζ*	128.0[c]	7.05−7.35[d]
N-Me	30.4	2.81 *s*	*N*-Me	30.6	2.83 s
Phe2			Val		
CO	172.1		CO	172.1	
α	51.4	4.90 *br*	α	55.8	4.27 t
β	37.2	a 3.17 *dd*	*β*	29.0	2.11 m
		b 2.84	*γ*	20.1	0.88 d
γ	136.9[b]		*γ′*	18.0	0.72 d
δ	127.0[c]	7.05−7.35[d]	NH		7.12
ϵ	127.0[c]	7.05−7.35[d]			
ζ	126.4[c]	7.05−7.35[d]			
NH		*e*			

[a] Where no multiplicity is specified, this is due to overlapping signals. [b,c,d] Chemical shifts with the same index may be interchanged. [e] The amide proton of *Phe2* was not detectable, possibly due to extensive broadening of the signal.

Compounds **2** (8.9 mg) and **1** (3.1 mg) were eluted at 5.9 and 10.5 min, respectively.

Hirsutide (1): white solid; $[\alpha]^{20}_D$ −192° (*c* 0.2, CH_2Cl_2); for ^{1}H and ^{13}C NMR data see Table 1, for selected HMBC correlations, see Figure 1; HRESIMS *m/z* 569.3133 $[M + H]^+$ (calcd for $C_{34}H_{41}N_4O_4$, 569.3128).

Cytochalasin Q (2): white solid; $[\alpha]^{20}_D$ −91° (*c* 0.2, $CHCl_3$); ^{1}H and ^{13}C NMR data and results from CIGAR, COSY, and NOESY were identical with, or consistent with, reported data;[8] ESIMS *m/z* 507 $[M + H]^+$.

Preparation and Analysis of Marfey Derivatives. Compound **1** (0.7 mg) was hydrolyzed by heating in HCl (6 M; 1 mL) at 110 °C for 24 h. After cooling, the solution was evaporated to dryness and redissolved in H_2O (50 μL). To the acid hydrolysate solution (or to 50 μL of a 50 mM solution of the respective amino acid) was added a 1% (w/v) solution (100 μL) of FDAA (Marfey's reagent, N^{α}-(2,4-dinitro-5-fluorophenyl)-L-alaninamide)[9] in acetone. After addition of $NaHCO_3$ solution (1 M; 20 μL) the mixture was incubated for 1 h at 40 °C. The reaction was stopped by addition of HCl (2 M; 10 μL), the solvents were evaporated to dryness, and the residue was redissolved in MeOH−H_2O (1:1; 1 mL). An aliquot of this solution (10 μL) was analyzed by HPLC (Phenomenex Luna C18, 250 × 4.6, 5 μm; solvents: (A) water + 0.05% TFA, (B) MeCN; linear gradient: 0 min 35% B, 30 min 45% B; 25 °C; 1 mL min^{-1}; 25 °C). Retention times (min) of the amino acid derivatives were as follows: L-Val (11.5), D-Val (16.3), L-Phe (17.4), D-Phe (22.3), *N*Me-L-Phe (16.6), and *N*Me-D-Phe (6.6).

Acknowledgment. This work was supported by a Fellowship within the Postdoctoral Programme of the German Academic Exchange Service (DAAD). We thank Mr. B. Clark for mass spectrometric analysis and Ms. G. Ellis for bioactivity assays.

References and Notes

(1) Cheng, Y.; Schneider, B.; Riese, U.; Schubert, B.; Li, Z.; Hamburger, M. *J. Nat. Prod.* **2004**, *67*, 1854−1858.
(2) Feng, Y.; Blunt, J. W.; Cole, A. L. J.; Munro, M. H. G. *J. Nat. Prod.* **2002**, *65*, 1681−1682.
(3) Vongvanich, N.; Kittakoop, P.; Isaka, M.; Trakulnaleamsai, S.; Vimuttipong, S.; Tanticharoen, M.; Thebtaranonth, Y. *J. Nat. Prod.* **2002**, *65*, 1346−1348.
(4) Isaka, M.; Rugseree, N.; Maithip, P.; Kongsaeree, P.; Prabpai, S.; Thebtaranonth, Y. *Tetrahedron* **2005**, *61*, 5577−5583.
(5) Krasnoff, S. B.; Gupta, S. *J. Chem. Ecol.* **1994**, 293−302.

(6) Mazet, I.; Vey, A. *Microbiology-UK* **1995**, *141*, 1343−1348.
(7) Liu, J.-C.; Boucias, D. G.; Pendland, J. C.; Liu, W.-Z.; Maruniak, J. *J. Invertebr. Pathol.* **1996**, *67*, 224−228.
(8) Abate, D.; Abraham, W.-R.; Meyer, H. *Phytochemistry* **1997**, *44*, 1443−1448.
(9) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591−596.
(10) Perry, N. B.; Benn, M. H.; Brennan, N. J.; Burgess, E. J.; Ellis, G.; Galloway, D. J.; Lorimer, S. D.; Tangney, R. S. *Lichenologist* **1999**, *31*, 627−636.
(11) Minter, D. W.; Brady, D. L. *T. Br. Mycol. Soc.* **1980**, *74*, 271−282.
(12) Hywel-Jones, N. L. *Mycol. Res.* **1997**, *101*, 1202−1206.

NP0501536