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New Diketopiperazines from the Entomopathogenic Fungus *Verticillium hemipterigenum* BCC 1449

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Two new diketopiperazines, bisdethiodi(methylthio)-1-demethylhyalodendrin and 1-demethylhyalodendrin tetrasulfide, together with two known cyclodepsipeptides, enniatins B and B_4, and two known pyrones, pyrenocines A and B, were isolated from a culture broth of the entomopathogenic fungus *Verticillium hemipterigenum* BCC 1449. These structures were elucidated using spectroscopic methods and X-ray crystallography. Antimalarial and cytotoxic activities of these compounds were evaluated.

The fungal genus *Verticillium* has been known to be a rich source of bioactive secondary metabolites of diverse structures, for examples, balanol (a protein kinase C inhibitor) from *V. balanoides*,[1] ES-242-1 (a bioxanthracene, NMDA receptor antagonist) from *Verticillium* sp. SPC-15898,[2] 11α,11'α-dihydroxychaetocin (antibiotic) from *V. tenerum*,[3] verticillins A~C (diketopiperazine dimers, antibiotics) from *Verticillium* sp. TM-759,[4] and (−)-vertinolide (a β-tetronic acid derivative, mycotoxin) and bisvertinols (dimeric vertinoids) from *V. intertextum*.[5,6] Recently we reported the isolation of two new enniatins H and I, together with known enniatins B and B_4, from a mycelial extract of *V. hemipterigenum* BCC 1449 as antimalarial constituents.[7] Further investigation on EtOAc extract of the culture filtrate from this strain led to the identification of two new diketopiperazines **1** and **2**, together with four known compounds, enniatins B (**3**) and B_4 (**4**), and pyrenocines A (**5**) and B (**6**). We report herein the isolation, structural elucidation, and biological activities of these compounds.

Fermentation and Isolation

V. hemipterigenum was collected from Khlong Nakha Wildlife Sanctuary, Phetchaboon province, northern Thailand, on Homoptera – adult leafhopper, and identified by Dr. NIGEL L. Hywel-Jones of Mycology Research Unit, BIOTEC. The fungus is deposited at the Thailand BIOTEC Culture Collection as BCC 1449.

A culture of BCC 1449 maintained on potato dextrose agar was inoculated into potato dextrose broth (4× 250 ml). After 7 days, the primary inoculum (total 1 liter) was transferred into 40×1 liter Erlenmeyer flasks, each containing 250 ml of potato dextrose broth medium, and incubated at 30°C for 14 days. The culture filtrate (10 liters) was extracted twice with equal volume of EtOAc, and the combined organic layer was concentrated under reduced pressure to obtain a brown gum (481 mg). Trituration in MeOH (2 ml) gave light yellow crystals (123 mg). The filtrate was concentrated to obtain a brown gum (320 mg). Recrystallization of the crystals from EtOH-H_2O gave pure enniatin B (**3**; colorless crystals, 80 mg). The dried filtrate was purified by Sephadex LH-20 column (MeOH eluent)

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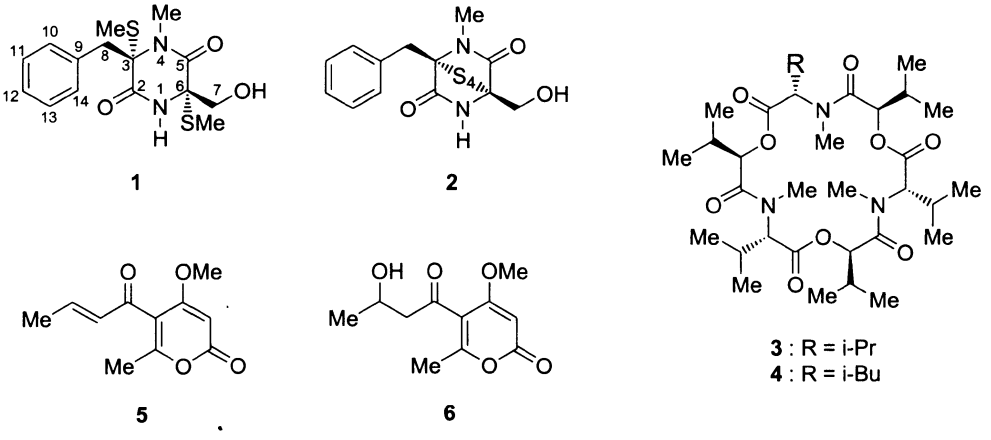
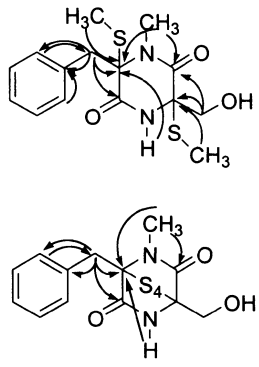
Structures of **1**~**6**.



followed by column chromatography on silica gel (0~20% MeOH in CH_2Cl_2, gradient elution) to obtain, in the order of elution, five fractions: Fr-1 (62 mg), Fr-2 (17 mg), Fr-3 (21 mg), Fr-4 (24 mg) and Fr-5 (33 mg). Each fraction was subjected to preparative HPLC using a reversed-phase column (Prep Nova-Pak® HRC_{18}, 6 μm, 40×100 mm) with MeCN/H_2O as eluent, followed by column chromatography on silica gel (5~40% EtOAc in hexane, gradient elution). Compound **5** (43 mg) was obtained from Fr-1. Compounds **2** (3.6 mg) and **6** (5.9 mg) were obtained from Fr-2. From Fr-3 was isolated compound **1** (8.1 mg). Compounds **3** (5.2 mg) and **4** (3.3 mg) were isolated from Fr-4. Fr-5 mainly provided enniatin B (**3**).

Fig. 1. Selected HMBC correlations for **1** and **2**.



Structure Elucidation

The ^{13}C NMR spectrum ($CDCl_3$) of compound **1** showed twelve carbon signals where two carbonyl carbon signals were superimposed at δ_C 164.9 ppm; this was evident by the detection of two separate signals at δ_C 167.3 and δ_C 167.4 ppm in the spectrum acquired in MeOH-d_4 (total 13 signals). Combined analyses of ^1H, ^{13}C, DEPTs, COSY and HMQC spectra revealed that this compound possesses a benzyl group, a hydroxymethyl group, two methylthio groups (δ_C 13.5, δ_H 2.23 ppm; and δ_C 14.4, δ_H 2.20 ppm), a methyl group attached to an amide nitrogen (δ_C 30.3, δ_H 3.28 ppm), a secondary amide proton (δ_H 6.39 ppm, exchangeable with D_2O), two quaternary carbons at δ_C 64.8 and 75.7 ppm, and two carbonyls. The molecular formula of **1**, $C_{15}H_{20}S_2N_2O_3$, was determined by HRMS (ESI-TOF) analysis. The presence of amide groups was evident by strong IR absorptions at ν_{max} 1693 and 1634 cm^{-1}. HMBC correlations (in MeOH-d_4) demonstrated that the benzyl group, one of the methylthio groups (δ_H 2.27 ppm), one carbonyl (δ_C 167.3 ppm), and the methylated amide nitrogen were attached to the quaternary carbon at δ_C 76.5 ppm. Considering also other HMBC correlations (Fig. 1), the structure of **1** was elucidated as depicted.

The structure of **1** was confirmed by X-ray crystallographic analysis and it revealed that the two methylthio groups of **1** are attached to the same side of the six-membered ring (Fig. 2). The absolute configuration of **1** was elucidated unambiguously to be (3*S*,6*S*) by the standard anomalous scattering method.

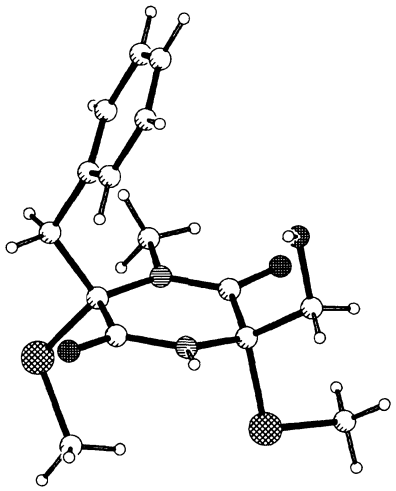
The NMR, IR and UV spectra of compound **2** were close to those of **1** except for the lack of the two NMR signals of

sulfur-connected methyl groups both in ^{1}H and ^{13}C spectra. The molecular formula of $C_{13}H_{14}S_4N_2O_3$, established by HRMS, requested a structure bearing $-S_4-$ bridge depicted as **2**. Since compound **2** was isolated from the same extract as that of **1**, it is more likely to possess (3*S*,6*S*)-configuration rather than its optical antipode.

Compounds **1** and **2** are new 1-demethyl analogs of the known bisdethiodi(methylthio)hyalodendrin[8~10] and hyalodendrin tetrasulfide,[9~11] respectively. (3*S*,6*S*)-bisdethiodi(methylthio)hyalodendrin and (3*S*,6*S*)-hyalodendrin tetrasulfide have previously been isolated from *Hyalodendron* spp.,[8,11] while the (3*R*,6*R*)-isomers of these compounds were isolated from *Penicillium turbatum*.[9] In the extract of *V. hemipterigenum* BCC 1449, these known compounds were not detected.

The spectral data (^{1}H and ^{13}C-NMR, IR, MS, and UV) of enniatins B (**3**: colorless crystals; mp 173~175°C; $[\alpha]_D^{29}$ −96, *c* 1.04, $CHCl_3$) and B_4 (**4**: amorphous solid; $[\alpha]_D^{27}$ −57, *c* 0.09, $CHCl_3$) were identical to those previously isolated from the mycelial extract by our group,[7] and also identical to literature data.[12~15] Structures of compounds **5** (colorless oil) and **6** (colorless oil) were elucidated by NMR, MS and IR analyses as known α-pyrones, pyrenocines A and B, respectively. Spectral data (^{1}H and ^{13}C NMR, IR, MS, UV) were consistent with those reported in literatures.[16~18]

Fig. 2. X-Ray crystal structure of **1**.



Biological Activities

Compounds **1**~**6** were tested for *in vitro* antimalarial activity and cytotoxicity against two cancer cell lines (KB and BC-1) and Vero cells (Table 2). Assay for activity against *P. falciparum* (K1, multi-drug resistant strain) was performed using the standard protocol[19] which follows the microculture radioisotope technique.[20] Cytotoxic activities against human epidermoid carcinoma (KB) and human breast cancer (BC-1) cell lines and African green monkey kidney fibroblast (Vero cells) were evaluated using the colorimetric method.[21] IC_{50} values of a standard compound ellipticine are 0.46 μg/ml for KB, and 0.60 μg/ml for BC-1.

A number of diketopiperazines from fungal strains have been reported to show variety of biological activities such as antibacterial,[9,22] antiviral,[9] and antifungal.[10] This class of compounds also exhibit inhibitory activities against plasminogen activator inhibitor-1,[23] calpain[24] and farnesyltransferase.[25] In the present research, however, we found first that compound **2** shows antimalarial activity.

Table 1. Physico-chemical properties of **1** and **2**.

	1	**2**
Appearance	Colorless crystals	Colorless crystals
MP	154~157 °C	152~156 °C
Molecular formula	$C_{15}H_{20}N_2O_3S_2$	$C_{13}H_{14}N_2O_3S_4$
HRMS (ESI-TOF, negative)		
Found (*m/z*)	339.0841 $[M-H]^-$	372.9820 $[M-H]^-$
Calcd.	339.0837	372.9808
$[\alpha]_D$	−70° (*c* 0.21, $CHCl_3$, 26 °C)	−123° (*c* 0.16, $CHCl_3$, 26 °C)
UV λ_{max} nm (log ε) in EtOH	205 (4.35), 258 (2.94)	204 (4.35), 299 (3.59)
IR ν_{max} (KBr) cm^{-1}	3399, 3205, 3108, 1693, 1634, 1435, 1389, 1042, 701	3290, 3102, 1694, 1634, 1436, 1436, 1388, 1054, 752, 711

Table 2. ^{1}H and ^{13}C NMR data of compounds **1** and **2**.

position	1			2	
	^{1}H ($CDCl_3$)	^{13}C ($CDCl_3$)	^{13}C (MeOH-d_4)	^{1}H ($CDCl_3$)	^{13}C ($CDCl_3$)
2		164.9[a](s)	167.3[c](s)		168.0[b](s)
3		75.7 (s)	76.5 (s)		78.1 (s)
5		164.9[a](s)	167.4[c](s)		168.0[b](s)
6		64.8 (s)	66.6 (s)		71.0 (s)
7	2.74 (brd, 11.6) 3.42 (d, 11.8)	65.2 (t)	66.3 (t)	3.79 (dd, 12.2, 8.7) 3.94 (dd, 12.2, 4.7)	65.4 (t)
8	3.15 (d, 13.8) 3.54 (d, 13.9)	42.3 (t)	43.1 (t)	3.30 (d, 14.6) 3.87 (d, 14.6)	39.4 (t)
9		133.7 (s)	135.6 (s)		133.4 (s)
10, 14	7.11-7.32 (m)	128.8 (d)	129.7 (d)	7.25-7.31 (m)	128.9 (d)
11, 13	7.11-7.32 (m)	130.0 (d)	131.1 (d)	7.16-7.19 (m)	129.4 (d)
12	7.11-7.32 (m)	128.0 (d)	128.7 (d)	7.25-7.31 (m)	127.9 (d)
N-CH_3	3.28 (s)	30.3 (q)	30.9 (q)	3.17 (s)	30.3 (q)
N-*H*	6.39 (brs)			6.60 (brs)	
3-SCH_3	2.20 (s)	14.4 (q)	14.1 (q)		
6-SCH_3	2.23 (s)	13.5 (q)	13.7 (q)		
7-O*H*	1.85 (brs)			2.71 (m)	

[a, b] Two ^{13}C signals are overlapping. [c]Assignment can be interchanged.

Table 3. Antimalarial and cytotoxic activities of compounds **1**~**6**.

compound	antimalarial activity *P. falciparum* K1 (IC_{50}, μg/ml)	cytotoxicity (IC_{50}, μg/ml)		
		KB cells[a]	BC-1 cells[b]	Vero cells[c]
compound **1**	>20	>20	>20	>20
compound **2**	2.5	15	3.9	8.9
enniatin B (**3**)	0.27	16	18	17
enniatin B_4 (**4**)	0.20	11	12	18
pyrenocine A (**5**)	7.1	3.2	1.2	2.3
pyrenocine B (**6**)	22	>20	4.3	7.2
chloroquine diphosphate[d]	0.16	>20	16	>20

[a] Human epidermoid carcinoma in the mouth (oral cavity). [b] Human breast cancer cells. [c] African green monkey kidney fibroblast. [d] Standard antimalarial compound.

Thus, compound **2** moderately inhibited the proliferation of *P. falciparum* K1 while the bisdimethylthio ether **1** was inactive (Table 3).

Enniatin B, the major secondary metabolite, was most active against the malaria parasite, thus, it is most likely that this compound was responsible for the antimalarial activity as detected in the extract of the fungus BCC 1449. It should be noted that compounds **2**~**6** exhibited both antimalarial activity and cytotoxicity, therefore, the *in vitro* antimalarial activity of these compounds may be due to their cytotoxicity.

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