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Unique Diketopiperazine Dimers from the Insect Pathogenic Fungus *Verticillium hemipterigenum* BCC 1449

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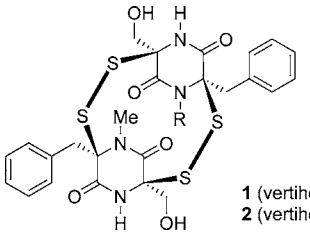
ABSTRACT



1 (vertihemiptellide A): R = Me
2 (vertihemiptellide B): R = H

Vertihemiptellides A (1) and B (2), unique diketopiperazine dimers, were isolated from the insect pathogenic fungus *Verticillium hemipterigenum* BCC 1449. Structures of these compounds were elucidated by NMR and mass spectral analysis, and the stereochemistry of 1 was determined by X-ray crystallography. The absolute stereochemistry of bisdethiodi(methylthio)-1-demethylhyalodendrin (3), previously isolated from the same fungus, was revised to the (3*R*,6*R*) configuration.

We report herein the isolation and structural elucidation of two novel diketopiperazine dimers, vertihemiptellide A (**1**) and B (**2**). Although a number of epipolythiodiketopiperazines have been isolated from fungal sources,[1] the dimeric structure via two disulfide bridges as shown in **1** and **2** has been hitherto unknown.

Recently, we isolated two new enniatins (cyclohexadepsipeptides)[2] and two new diketopiperazines (compound **3** and its tetrathio derivative)[3] from the insect pathogenic fungus *Verticillium hemipterigenum* BCC 1449. Studies on fermentation conditions focused on enniatin production resulted in the conclusion that yeast extract sucrose (YES) medium was efficient for BCC 1449, giving rapid mycelial growth and high amounts of enniatins.[4] Interestingly, the fermentation conditions were also suitable for production of epipolythiodiketopiperazines. Two novel diketopiperazine dimers, **1** (18.7 mg) and **2** (18.2 mg), were isolated together with the major constituent, **3** (bisdethiodi(methylthio)-1-demethylhyalodendrin; 379 mg), a known minor derivative, **4** (7.7 mg),[5] and a plausible biosynthetic precursor, **5** (*cyclo*-L-Ser-L-Phe; 7.0 mg; $[\alpha]^{25}_{D}$ −56, *c* 0.20, MeOH), from the EtOAc

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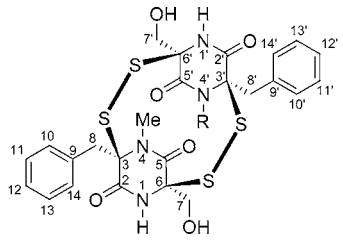
extract of the culture filtrate (10 L) by Sephadex LH20 column (MeOH), repeated silica gel column chromatography ($EtOAc/CH_2Cl_2$, and $MeOH/CH_2Cl_2$), followed by preparative HPLC (reverse-phase column, $MeOH/H_2O$).[6]



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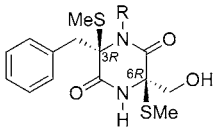
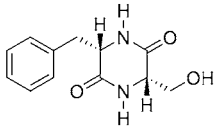


3 : R = Me
4 : R = H



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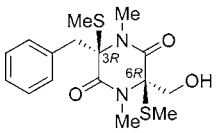

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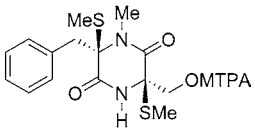

7a : (*R*)-MTPA ester
7b : (*S*)-MTPA ester

Vertihemiptellide A (**1**)[7] was obtained as a colorless solid (mp 233−234 °C, dec). The molecular formula of **1** was determined by HRMS as $C_{26}H_{28}N_4O_6S_4$. The presence of only 11 carbon signals in the ^{13}C NMR spectrum indicated the symmetric, homo-dimer structure of this compound. Analysis of ^1H and ^{13}C NMR, DEPTs, and HMQC spectra revealed that one-half of the molecule, $C_{13}H_{14}N_2O_3S_2$, possessed two amides (two carbonyls, δ_C 165.0 and 163.6; N*H*, δ_H 7.81; N*CH*$_3$, δ_C 29.8, δ_H 2.84), a benzyl group, a hydroxymethyl group, and two quaternary carbons (δ_C 78.2 and 70.5). HMBC correlation data were used to elucidate the structure of the half unit: the same structure as that of compound **3**, but lacking methylthio groups. Considering the symmetry and the requirement of incorporation of four sulfur atoms (by HRMS), a dimer structure via two −S−S− bridges was proposed for vertihemiptellide A (**1**). Finally, X-ray crystallographic analysis (Figure 1)[8] revealed the head-to-tail

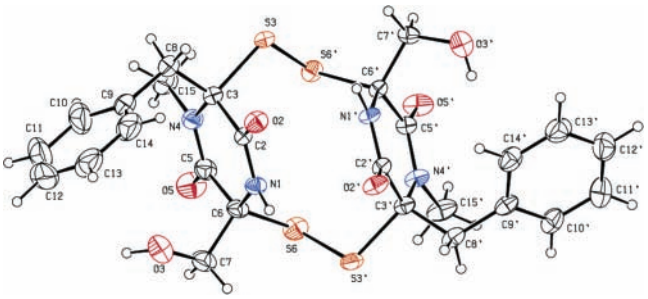

Figure 1. Crystal structure of vertihemiptellide A (**1**).

dimeric structure as shown in **1**. The absolute stereostructure of **1** was established as 3*R*,6*R*,3′*R*,6′*R* by anomalous dispersion method.

The molecular formula of vertihemiptellide B (**2**)[9] was determined by HRMS and ^{13}C NMR as $C_{25}H_{26}N_4O_6S_4$. ^{13}C NMR spectral data indicated a non-symmetrical structure for this compound, lacking a N(4′)-methyl group in **1**. Assignment of protons and carbons were established by analysis of 2D-NMR data, especially HMBC correlations (Table 1).

In the previous report,[2] we proposed the (3*S*,6*S*) configuration for compound **3** ($[\alpha]^{26}_D$ −70; *c* 0.21, $CHCl_3$), isolated from the same fungus (BCC 1449) cultured in potato dextrose broth (PDB) medium, based on X-ray crystallographic analysis. However, it was not consistent with the above-mentioned absolute stereochemistry of **1**. Compound **3** ($[\alpha]^{24}_D$ −63; *c* 0.30, $CHCl_3$), isolated in the present study (from culture of BCC 1449 grown in YES medium) was converted to the known compound **6**[10−12] by *N*-methylation (MeI, K_2CO_3, 2-butanone). Optical rotation data for this compound, $[\alpha]^{24}_D$ −39 (*c* 0.20, MeOH) and $[\alpha]^{24}_D$ −43 (*c*

(6) *Verticillium hemipterigenum* was collected from Khlong Naka Wildlife Sanctuary, Ranong province, southern Thailand, on Homoptera-adult leafhopper and identified by Dr. Nigel L. Hywel-Jones of the Mycology Research Unit, BIOTEC. This fungus is deposited in the BIOTEC Culture Collection (BCC) as BCC 1449.

(7) Vertihemiptellide A (**1**): colorless solid; mp 233−234 °C (dec); $[\alpha]^{25}_D$ −393 (*c* 0.05, dioxane); UV (MeOH) λ_{max} (log ϵ) 204 (4.61) nm; IR (KBr) ν_{max} 3523, 3444, 1696, 1683, 1654, 1645, 1436, 1395, 1083, 704 cm^{-1}; HRMS (ESI-TOF) *m*/*z* 643.0781 (calcd for $C_{26}H_{28}N_4O_6S_4Na$, 643.0789) $[M + Na]^+$; ^1H NMR (500 MHz, DMSO-d_6) δ 7.81 (2H, s, 1(N)-*H*, 1′(N)-*H*), 7.18−7.21 (10H, m, H-10, H-10′, and H-11, H-11′, and H-12, H-12′, and H-13, H-13′, and H-14, H-14′), 4.85 (2H, dd, *J* = 7.0, 6.4 Hz, 7-O*H*, 7′−O*H*), 3.67 (2H, d, *J* = 14.4 Hz, Ha-8, Ha-8′), 3.48 (2H, dd, *J* = 11.2, 7.5 Hz, Ha-7, Ha-7′), 3.40 (2H, dd, *J* = 11.3, 6.1 Hz, Hb-7, Hb-7′), 3.31 (2H, d, *J* = 14.6 Hz, Hb-8, Hb-8′), 2.84 (6H, s, 4(N)-C*H*$_3$, 4′(N)-C*H*$_3$); ^{13}C NMR (125 MHz, DMSO-d_6) δ 165.0 (s, C-5 and C-5′), 163.6 (s, C-2, C-2′), 133.9 (s, C-9, C-9′), 129.6 (d, C-10, C-10′, and C-14, C-14′), 128.2 (d, C-11, C-11′, and C-13, C-13′), 127.1 (d, C-12, C-12′), 78.2 (s, C-3, C-3′), 70.5 (s, C-6, C-6′), 68.9 (t, C-7, C-7′), 39.3 (t, C-8, C-8′), 29.8 (q, 4(N)-*C*H$_3$, 4′(N)-*C*H$_3$).

(8) Compound **1** was recrystallized by slow evaporation in dioxane, which is also present in the crystal structure. Crystal data for compound (**1**) at 298(2) K: $C_{26}H_{28}N_4O_6S_4$ï$C_4H_8O_2$, M_r = 708.89, monoclinic, space group $P2_1$ (No. 4), *a* = 10.1387(2) Å, *b* = 10.6986(4) Å, *c* = 15.7571(11) Å, β = 95.362(2)°, *V* = 1701.7(1) Å3, *Z* = 2, D_x = 1.383 Mg m^{-3}. F_{000} = 744, λ (Mo Kα) = 0.71073 Å, μ = 0.333 mm^{-1}. Data collection and reduction: crystal size 0.15 × 0.20 × 0.20 mm^3, θ range 0.998−24.72°, 14115 reflections collected, 5560 independent reflections, 5168 observed ($I > 2\sigma(I)$ (R_{int} = 0.055), final *R* indices: R_1 = 0.0560, wR_2 = 0.1502 for 415 parameters, GOF = 1.065. Flack parameter = 0.13(10). The coordinates were deposited with the Cambridge Crystallographic Data Centre with reference code CCDC 266624. These data can be obtained free of charge via the Internet at www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB21EZ, UK; fax: (+44)1223-336-033; e-mail: deposit@ccdc.cam.ac.uk.

(9) Vertihemiptellide B (**2**): colorless solid; mp 224−226 °C (dec); $[\alpha]^{25}_D$ −182 (*c* 0.05, dioxane); UV (MeOH) λ_{max} (logϵ) 207 (4.72) nm; IR (KBr) ν_{max} 3537, 3417, 3324, 1701, 1678, 1656, 1434, 1401, 1079, 1058, 705 cm^{-1}; HRMS (ESI-TOF) *m*/*z* 629.0640 (calcd for $C_{25}H_{26}N_4O_6S_4Na$, 629.0633) $[M + Na]^+$; NMR data in DMSO-d_6, Table 1.

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Table 1. NMR Data for Vertihemiptellide B (**2**) in DMSO-d_6.

position	^{1}H (mult, *J* in Hz)	^{13}C (mult)	HMBC (H to C)
1(N)-*H*	7.70 (s)		C-3,5
2		164.6 (s)	
3		78.7 (s)	
4(N)-*CH*$_3$	2.86 (s)	30.2 (q)	C-3,5
5		165.67 (s)[b]	
		70.9 (s)[c]	
7	3.40 (m)	69.3 (t)	C-5
	3.54 (dd, 11.4, 7.7)		C-6
7-O*H*	4.84 (dd, 7.2, 6.4)		C-7
8	3.29 (d, 14.7)	40.0 (t)	C-2,3,9,10,14
	3.66 (d, 14.4)		C-2.3,9,10,14
9		134.4 (s)	
10, 14	7.18−7.21 (m)[a]	130.0 (d)	
11, 13	7.18−7.21 (m)[a]	128.4 (d)[d]	
12	7.18−7.21 (m)[a]	127.5 (d)[e]	
1′(N)-*H*	7.39 (s)		C-3′,5′
2′		164.4 (s)	
3′		73.0 (s)	
4′(N)-*H*	9.59 (s)		C-2′,6′
5′		165.68 (s)[b]	
6′		71.0 (s)[c]	
7′	3.29 (m)	69.1(t)	C-5′
	3.35 (m)		C-6′
7′−O*H*	4.61 (dd, 7.0, 6.5)		
8′	2.98 (d, 14.2)	42.4 (t)	C-2′,3′,9′,10′,14′
	3.49 (d, 14.5)		C-2′,3′,9′,10′,14′
9′		134.6 (s)	
10′, 14′	7.18−7.21(m)[a]	130.8 (d)	
11′, 13′	7.18−7.21(m)[a]	128.7 (d)[d]	
12′	7.18−7.21(m)[a]	127.6 (d)[e]	

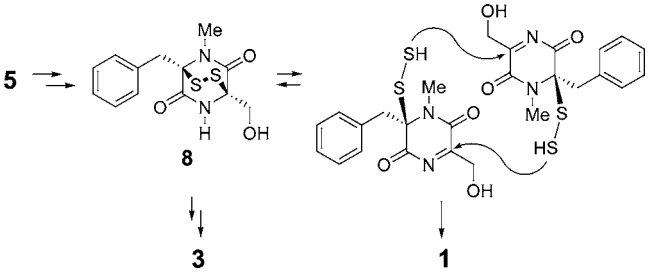
[a] The proton signals of the phenyl group(s) are overlapping. [b−e] Assignments of carbons are interchangeable.

0.235, $CHCl_3$), were consistent with the literature data for (3*R*,6*R*)-**6** (A26771E; $[\alpha]^{27}_D$ −47, *c* 0.13, MeOH)[10] and opposite to that for (3*S*,6*S*)-**6** (bisdethiodi(methylthio)-hyalodendrin; $[\alpha]^{23}_D$ +64; *c* 1.071, $CHCl_3$).[12] Compound **3** (from YES culture) was also converted to its (*R*)- and (*S*)-MTPA esters, **7a** and **7b**, by treatment with (*S*)- and (*R*)-MTPA-Cl, respectively. Each ester was obtained as a single product and was clearly distinguishable from each other in the ^{1}H NMR spectrum, which indicated that compound **3** was enantiomerically pure. Finally, we reexamined the X-ray diffraction analysis of **3**, of the compounds both from previous isolation (culture in PDB medium) and from the present isolation (culture in YES medium), which indicated the (3*R*,6*R*) configuration.[13] On the basis of these experimental data, we wish to revise the absolute stereochemistry of compound **3**, produced by *V. hemipterigenum* BCC 1449, to the (3*R*,6*R*) configuration.

Spectral data (^{1}H and ^{13}C NMR, IR, MS) for compound **4** ($[\alpha]^{25}_D$ −34; *c* 0.30, dioxane) were consistent with those of bis-*N*-norgliovictin ($[\alpha]_D$ −32; *c* 0.1, MeOH) previously isolated together with gliotoxin and several related compounds from *Gliocladium virens*.[5]

A plausible biosynthetic pathway for **1** is proposed in Scheme 1. The epidithiodiketopiperazine **8** (1-demethyl analogue of hyalodendrin[14]/A26771A[10]) could well be the precursor for the dimer **1**, although we did not detect this hypothetical compound. Because compounds **3** and **4** are stable upon extensive silica gel chromatography, it seems very unlikely that the conversion from **8** to **1** occurred during the isolation. The presence of the diketopiperazine **5** as a co-metabolite in BCC 1449 suggested that replacement of the α-protons with sulfur atoms (**5** to **8**) should take place with retention of configuration. Since these biosynthetic pathways (Scheme 1) are highly speculative, other possible mechanisms for the formation of compound **1** have been considered, for example, (a) a radical pathway, instead of the ionic dimerization shown in Scheme 1, and (b) dimerization of bis-thioradical intermediates, genarated by the cleavage of S−S bond in **8** or directly from **5**.

Scheme 1. Possible Biogentic Pathways



Compounds **1**−**4** exhibited growth inhibitory activity against *Mycobacterium tuberculosis* H_{37}Ra but also showed moderate cytotoxic activities (Table 2). These compounds

Table 2. Antimycobacterial and Cytotoxic Activities of Compounds **1**−**4**

compd	anti-TB (MIC, μg/mL)	cytotoxicity (IC_{50}, μg/mL) KB[b]	BC[c]	NCI−H187[d]	Vero[e]
1	12.5	>20	8.3	4.4	4.9
2	12.5	>20	16.8	3.5	9.7
3	100	>20	>20	>20	>50
4	25	>20	>20	6.6	49.9
isoniazid[a]	0.06	*f*	*f*	*f*	*f*

[a] Standard antitubercular drug. [b] Human epidermoid carcinoma in the mouth. [c] Human breast cancer cells. [d] Human small cell lung cancer. [e] African green monkey kidney fibroblast. [f] Not tested.

(13) Compound **3** was recrystallized in EtOAc−hexane by slow evaporation. Crystal data for compound (**3**) at 298(2) K: $C_{15}H_{20}N_2O_3S_2$, M_r = 340.46, monoclinic, space group $P2_1$ (No. 4), a = 10.9060(5) Å, b = 8.0074-(2) Å, c = 19.0249(8) Å, β = 94.790(8)°, V = 1655.6(1) Å3, Z = 4, D_x = 1.360 Mg m^{-3}. F_{000} = 720, λ (Mo Kα) = 0.71073 Å, μ = 0.333 mm^{-1}. Data collection and reduction: crystal size 0.20 × 0.25 × 0.30 mm^3, θ range 2.15−27.49°, 15288 reflections collected, 7165 independent reflections, 6406 observed ($I > 2\sigma(I)$) (R_{int} = 0.036), final R indices: R_1 = 0.0410, wR_2 = 0.0972 for 397 parameters, GOF = 1.077, Flack parameter = −0.02-(5). The coordinates were deposited with the Cambridge Crystallographic Data Centre with reference code CCDC 266625. These data can be obtained free of charge via the Internet at www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB21EZ, UK; fax: (+44)1223-336-033; e-mail: deposit@ccdc.cam.ac.uk.

were inactive against the malarial parasite *Plasmodium falciparum* K1 (at a concentration of 20 μg/mL) and the fungus *Candida albicans* (at 50 μg/mL).

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Supporting Information Available: Experimental procedures, ^{1}H and ^{13}C NMR spectra of compounds **1** and **2**, and crystallographic data of **1** and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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