Table 1. NMR Spectral Data for Aigialone (**1**) and Aigialospirol (**2**)

aigialone (**1**) ($CDCl_3$)				aigialospirol (**2**) (acetone-d_6/D_2O, 4:1)			
position	δ_C (mult.)	δ_H (mult., J in Hz)	HMBC (H to C)	position	δ_C (mult.)	δ_H (mult., J in Hz)	HMBC (H to C)
2	84.0 (d)	4.71 (q, 7.1)	3, 7a	1	105.1 (s)		
3	197.9 (s)			2	158.4 (s)		
3a	90.7 (s)			3	102.3 (d)	6.42 (d, 1.9)	1, 2, 4, 5
4	67.5 (d)	4.44 (brs)	5, 7a	4	167.1 (s)		
5	71.7 (s)			5	100.6 (d)	6.61 (dd, 1.9, 0.9)	1, 3, 4, 1′
6	89.6 (d)	4.32 (brd, 10.1)	4, 5, 7a, 9, 10	6	151.8 (s)		
7a	180.8 (s)			1′	81.4 (d)	5.33 (d, 5.2)	6, 2′, 3′, −*C*OO−
8	16.0 (q)	1.47 (d, 7.1)	2, 3	2′	66.3 (d)	4.27 (ddd, 12.0, 5.2, 2.6)	
9	18.3 (q)	1.35 (s)	4, 5, 6	3′	32.9 (t)	1.75 (ddd, 13.9, 12.0, 2.9)	2′
10	28.6 (t)	1.92 (m), 1.78 (m)	6, 12			1.65 (ddd, 13.9, 3.4, 2.5)	4′, 5′
11	26.5 (t)	1.60 (m), 1.39 (m)		4′	68.3 (d)	4.02 (ddd, 3.4, 3.3, 3.1)	
12	29.13 (t)[a]	1.35−1.25 (m)[b]		5′	70.9 (d)	3.48 (d, 3.6)	7′
13	29.10 (t)[a]	1.35−1.25 (m)[b]		6′	99.1 (s)		
14	31.7 (t)	1.35−1.25 (m)[b]		7′	127.2 (d)	5.52 (ddd, 10.1, 2.0, 2.0)	6′, 9′
15	22.6 (t)	1.35−1.25 (m)[b]		8′	131.3 (d)	6.04 (ddd, 10.0, 4.3, 3.6)	6′
16	14.0 (q)	0.88 (t, 6.8)	14, 15	9′	31.4 (t)	1.92−1.93 (2H, m)	7′, 8′, 10′
O*H*-4		3.99 (brs)	3a, 4, 5	10′	65.2 (d)	3.93 (m)	*C*H_3-10′
O*H*-5		4.57 (brs)	5, 6, 9	−*C*OO−	170.0 (s)		
				CH_3-10′	20.7 (q)	1.14 (3H, d, 6.3)	9′, 10′
				O*C*H_3-4	56.2 (q)	3.76 (3H, s)	4

[a] Interchangeable assignments. [b] Overlapping signals.

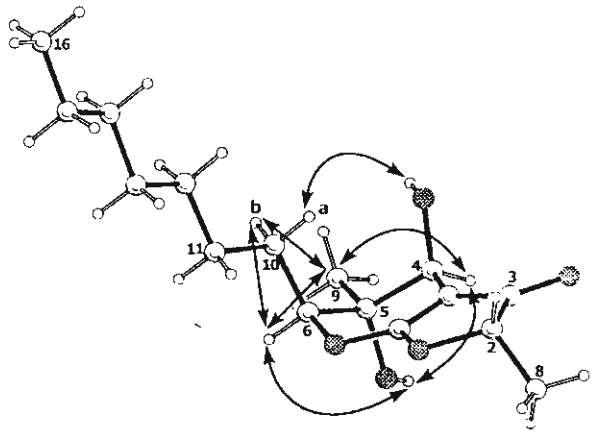
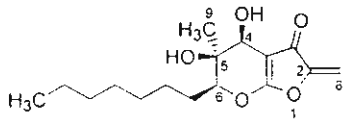


Figure 1. Crystal structure of aigialone (**1**). Selected NOESY correlations are illustrated with solid arrows.

Unfortunately attempts to prepare α-methoxy-α-trifluoromethylphenylacetic acid (MTPA) esters[7] of **1** were not successful, and the absolute configuration of **1** remains questionable.

Compounds possessing a ketene acetal functionality are very rare in nature.[8-10] The structure of aigialone (**1**) is closely related to that of benesudon (**5**), isolated from the fungus *Mollisia benesuada* A26-93.[8] Benesudon possesses a C-2−C-8 double bond (exomethylene), and its relative configuration differs from that of **1**. The relative stereochemistry (C-4, C-5, and C-6) of benesudon (**5**) was determined on the basis of the NOESY correlation data, which were in good agreement with pseudo-equatorial orientations of OH-4, the 5-methyl and 6-heptyl substituents, and a pseudo-axial OH 5.[8] However, the stereochemistry shown in Figure 1 also accounts well for the NOESY correlation data recorded for **5**. Therefore our finding casts some doubts on the relative assignment of stereochemistry of benesudon (**5**). The ^1H and ^{13}C NMR spectral data of **1** in CD_3OD were very similar to those described for compound **5** except for those of the five membered-ring moiety. In addition, a W coupling of H-4 and H-6 (J = 1.5 Hz), recorded for **5**, was suggestive for the pseudo-equatorial orientations of these two protons in **5**.[11]



5

Aigialospirol (**2**) was isolated as a pale yellow amorphous solid, and its molecular formula of $C_{16}H_{22}O_8$ was suggested by HRMS and its ^1H and ^{13}C NMR spectral data. Initially the NMR spectra of **2** were taken in $CDCl_3$; however, better peak resolution was achieved in acetone-d_6/D_2O (4:1). NMR spectral analysis revealed that aigialospirol (**2**) is somewhat related structurally to hypothemycin (**3**) and aigialomycins (e.g., **4**), possessing a resorcylic ester connected at C-6 with an aliphatic polyketide chain. Unlike the 14-membered macrolides, this compound possesses a dihydroisobenzofuranone structure, as indicated by the low-field (δ_H 5.33) shift of H-1′ and also by the HMBC correlation from this proton to the ester carbonyl. The linkage from C-1′ to C-10′, through a quaternary carbon (C-6′, δ_C 99.1), was established by analysis of the COSY, HMQC, and HMBC correlations. In addition to HMBC correlation data acquired in acetone-d_6/D_2O (Table 1), the spectrum taken in $CDCl_3$ showed a correlation from H-10′ to C-6′. Although a HMBC correlation from H-2′ to C-6′ was not observed, the molecular formula ($C_{16}H_{22}O_8$) of this compound required a spiroacetal-type structure.

Analysis of the vicinal coupling constant values and NOESY spectral correlations of **2** revealed the relative configuration from C-2′ to C-6′, demonstrating that the inner tetrahydropyran ring adopts a chair conformation. Thus, an intense NOESY interaction between one of the C-3′ methylene protons at δ_H 1.75 (H-3′ax) and H-5′ indicated the 1,3-diaxial relationship of these protons. The axial orientation of H-2′ was indicated by its large coupling (J = 12.0 Hz) to H-3′ax. The H-4′ signal appeared as a doublet of doublets of doublets with small J values (3.4, 3.3, and 3.1 Hz), and this proton showed NOESY correlations to H-3′ax, H-3′eq, and H-5′, which placed it in an equatorial position. The olefinic proton, H-7′, showed an intense NOESY correlation signal to the axial proton, H-5′, which clearly indicated the configuration of the acetal carbon (C-6′), as shown in Figure 2. Configurations of C-1′ and C-10′ relative to the C-2′−C-6′ asymmetric centers

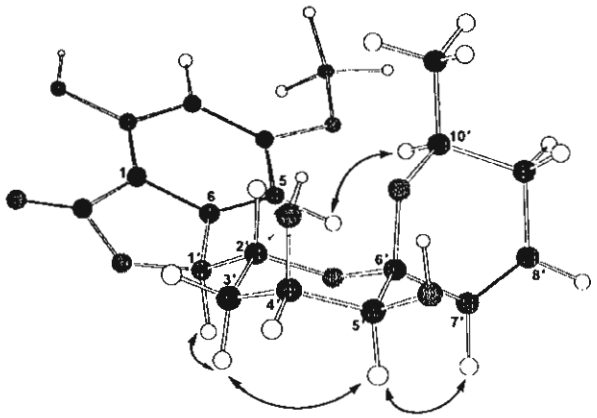


Figure 2. Probable conformation of aigialospirol (**2**). Selected NOESY correlations are illustrated with solid arrows.

were also established by NOESY spectral analysis. The H-1′ proton showed a clear NOESY correlation to H-3′ax, but was only very weakly correlated to H-3′eq. On the other hand, one of the aromatic protons, H-5, showed a NOESY correlation to H-10′, despite the long through-bond distance between these protons. These data demonstrated the relative configuration and plausible conformation of **2**, as shown in Figure 2.

The structure of aigialospirol (**2**) is related to hypothemycin (**3**), the major metabolite in the fungal extract. The relative stereochemistry of the asymmetric carbon centers of **2** was identical to that of **3** except for the opposite configuration at C-1′. Since compound **2** was not detected in the extract from the earlier harvested fermentation culture, it is not unreasonable to assume that this compound may be derived from **3**, and this correlation suggested that the absolute configuration of aigialospirol (**2**) should be that as depicted. It must be noted that aigialospirol (**2**) was not obtained as an isolation artifact. Compound **2** was detected in the HPLC/UV analysis (ODS column, MeCN/H_2O) of the crude extract, and this was confirmed by co-injection experiments with the standard.

A plausible biosynthetic pathway from **3** to **2** is the initial epoxide cleavage (hydration) of **3** by attack of a hydroxyl oxygen to the benzylic carbon (C-1′) with stereoinversion (S_N2), giving a 1′,2′-diol, followed by γ-lactone formation. Subsequent spiroacetal formation may well proceed smoothly due to the favorable *cis*-olefinic geometry. An alternative pathway, involving the hydrolysis of the ester linkage and subsequent attack at the epoxide (C-1′), may also be plausible.

Compounds **1** and **2** were inactive in our in vitro biological protocols, inclusive of antimalarial (*Plasmodium falciparum* K1), antitubercular (*Mycobacterium tuberculosis* H37Ra), antiviral (HSV-1), antifungal (*Candida albicans*), and cytotoxicity (KB cells, BC-1 cells) assays. In contrast, hypothemycin (**3**) has been shown to exhibit significant antimalarial[3] and antitumor[3,6] activities.

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 DIP-370 digital polarimeter. UV spectra were recorded on a Varian CARY 1E UV−visible spectrophotometer. FT-IR spectra were taken on a Perkin-Elmer 2000 spectrometer. NMR spectra were taken on Bruker DRX400 and AV500D spectrometers. ESI-TOF mass spectra were measured with a Micromass LCT mass spectrometer.

Fungal Material. *Aigialus parvus* was collected, identified, and isolated from mangrove wood by Prof. E. B. G. Jones in June 1999. This fungus was deposited at the Thailand BIOTEC Culture Collection as BCC 5311.[3]

Fermentation and Isolation. *A. parvus* BCC 5311 was grown on potato dextrose agar (PDA) at 22 °C for 35 days, before inoculation into 20 × 1 L Erlenmeyer flasks each containing 250 mL of potato dextrose broth (PDB). Fermentation was conducted under stationary conditions at 22 °C for 80 days. The flask cultures were filtered, and the filtrate (5 L) was extracted with an equal volume of EtOAc to obtain a light brown solid (1.23 g). This crude extract was passed through a Sephadex LH-20 column (elution with MeOH), where an early-eluting fraction (Fr-A, 160 mg) consisted mainly of aigialone (**1**), and a later fraction (Fr-B, 1.05 g) contained aigialospirol (**2**) and 14-membered macrolides. Fr-A was subjected to column chromatography on silica gel (MeOH/CH_2Cl_2, 3:97, then 5:95) to obtain aigialone (**1**, 124 mg; R_f 0.20, MeOH/CH_2Cl_2, 5:95) as a colorless solid. Fr-B was fractionated by repeated silica gel column chromatography (MeOH/EtOAc, then MeOH/CH_2Cl_2), and the following compounds were obtained in the order of elution: **3** (195 mg); **2** (16 mg); and **4** (80 mg). Compound **2** was further purified by preparative HPLC using a reversed-phase column (4.0 × 10.0 cm; MeCN/H_2O, 20:80; flow rate, 20 mL/min): t_R 15 min, 13.9 mg.

Aigialone (1): colorless crystals (EtOAc/hexane); mp 132.5−133.0 °C; $[\alpha]^{26}_D$ −193° (*c* 0.65, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 254 (4.35) nm; IR (KBr) ν_{max} 3492, 3245, 2921, 1677, 1574, 1493, 1039, 934 cm^{-1}; NMR data in $CDCl_3$, Table 1; ^1H NMR (CD_3OD, 400 MHz) δ 4.84 (1H, q, *J* = 7.0 Hz, H-2), 4.42 (1H, brd, *J* = 11.6 Hz, H-6), 4.20 (1H, d, *J* = 1.3 Hz, H-4), 2.03 (1H, m, H-10a), 1.74 (1H, m, H-10b), 1.61 (1H, m, H-11a), 1.49 (3H, d, *J* = 7.0 Hz, H-8), 1.42 (1H, m, H-11b), 1.39 (3H, s, H-9), 1.39−1.33 (8H, m, H-12, H-13, H-14, and H-15), 0.95 (3H, t, *J* = 7.0 Hz, H-16); ^{13}C NMR (CD_3OD, 100 MHz) δ 200.0 (s, C-3), 182.2 (s, C-7a), 91.9 (d, C-6), 91.6 (s, C-3a), 84.9 (d, C-2), 72.4 (s, C-5), 67.1 (d, C-4), 33.0 (t, C-14), 30.4 (t, C-10), 30.29 and 30.26 (t, C-12 and C-13), 28.0 (t, C-11), 23.7 (t, C-15), 20.8 (q, C-9), 16.3 (q, C-8), 14.4 (q, C-16); HRMS (ESI-TOF, positive) *m/z* 299.1839 (calcd for $C_{16}H_{27}O_5$, 299.1858) $[M + H]^+$.

X-ray Crystal Structure Determination of Aigialone (1). Crystal data of aigialone (**1**): $C_{16}H_{26}O_5$, MW 298.38, monoclinic, $P2_1$ (No. 4), *a* = 5.4952(2) Å, *b* = 8.6872(4) Å, *c* = 17.1402(8) Å, β = 93.979(2)°, *V* = 816.27(6) Å^3, D_x = 1.214 g/cm^3, *Z* = 2. A total of 19 048 reflections, of which 2028 were unique (1882 observed, $|F_o| > 4\sigma(F_o)$), were measured at room temperature from a 0.20 × 0.15 × 0.10 mm^3 colorless crystal using graphite monochromated Mo Kα radiation (λ = 0.71073 Å) on a Bruker-Nonius kappaCCD diffractometer. The crystal structure was solved by direct methods using SIR-97, and then all atoms except hydrogen atoms were refined anisotropically by full-matrix least-squares methods on F^2 using SHELXL-97 to give a final *R*-factor of 0.0392 (R_w = 0.1001 for all data) with a data-to-parameter ratio of 10.6:1.[12]

Aigialospirol (2): pale yellow amorphous solid; mp 85−89 °C; $[\alpha]^{25}_D$ +47° (*c* 0.50, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 217 (4.29), 257 (3.92), 292 (3.51) nm; IR (KBr) ν_{max} 3453, 1741, 1615, 1455, 1218, 1159, 1167, 1001 cm^{-1}; NMR data in acetone-d_6/D_2O, Table 1; ^1H NMR ($CDCl_3$, 500 MHz) δ 6.59 (1H, dd, *J* = 1.7, 1.0 Hz, H-5), 6.47 (1H, d, *J* = 1.8 Hz, H-3), 6.16 (1H, ddd, *J* = 10.0, 4.2, 3.7 Hz, H-8′), 5.66 (1H, ddd, *J* = 10.1, 2.1, 1.9 Hz, H-7′), 5.36 (1H, d, *J* = 6.0 Hz, H-1′), 4.15 (1H, ddd, *J* = 12.0, 6.0, 2.3 Hz, H-2′), 4.13 (1H, m, H-4′), 4.01 (1H, m, H-10′), 3.86 (3H, s, OCH_3-4), 3.52 (1H, d, *J* = 3.6 Hz, H-5′), 2.05−2.03 (3H, m, H-3′a and H-9′), 1.88 (1H, ddd, *J* = 13.9, 12.1, 3.0 Hz, H-3′b), 1.26 (3H, d, *J* = 6.2 Hz, CH_3-10′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.3 (s, −COO−), 167.3 (s, C-4), 157.7 (s, C-2), 149.1 (s, C-6), 130.5 (d, C-8′), 126.5 (d, C-7′), 104.3 (s, C-1), 101.6 (d, C-3), 101.2 (d, C-3), 99.1 (s, C-6′), 82.3 (d, C-1′), 70.7 (d, C-5′), 68.3 (d, C-4′), 65.8 (d, C-2′), 65.0 (d, C-10′), 56.0 (q, OCH_3-4), 33.6 (t, C-3′), 31.4 (t, C-9′), 21.1 (q, CH_3-10′), HRMS (ESI-TOF, positive) *m/z* 401.1228 (calcd for $C_{19}H_{22}O_8$-Na, 401.1212) $[M + Na]^+$.

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Supporting Information Available: NMR and IR spectra of compounds **1** and **2** and ORTEP diagram and crystallographic data of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) Crystallographic data of compound **1** have been deposited at the Cambridge Crystallographic Data Centre under the reference number CCDC 218502. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (e-mail: deposit@ccdc.cam.ac.uk).

NP030344D

Isolation and Structure Elucidation of Enniatins L, M_1, M_2, and N: Novel Hydroxy Analogs

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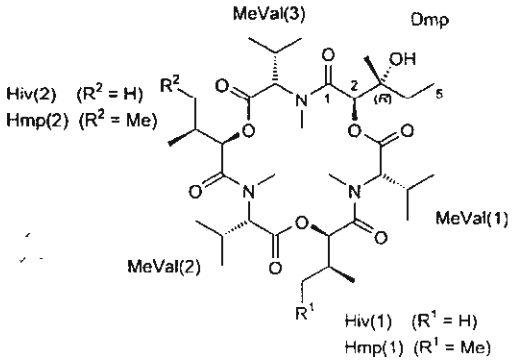
Four new cyclohexadepsipeptides, enniatins L (**1**), M_1 (**2**), M_2 (**3**), and N (**4**), have been isolated from an unidentified fungus (BCC 2629), together with the known enniatins B (**5**), H (**6**), and I (**7**), MK1688 (**8**), and enniatin B_4 (**9**). Compounds **1**–**4** are the first enniatin analogs with an OH group at the side chain of one of the 2-hydroxycarboxylic acid residues. The structures of **1**–**4** were elucidated by spectroscopic means and by X-ray crystallography.

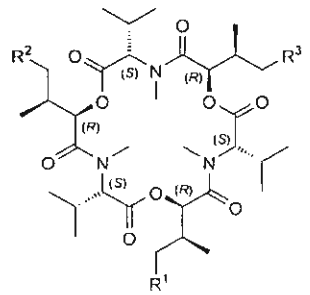
Introduction. – Enniatins are well-known cyclohexadepsipeptide antibiotics produced by various *Fusarium* species [1]. These compounds have been known to exhibit antibiotic [2][3], insecticidal [4][5], and phytotoxic activities [6][7]. They also inhibit acyl-CoA cholesterol acyltransferase (ACAT) [8]. Generally, enniatins consist of three D-configured 2-hydroxyisovaleric acid (Hiv) and three L-configured *N*-methyl amino acid residues linked alternately to furnish an 18-membered macrocycle.

Recently, we reported the isolation of the two new enniatins H and I, which bear one and two 2-hydroxy-3-methylpentanoic acid (Hmp) residues instead of Hiv, respectively, together with the known enniatins B and B_4, and their antimalarial and antituberculous activities were also evaluated [9]. In our further search for enniatin-producing fungi from the Thailand BIOTEC Culture Collection (BCC), we came across the strain BCC 2629, an unidentified fungus, from which four new compounds, enniatins L (**1**), M_1 (**2**), M_2 (**3**), and N (**4**), were isolated, along with the known enniatins B (**5**), H (**6**), I (**7**), MK1688 (**8**), and enniatin B_4 (**9**). The new compounds **1**–**4** are the first representatives of naturally-occurring enniatins possessing an OH group at one of the 2-hydroxy carboxylic acid residue side chains. Herein, we report the isolation, structure elucidation, and biological activities of these new analogs.

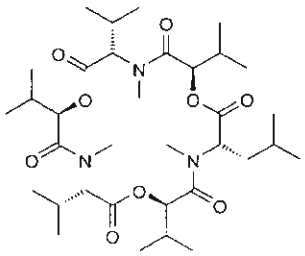
Results and Discussion. – Enniatins **1**–**9** were isolated by chromatographic fractionation of the MeOH extract of mycelia of the BCC-2629 fermentation broth. The four new compounds **1**–**4** and enniatin B_4 (**9**) were obtained as minor components, major metabolites being enniatins B (**5**), H (**6**), I (**7**), and MK 1688 (**8**). Unfortunately, attempted purification of **2** and **3** had met with failure, therefore, they were isolated as an inseparable *ca.* 1:1 mixture. The spectral data of the known enniatins **5**–**9** were identical to those reported in the literature [9–11].



1 $R^1 = R^2 = H$
2 $R^1 = Me$, $R^2 = H$
3 $R^1 = H$, $R^2 = Me$
4 $R^1 = R^2 = Me$



5 $R^1 = R^2 = R^3 = H$
6 $R^1 = Me$, $R^2 = R^3 = H$
7 $R^1 = R^2 = Me$, $R^3 = H$
8 $R^1 = R^2 = R^3 = Me$



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The ^{1}H- and ^{13}C-NMR spectra of enniatin L (**1**) suggested that this compound is an enniatin analog, possessing three *N*-methyl amino acid and three 2-hydroxycarboxylic acid units. The IR spectrum of **1** was very similar to those of the known enniatins **5**–**9**, showing ester (1741 cm^{-1}) and amide (1660 cm^{-1}) resonances, except for an additional OH absorption at 3442 cm^{-1}. High-resolution mass spectral (HR-MS) analysis gave rise to the molecular formula $C_{34}H_{59}N_3O_{10}$, in accord with an additional O-atom. NMR analysis (^{1}H, ^{13}C, DEPT, COSY, HMQC, and HMBC; in $CDCl_3$) revealed that all three *N*-methyl amino acid units were *N*-methylvaline (MeVal), whereas two of the three 2-hydroxycarboxylic acid units were 2-hydroxyisovaleric acid (Hiv). The remaining acid residue was elucidated as 2,3-dihydroxy-3-methylpentanoic acid (Dmp), which, so far, has not been found in previously reported enniatins. The ^{1}H-NMR signal for H–C(2), the α-H-atom, a sharp *singlet* at δ(H) 5.08, indicated that C(3) of Dmp was quaternary. HMBC Correlations of **1** clearly indicated that C(3) at δ(C) 73.6 was connected with a Me, an Et, and an OH group (*Fig. 1*).

The alternating connectivity pattern of the six acid residues was revealed by the analysis of HMBC and NOESY spectral data (*Fig. 1*). Most importantly, intense

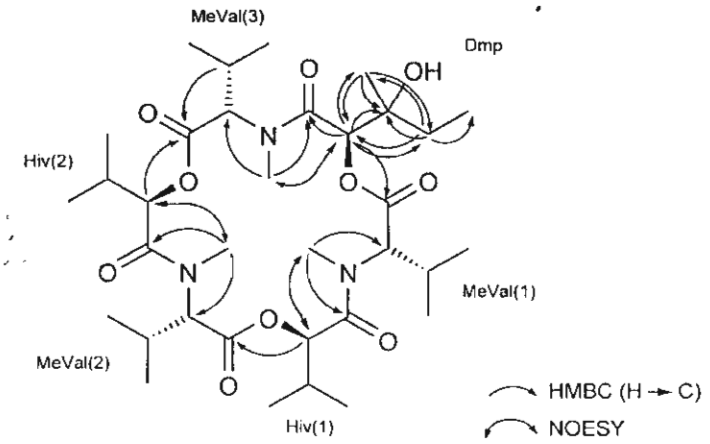

Fig. 1. *Selected HMBC and NOESY correlations for enniatin L* (**1**), *showing the connectivity of the six residues of the macrocycle as well as the partial structure of the Dmp residue*

NOESY correlations were observed for the three N-Me H-atoms at δ(H) 3.26 (MeVal(3)), 2.90 (MeVal(1)), and 3.37 (MeVal(2)) with respect to the three α-H-atoms of the 2-hydroxy acid units at δ(H) 5.08 (Dmp), 5.55 (Hiv(1)), and 5.20 (Hiv(2)). In contrast, the NOESY cross-signals between the N-Me and the α-H resonances of the corresponding MeVal units were absent or very weak relative to the above-mentioned correlations. These data are consistent with the established relative configurations and conformations of known enniatins, such as enniatin B [12], wherein the three N-Me groups are co-planar to the α-H-atoms of the neighboring (2*R*)-Hiv moiety in the macrocyclic ring, while the N-Me groups are antiperiplanar to the corresponding α-H-atoms of (2*S*)-MeVal. We also observed similar NOESY-correlation data for enniatins **5**–**9** in the same solvent ($CDCl_3$). The α-H-atoms and the C-atoms of the six units were clearly distinguished in the ^{1}H- and ^{13}C-NMR spectra (*Table 1*).

Enniatin N (**4**), which had the molecular formula $C_{36}H_{63}N_3O_{10}$ according to HR-MS, showed UV and IR spectra similar to those of **1**. Analysis of 2D-NMR (COSY, NOESY, HMQC, HMBC) spectra revealed that **4** consists of three MeVal, one Dmp, and two 2-hydroxy-3-methylpentanoic acid (Hmp) residues. The structural information derived from HMBC and NOESY data were very similar to those of **1**. Again, NOESY correlations of the three N-Me resonances at δ(H) 3.28 (MeVal(3)), 2.92 (MeVal(1)), and 3.38 (MeVal(2)), respectively, to the three α-H-atoms of the 2-hydroxy acid units at δ(H) 5.11 (Dmp), 5.65 (Hmp(1)), and 5.30 (Hmp(2)) strongly supported the proposed structure.

Attempts to separate compounds **2** and **3** by means of various chromatographic techniques had met with failure. Therefore, spectroscopic analyses were conducted for a *ca.* 1:1 mixture. The IR and UV Spectra of the mixture **2**/**3** were very similar to those of **1** and **4**. The ESI-TOF mass spectrum of the mixture showed two intense signals at *m*/*z* 684 (100%) and 706 (65%), correponding to $[M+H]^+$ and $[M+Na]^+$, respectiverly, which indicated that the two compounds had identical molecular

Table 1. *NMR Data of Enniatins L* (**1**) *and N* (**4**). At 500/125 MHz in $^{*}CDCl_3$; δ in ppm, J in Hz. For abbreviations, see text and chemical formulae.

Residue	Group	**1**		**4**	
		^{13}C	^{1}H	^{13}C	^{1}H
DMP	C(1)	168.3	–	168.4	–
	H–C(2)	75.1	5.08 (*s*)	75.0	5.11 (*s*)
	C(3)	73.6	–	73.7	–
	CH_2(4)	34.9	1.59 (*q*, *J* = 7.3)	34.8	1.61 (*q*, *J* = 7.5)
	Me(5)	7.7	0.97 (*t*, *J* = 7.5)	7.9	0.99 (*t*, *J* = 7.4)
	Me(4')	22.6	1.32 (*s*)	22.6	1.34 (*s*)
	OH	–	4.72 (br. *s*)	–	n.d.[c])
MeVal(1)	C(1)	169.7	–	169.7	–
	H–C(2)	61.2	4.87 (*d*, *J* = 10.5)	61.4	4.88 (*d*, *J* = 10.8)
	H–C(3)	25.7	2.18 (*m*)	25.8	2.20 (*m*)
	Me(4)	19.8	0.99 (*d*, *J* = 6.8)	19.9[a])	1.02 (*d*, *J* = 6.6)
	Me(4')	18.4	0.83 (*d*, *J* = 6.7)	18.4	0.85 (*d*, *J* = 6.7)
	N–Me	31.8	2.90 (*s*)	31.9	2.92 (*s*)
Hiv(1) or Hmp(1)	C–(1)	168.8	–	168.8	–
	H–C(2)	74.0	5.55 (*d*, *J* = 8.9)	73.1	5.65 (*d*, *J* = 9.1)
	H–C(3)	30.5	2.33 (*m*)	36.8	2.10 (*m*)
	Me(4), CH_2(4)	18.8	0.98 (*d*, *J* = 6.9)	24.4	1.52, 1.17 (2*m*)
	Me(5)	–	–	11.3[b])	0.92 (*t*, *J* = 7.4)
	Me(4')	18.0	0.93 (*d*, *J* = 6.9)	14.8	0.95 (*d*, *J* = 6.6)
MeVal(2)	C(1)	170.7	–	170.7	–
	H–C(2)	61.0	5.00 (*d*, *J* = 9.8)	61.1	5.03 (*d*, *J* = 9.8)
	H–C(3)	28.6	2.24 (*m*)	28.7	2.26 (*m*)
	Me(4)	20.0	1.12 (*d*, *J* = 6.6)	20.0[a])	1.13 (*d*, *J* = 6.6)
	Me(4')	19.8	0.92 (*d*, *J* = 6.9)	19.8[a])	0.97 (*d*, *J* = 6.7)
	N–Me	32.4	3.37 (*s*)	32.4	3.38 (*s*)
Hiv(2) or Hmp(2)	C(1)	170.7	–	170.7	–
	H–C(2)	74.0	5.20 (*d*, *J* = 9.9)	73.1	5.30 (*d*, *J* = 9.6)
	H–C(3)	29.7	2.24 (*m*)	36.0	2.07 (*m*)
	Me(4), CH_2(4)	18.6	0.95 (*d*, *J* = 6.9)	24.6	1.38, 1.05 (2*m*)
	Me(5)	–	–	11.2[b])	0.92 (*t*, *J* = 7.4)
	Me(4')	17.7	[illegible]	14.6	0.93 (*d*, *J* = 6.8)
MeVal(3)	C(1)	169.4	–	169.5	–
	H–C(2)	67.1	3.27 (br.)	67.7	3.43 (br.)
	H–C(3)	28.3	2.34 (*m*)	28.4	2.37 (*m*)
	Me(4)	21.3	1.05 (*d*, *J* = 6.5)	21.3	1.08 (*d*, *J* = 6.5)
	Me(4')	19.1	0.95 (*d*, *J* = 7.0)	19.1	0.98 (*d*, *J* = 6.2)
	N–Me	38.7	3.26 (*s*)	38.8	3.28 (*s*)

[a]) [b]) Assignments may be interchanged. [c]) Not detected.

formulae, *i.e.*, $C_{35}H_{61}N_5O_{10}$. 2D-NMR Spectral analysis revealed that **2**/**3** consists of six MeVal, two Dmp, two Hmp, and two Hiv residues. These informations, as well as the observation that **1**–**4** were only minor components (in contrast to the major metabolites **5**–**8**) suggested that both **2** and **3** consist of three MeVal, one Dmp, one Hmp, and one Hiv residues each, and that only the connectivity pattern differs. ^{1}H- and ^{13}C-NMR assignments of each residue for **2** and **3** could not be distinguished due to strong signal overlapping. Therefore, these data are presented separately as sets of signals (*Table 2*).

Table 2. *NMR Data of a 1:1 Mixture of* **2**/**3**. At 500/125 MHz in $CDCl_3$; δ in ppm, J in Hz.

Residue	C-Atom	^{13}C	^1H	Residue	C-Atom	^{13}C	^1H
Dmp	C(1)	168.3	–	MeVal(2)	C(1)	170.6	–
(two units)	H–C(2)	74.9	5.09, 5.08 (2*s*)	(two units)	H–C(2)	61.0	5.01 (*d*, *J* = 9.8)
	C(3)	73.6	–		H–C(3)	28.6	2.24 (*m*)
	CH_2(4)	34.8, 34.7	1.60 (*q*, *J* = 7.4)		Me(4)	19.9, 19.7[a])	1.12, 1.11 (2*d*, *J* = 6.5 each)
	Me(5)	7.7	0.97 (*t*, *J* = 7.3)		Me(4′)	19.8[a])	0.98–0.92[c])
	Me(4′)	22.5	1.32 (*s*)		N–Me	32.4, 32.3	3.36, 3.35 (2*s*)
MeVal(1)	C(1)	169.7	–	Hiv(2)	C(1)	170.6	–
(two units)	H–C(2)	61.3, 61.2	4.87 (*d*, *J* = 10.2), 4.85 (*d*, *J* = 9.8)	(for **2**)	H–C(2)	74.0	5.20 (*d*, *J* = 9.9)
	H–C(3)	25.7	2.18 (*m*)		H–C(3)	29.7	2.24 (*m*)
	Me(4)	19.9[a])	0.99 (*d*, *J* = 7)		Me(4)	18.8[b])	0.98–0.92[c])
	Me(4′)	18.4, 18.3	0.83 (*d*, *J* = 6.7)		Me(4′)	17.7	0.88 (*d*, *J* = 6.7)
	N–Me	31.8	2.90 (*s*)				
Hiv(1)	C(1)	168.8[b])	–	Hmp(2)	C(1)	170.6	–
(for **3**)	H–C(2)	74.1	5.55 (*d*, *J* = 8.9)	(for **3**)	H–C(2)	73.1	5.27 (*d*, *J* = 9.7)
	H–C(3)	30.4	2.33 (*m*)		H–C(3)	35.9	2.05 (*m*)
	Me(4)	18.6[c])	0.98–0.92[c])		CH_2(4)	24.3	1.37, 1.03 (2*m*)
	Me(4′)	18.0[c])	0.98–0.92[c])		Me(5)	11.2[d])	0.89 (*t*, *J* = 7.3)
					Me(4′)	14.5	0.98–0.92[c])
Hmp(1)	C(1)	168.7[b])	–	MeVal(3)	C(1)	169.5	–
(for **2**)	H–C(2)	73.0	5.62 (*d*, *J* = 8.2)	(two units)	H–C(2)	67.8, 67.6	3.41 (br.)
	H–C(3)	36.7	2.07 (*m*)		H–C(3)	28.4, 28.3	2.34 (*m*)
	CH_2(4)	24.5	1.51, 1.15 (2*m*)		Me(4)	21.2	1.06, 1.05 (2*d*, *J* = *ca.* 6.5 each)
	Me(5)	11.1[d])	0.89 (*t*, *J* = 7.3)		Me(4′)	19.1, 19.0[c])	0.98–0.92[c])
	Me(4′)	14.7	0.98–0.92[c])		N–Me	38.9, 38.8	3.26, 3.25 (2*s*)

[a])–[d]) Assignments may be interchanged. [c]) Overlapping signals.

The structure of enniatin N (**4**) was confirmed by X-ray single-crystal structure analysis (*Fig. 2*). The relative configuration in α-position (C(2)) of the amino- and hydroxy acid residues was found to be identical to that of other known enniatins. By means of correlation between **4** and the co-metabolites **5**–**9**, the (2*S*)-configuration for the three MeVal residues of **4**, and the (2*R*)-configuration for Dmp and the two Hmp residues were assigned. Hence, the configuration at the quaternary C(3)-atom in the Dmp unit of **4**, which could not be determined by NMR methods, was (*R*). The very similar ^1H- and ^{13}C-NMR data for the single Dmp moieties of compounds **1**–**4** (*Tables 1* and *2*) strongly suggested that **1**–**3** should also possess (3*R*)-configuration. It should also be noted that the (3*S*)-configuration of the two Hmp units of **4** is the same as in enniatins H (**6**), I (**7**), and MK1688 (**8**), as previously and independently established by means of precursor-directed biosynthesis [9].

The new enniatins L (**1**), M_1/M_2 (**2**/**3**), and N (**4**) were tested for their antimalarial, antituberculosis, and cytotoxic activities realtive to the known enniatin B (**5**). The new hydroxy congeners displayed similar growth-inhibitory activities as the reference compound, suggesting that the OH group does not play an important role in these assays (*Table 3*).

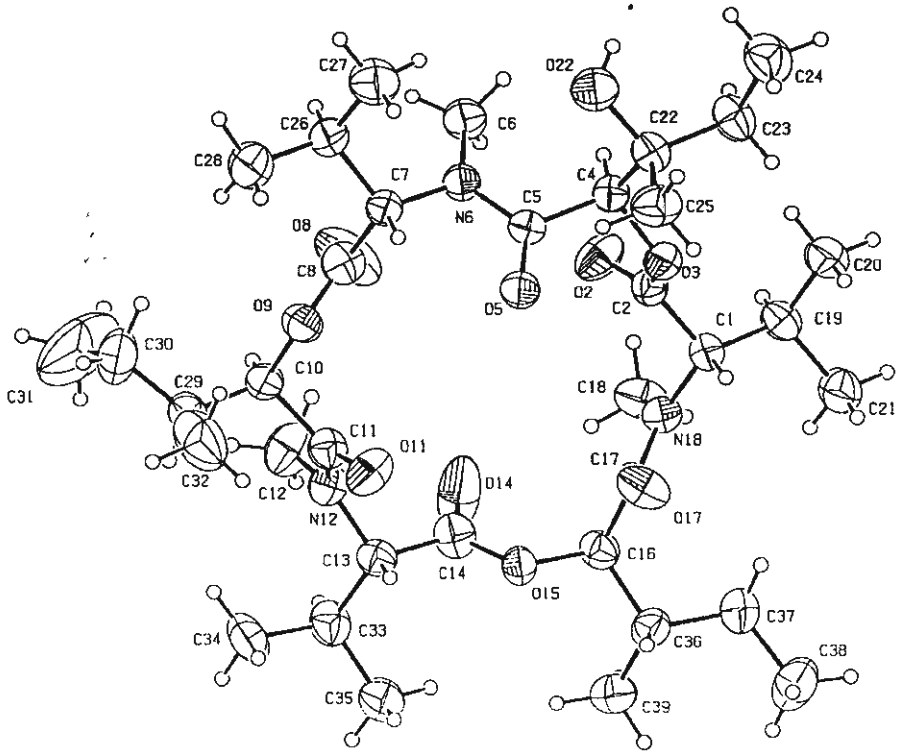

Fig. 2. *X-Ray crystal structure* (ORTEP plot) *of Enniatin N* (**4**)

Table 3. *Biological Activities of* **1**–**5** *against Malaria- and Tuberculosis-Inducing Microorganisms, as well as Cytotoxic Activities towards Different Cancer and Fibroblast Cells.* KB: oral human epidermal carcinoma cells; BC: human breast cancer cells; NCI-H187: human small cell lung-cancer cells; Vero: African-green-monkey kidney-fibroblast cells. For details, see the *Exper. Part*.

Compound	Malaria[a])	Tuberculosis[b])	Cytotoxicity[c])			
	IC_{50} [μg/ml]	*MIC* [μg/ml]	KB	BC	NCI-H187	Vero
1	3.3	12.5	3.6	3.8	2.1	6.4
2/**3**[d])	3.4	6.25	3.1	2.0	2.2	3.7
4	3.4	6.25	4.0	0.78	1.2	4.7
5	3.4	3.13	5.8	2.7	0.96	5.9

[a]) Against *P. falciparum* K1; the standard antimalarial drug dihydroartemisinin had an IC_{50} value of 0.0012 μg/ml. [b]) Against *M. tuberculosis* H37Ra; the standard antituberculosis drugs *isoniazid* and *kanamycin* had *MIC* values of 0.06 and 2.5 μg/ml, resp. [c]) The standard drug *ellipticine* exhibited IC_{50} values of 1.3, 1.5, 0.39, and 0.40 μg/ml for KB, BC, NCI-H187, and Vero cells, resp. [d]) Approximately a 1:1 mixture.

Experimental Part

General. Column chromatography (CC): *Sephadex LH-20* (*Pharmacia*) or *Silica Gel 60 H* (*Merck*). HPLC: *Waters 600* controller; *Waters 996* photodiode-array detector; *Nova-Pak HR-C18* (40 × 100 mm; 6 μm) column.

M.p.: Electrothermal *IA9100* digital melting-point apparatus; uncorrected. Optical rotations: *JASCO DIP-370* digital polarimeter. UV Spectra: *Varian CARY-1E* spectrophotometer; λ_{max} in nm ($\log \varepsilon$). IR Spectra: *Bruker VECTOR-22* spectrophotometer; in cm^{-1}. ^1H-, ^{13}C-, and 2D-NMR: *Bruker AV500* spectrometer; in $CDCl_3$; δ in ppm rel. to $SiMe_4$ as internal standard. *J* in Hz. MS: *Micromass LCT* spectrometer.

Fungal Material. The fungus used in this study had been isolated on May 27, 2000, by Dr. *Nigel L. Hywel-Jones* (Mycology Research Unit, BIOTEC) from spore attached to the synnema of *Hirsutella fomicarum* on ant collected at Khao Sok National Park, Surat Thani, southern Thailand. This fungus was deposited at the Thailand BIOTEC Culture Collection (BCC), registered as BCC 2629. The colony-growth rate was moderate on potato dextrose agar, reaching a diameter of 50 mm in 20 d, pure white, diffuse, thin and immersed with aerial mycelium, hyaline, septate and branching, up to 3 μm with sporulation of the fungus at 25° under normal daylight illumination. Anamorphic stage formed-genus *Acremonium*, however, it is not possible to identify into species level as this fungal genera is revising at the moment.

Fermentation, Extraction, and Isolation. The fungus BCC 2629 was maintained on potato dextrose agar at 25° for 21 d, after which the mycelium was cut into pieces and inoculated in four 250-ml *Erlenmeyer* flasks, each containing 25 ml of potato-dextrose broth (PDB). After incubation at 25° for 8 d on a rotary shaker (200 r.p.m.), these primary seed cultures were transferred into four 1000-ml *Erlenmeyer* flasks, each containing 250 ml of PDB, and incubated at 25° for another 8 d on a rotary shaker (200 r.p.m.). Each 25-ml portion of the secondary seed cultures was transferred into forty 1000-ml *Erlenmeyer* flasks containing the same liquid medium, and the fermentation was carried out at 25° for 40 d under stationary condition. Then, the cultures were filtered, and the residual mycelial cakes were extracted at r.t. with MeOH (1500 ml) for 2 d. To the filtrate was added H_2O (100 ml), and the mixture was washed with hexane (800 ml). The aq. MeOH layer was concentrated under reduced pressure. The residue was dissolved in AcOEt (300 ml), washed with H_2O (100 ml), and the org. layer was concentrated under reduced pressure, leaving a deep-yellow amorphous solid (2.2 g). This extract was purified by CC (1. *Sephadex LH-20*; CH_2Cl_2/MeOH; 2. SiO_2; MeOH/CH_2Cl_2) to obtain a mixture of enniatins (1.99 g). This mixture was subjected to repeated prep. HPLC (*ODS* column; MeCN/H_2O 70:30). The compounds were eluted in the following order: **1** > **2**, **3**, **5** > **9** > **4**, **6** > **7** > **8**. Repeated HPLC purification of each fraction with MeOH/H_2O 80:20 then afforded **1** (21 mg), **5** (126 mg), an inseparable 1:1 mixture of **2**/**3** (63 mg), **9** (27 mg), **4** (25 mg), **6** (246 mg), **7** (224 mg), and **8** (173 mg).

Enniatin L (**1**[1]). Colorless powder. M.p. 132–133°. $[\alpha]_D^{25} = -102$ ($c = 0.22$, $CHCl_3$). UV (MeOH): 206 (4.11). IR (KBr): 3542, 2970, 1741, 1660, 1471, 1191, 1016. ^1H- and ^{13}C-NMR: see *Table 1*. HR-ESI-TOF-MS: 692.4908 ($[M + Na]^+$, $C_{34}H_{59}N_3NaO_{10}^+$; calc. 692.4098).

1:1 Mixture of Enniatins M_1 and M_2 (**2**/**3**). Colorless powder. M.p. 114–115°. $[\alpha]_D^{25} = -102$ ($c = 0.22$, $CHCl_3$). UV (MeOH): 207 (4.20). IR (KBr): 3442, 2968, 1744, 1659, 1469, 1189, 1012. ^1H- and ^{13}C-NMR: see *Table 2*. HR-ESI-TOF-MS: 684.4445 ($[M + H]^+$, $C_{35}H_{62}N_3O_{10}^+$; calc. 684.4436).

Enniatin N (**4**). Colorless crystals. M.p. 98–99°. $[\alpha]_D^{25} = -102$ ($c = 0.22$, $CHCl_3$). UV (MeOH): 206 (4.17). IR (KBr): 3416, 2968, 1743, 1659, 1467, 1193, 1010. ^1H- and ^{13}C-NMR: see *Table 1*. HR-ESI-TOF-MS: 676.4121 ($[M - H]^-$, $C_{36}H_{62}N_3O_{10}^-$; calc. 698.4591).

X-Ray Crystal Structure of Enniatin N (**4**)[2]. Crystals of compound **4** were grown from EtOH at r.t. Diffraction data were acquired from a colorless prism of size 0.25 × 0.30 × 0.50 mm on a *Bruker-Nonius kappaCCD* diffractometer (graphite-monochromated MoK_α radiation; $\lambda = 0.71073$ Å) over a θ-range of 1.02–21.97°. No significant decay was observed during the data collection. From a total of 4859 unique reflection ($R_{int} = 0.045$), 4065 reflections were observed with $I \leq 2\sigma(I)$. The structure of **4** was solved by direct methods using SIR97 [13], and refined by full-matrix least-square optimization on F^2 using SHELXL97 [14]. All non-H atoms were refined anisotropically, and the H-atoms were fixed at calculated positions, and refined by means of a riding model. The final indices were $R = 0.0707$ and $wR = 0.1818$, with a goodness-of-fit on F^2 of 1.12 and a data-to-parameter ratio of 10.54. The final difference-electron-density map showed a maximum of + 0.24 and a minimum of − 0.15 $Å^{-3}$, resp. The following data were generated: molecular formula $C_{36}H_{67}N_3O_{12}$ (**4**·2 H_2O);

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[1]) Systematic name: (3*S*,6*R*,9*S*,12*R*,15*S*,18*R*)-6-[(1*R*)-1-hydroxy-1-methylpropyl]-3,9,12,15,18-pentaisopropyl-4,10,16-trimethyl-1,7,13-trioxa-4,10,16-triazacyclooctadecane-2,5,8,11,14,17-hexone.

[2]) Crystallographic data have been deposited with the *Cambridge Crystallographic Data Centre* as deposition No. CCDC-233901. Copies of the data can be obtained, free of charge, on application to the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-1223-336033; e-mail: deposit@ccdc.cam.ac.uk) or *via* internet (http://www.cam.ac.uk/conts/retrieving.html).

M_r 733.94; trigonal system, space group $P\,3_1$; unit-cell parameters: $a = b = 14.8277(3)$, $c = 16.6565(3)$ Å; $V = 3171.5(1)$ Å^3; $D_x = 1.149$ g cm^{-3}; $Z = 3$, $\mu = 0.85$ cm^{-1}.

Biological Assays. The antimalarial assay for activity against *P. falciparum* K1 was performed according to a standard protocol [15], which follows the microculture radioisotope technique described by *Desjardins et al.* [16]. Growth inhibition against *M. tuberculosis* H37Ra was performed by means of the Microplate Alamar-Blue Assay (MABA) [17]. Cytotoxic activities of the pure compounds (or the 1 : 1 mixture in case of **2/3**) against oral human epidermal carcinoma (KB) cells, human breast-cancer (BC) cells, human small-cell lung-cancer (NCI-H187) cells, and African-green-monkey kidney fibroblasts (Vero cells) were evaluated colorimetrically [18].

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Enniatin Production by the Entomopathogenic Fungus *Verticillium hemipterigenum* BCC 1449

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Optimal fermentation conditions for enniatin production using the entomopathogenic fungus *Verticillium hemipterigenum* BCC 1449 have been investigated. Among various liquid media tested, highest efficiency of enniatin production was achieved by fermentation in yeast extract sucrose. Application of this condition to large-scale fermentation resulted in the isolation of three new analogs, O_1, O_2 and O_3, which are closely related isomers that were characterized as an inseparable mixture, along with seven known enniatins.

Recently, we reported the isolation and structure elucidation of two new enniatins[1] and two new diketopiperazines[2] from the entomopathogenic fungus *Verticillium hemipterigenum* BCC 1449. This fungus has also been shown to be useful for the studies on precursor-directed biosynthesis of enniatin analogs. Feeding L-leucine resulted in the selective uptake of this precursor in the *N*-methylamino acid residue of the enniatin molecule, while L-isoleucine was used as precursor for the 2-hydroxycarboxylic acid residue. In the above mentioned preliminary studies,[1] however, the low efficiency of enniatin production by BCC 1449 was a problem. Some minor enniatin analogs eluded the isolation/characterization process due to their low relative and absolute amounts in the crude extract from large-scale fermentation. This technical limitation in the precursor-feeding experiments has also been responsible for the problems in characterizing minor enniatin analogs. Considering the significant biological activities of this class of cyclodepsipeptide antibiotics as well as the aim to create a mini-library of enniatin analogs by precursor-directed biosynthesis[1,3,4] we decided to optimize the fermentation conditions for enniatin production using strain BCC 1449. In this paper, we report the results of the studies on fermentation conditions for BCC 1449 and the isolation of enniatins from a large-scale fermentation broth.

Results and Discussion

Fungal Material

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 the Mycology Research Unit, BIOTEC.[5] The fungus was deposited at the Thailand BIOTEC Culture Collection as BCC 1449.

Fermentation, Extraction, and Metabolites Analysis for Optimization

For the optimization of enniatin production, small scale cultivations were conducted in six liquid media (25°C, 21 days); 1) PDB (potato dextrose broth: potato infusion 200 g, Bacto dextrose 20 g, per liter distilled water), 2) SDB (Sabouraud's dextrose broth: peptone 15 g, glucose 20 g, per liter distilled water), 3) MSM (minimum salt medium: glucose 20 g, yeast extract 1.0 g, NH_4NO_3 3.0 g, KH_2PO_4 0.5 g, NaH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCl_2$ 0.5 g, per liter distilled water), 4) FDM (sucrose 25 g, $NaNO_3$ 4.25 g, NaCl 5.0 g, $MgSO_4 \cdot 7H_2O$ 2.5 g, KH_2PO_4 1.36 g, $FeSO_4 \cdot 7H_2O$ 0.1 g, $ZnSO_4 \cdot 7H_2O$ 0.0029 g, per liter distilled water), 5) YES (yeast extract sucrose: yeast extract 20 g, sucrose 150 g, per liter distilled water), and 6) coconut

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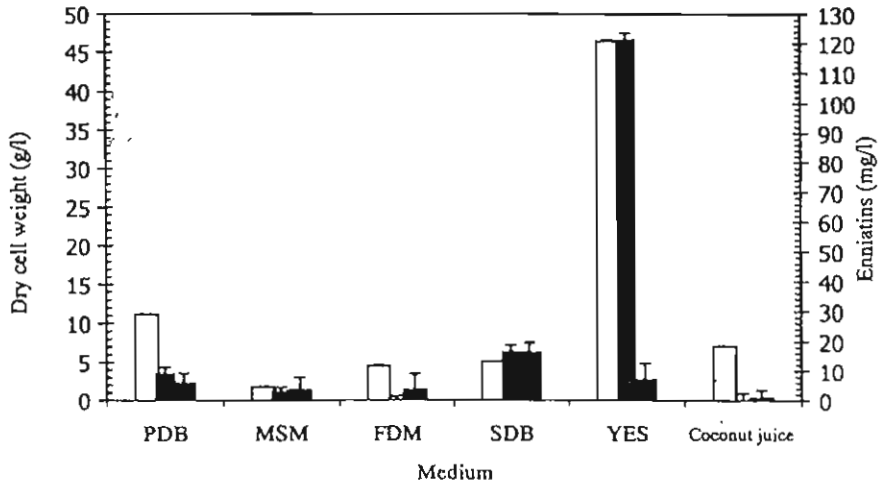
Fig. 1. Comparison of liquid medium type for enniatins production by BCC 1449.



The strain BCC 1449 was maintained on potato dextrose at 25°C for 10 days. The agar plate culture was cut pieces (1×1 cm) with a sterilized surgical knife and transferred into 250 ml Erlenmeyer flasks (2 pieces per flask) each containing 50 ml of PDB medium. The seed cultures were incubated on a rotary shaker (200 rpm) at 25°C for 4 days. A 5 ml portion of the seed culture was inoculated into twenty-five 250 ml Erlenmeyer flasks containing 45 ml of the production medium (PDB) and incubated without shaking at 25°C. After 7, 14, 21, 28 and 35 days, 5 flasks (total 250 ml broth) were harvested and subjected to extraction and analysis of the metabolites. This set of experiment was performed in triplicate. Cultivations in five other media were also carried out using the PDB seed culture.

The cultures in five Erlenmeyer flasks were filtered to separate filtrate and wet mycelia. The combined filtrate (1250 ml) was extracted twice with EtOAc (250 ml each). The EtOAc solution was dried over $MgSO_4$, filtered and concentrated under reduced pressure to obtain a crude extract. The wet mycelia were freeze-dried to measure dry weight, and were extracted with MeOH (50 ml, room temp., 2 days). The methanol extract of mycelia was filtered and 25 ml of H_2O was added to the filtrate and washed with 30 ml of hexane. The aqueous MeOH layer was separated from the hexane layer, and partially concentrated under reduced pressure. The residue was dissolved in EtOAc (50 ml) and washed with H_2O (30 ml), and concentrated to yield a crude mycelial extract.

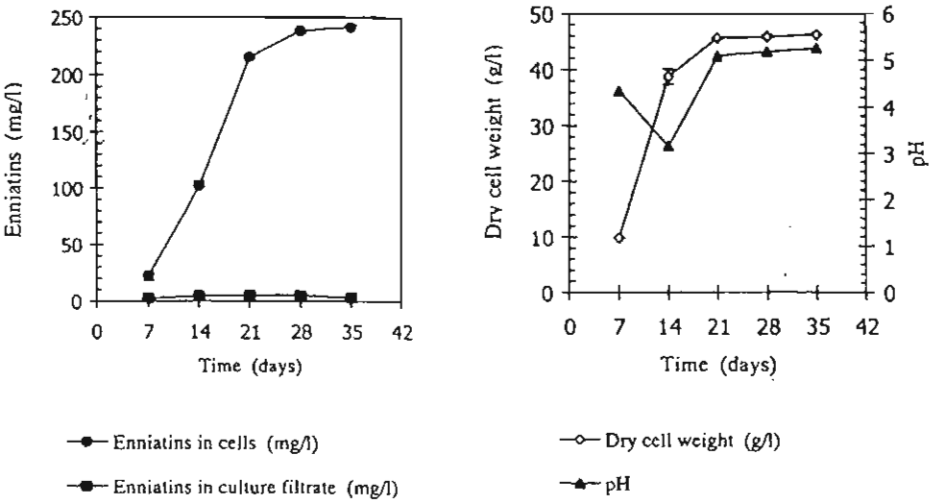
Quantitative analysis of enniatins in the extracts both from culture filtrate and mycelia was carried out by HPLC/UV (detection at 210 nm) using a reverse-phase column (NovaPak C_{18}, 4 μm, 3.9×150 mm; MeCN/H_2O=65:35; flow rate, 1 ml/minute). Ethyl 4-phenylbenzoate was used as an internal standard.

Amount of enniatins from a total 250 ml culture (5×50 ml; calculated in mg/liter), and cell dry-weights (calculated in g/liter) are shown in Fig. 1. Enniatins were produced best in YES medium, which corresponded to the efficiency of cell growth. Incubation in SDB medium also gave better production of enniatins compared to PDB, while other liquid media (MSM, FDM, coconut juice) were not sufficient for enniatin production. Compositions of enniatin isomers in the crude extracts were similar in all cases. In YES cultures, enniatins were present mainly in mycelia (Fig. 1). Examination of the time-course in YES medium revealed that enniatin production reached the maximum level within 4 weeks incubation (Fig. 2). Therefore, we set up the optimal liquid-medium fermentation conditions in laboratory scale: static fermentation in YES, at 25°C, for 28 days.

Large-scale Fermentation and Isolation

Large-scale cultivation was carried out under the

Fig. 2. Time course study on enniatins production by BCC 1449 in YES medium.



conditions optimized for enniatin production. The strain BCC 1449 in 40×1 liter Erlenmeyer flasks each containing 250 ml of YES medium were incubated for 28 days. The flask cultures were filtered, and the residual wet mycelia were extracted with MeOH (1000 ml, 2 days), then filtered. To the filtrate was added 50 ml of H_2O and washed with 500 ml of hexane. The aqueous MeOH layer was separated from the hexane layer, and partially concentrated under reduced pressure. The residue was dissolved in EtOAc (500 ml), washed with H_2O (150 ml), and concentrated to yield a brown gum (2.0 g). This extract was passed through a Sephadex LH-20 column ($MeOH/CH_2Cl_2=1:2$) and the fractions containing enniatins were combined (1.29 g) and subjected to silica gel column chromatography ($EtOAc/CH_2Cl_2$). The enniatin-containing fractions were combined (673 mg), and subjected to preparative HPLC using a reversed phase column (NovaPak HR C_{18}, 40×100 mm, 6 μm) with $MeCN/H_2O=68:32$ as eluent at a flow rate of 20 ml/minute to separate into six fractions in the following elution order: **1**, **2**, (**3** and **5**), (**4**, **8a**, **8b** and **8c**), **6**, **7**. Each fraction was further separated by repeated preparative HPLC using an eluent of $MeOH/H_2O=80/20$ to afford pure compounds; **1** (286 mg), **2** (77 mg), **3** (13 mg), **5** (65 mg), **8a/8b/8c** mixture (19 mg), **4** (0.3 mg), **6** (4 mg), and **7** (0.3 mg).

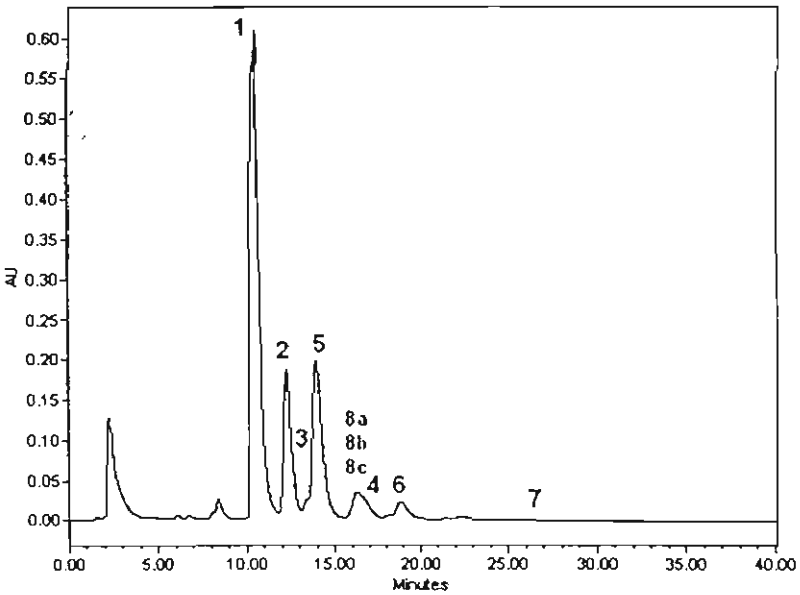
When the fermentation was repeated under the same conditions and scale, higher quantity of enniatin mixture (2.70 g) was obtained after partial purification by Sephadex LH20 and silica gel column (0.673 g, for the previous isolation). However, composition of enniatin analogs was similar in both of these fermentations. Presumably, the efficiency of the enniatin production may be affected by the seed culture preparation stage (on PDA) or other factors.

Structure Elucidation

Spectral data of known enniatins, **1**~**7**, were identical to those of our previous isolation from the same fungus[1] as well as those reported in the literature. It should be noted that enniatin C (**4**) was previously reported as a synthetic compound, and we recently obtained this compound by precursor-directed biosynthesis (feeding L-leucine) employing the same fungal strain.[1] Now it has been isolated, although in very low amount (0.3 mg), as a *bona fide* natural product.

A mixture obtained together with **1**~**7** composed of three new enniatins with an approximate ratio of 1:1:1, as indicated by its NMR spectra (^1H and ^{13}C). Attempts to separate each compound from this mixture using various chromatographic conditions met with failure, therefore, characterization was conducted for the mixture. The ESI-TOF mass spectrum indicated that the three inseparable compounds possess identical molecular formula of $C_{35}H_{61}N_3O_9$ (*m/z* 668.4502, $[M+H]^+$, $\Delta=1.6$ mmu), the same as that of enniatin G (**3**) and I (**6**). IR and UV spectral data and the specific rotation value were very similar to those of other enniatins. NMR analysis was supported by the full spectral data of related compounds **1**~**7** (all taken in $CDCl_3$), which revealed that the mixture of three compounds consists of six *N*-methylvaline, three *N*-

Fig. 3. HPLC chromatogram of enniatins mixture partially purified by silica gel column chromatography.



(NovaPak HR C_{18}, 3.9×150 mm; MeCN/H_2O=65 : 35; flow rate, 1 ml/minute).

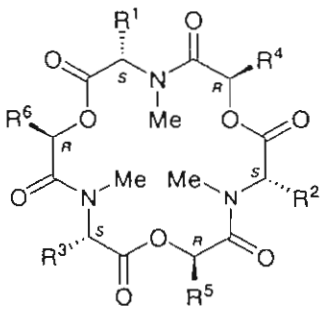
Table 1. NMR data for enniatins O_1, O_2, O_3 mixture.

	position	^{13}C NMR (125 MHz, $CDCl_3$)[a,b,c]	^1H NMR (500 MHz, $CDCl_3$)[d]
*N*MeVal	1 *C*=O	170.57, 170.51×2, 170.43×3	-
(6 units)	2	62.91, 62.73, 62.64, 61.36, 61.33×2	4.95 (2H, d, ca 9), 4.93 (d, ca 9), 4.59 (d, 9.3), 4.57 (d, 8.0), 4.53 (d, 10.0)
	3	27.92, 27.84×2, 27.76, 27.68, 27.59	2.28-2.23 (6H, m)[e]
	4	20.16, 20.13, 20.68, 19.91×2, 19.85	1.06-1.01 (18H, m)
	4'	19.76, 19.62, 19.55, 19.24×2, 19.03	0.93-0.88 (18H, m)[f]
	N-*CH*$_3$	33.22×3, 32.54, 32.49, 32.36	3.18 (3H, s), 3.16 (6H, s), 3.15 (3H, s), 3.13 (6H, s)
*N*MeLeu	1 *C*=O	170.85×3	-
(3 units)	2	56.79×2, 56.63	4.83-4.78 (3H, m)
	3	37.79, 37.66×2	1.85-1.83 (3H, m), 1.75-1.73 (3H, m)
	4	25.35, 25.31, 25.27	1.53-1.52 (3H, m)
	5	23.29, 23.25, 23.25	0.96-0.92 (9H, m)[f]
	5'	21.33×3	0.96-0.92 (9H, m)[f]
	N-*CH*$_3$	31.71×2, 31.39	3.13 (3H, s), 3.12 (3H, s), 3.09 (3H, s)
Hiv	1 *C*=O	169.69, 169.55×2, 169.48, 169.37×2	-
(6 units)	2	75.31, 75.24, 75.17×3	5.22 (d, 8.5), 5.15 (d, ca.9), 5.04 (2H, d, ca 9), 5.02 (d, 7.0), 4.98 (d, 9.1)
	3	30.31, 30.28, 29.82, 29.81, 29.74, 29.70	2.31-2.25 (6H, m)[e]
	4	18.98, 18.90, 18.70, 18.65, 18.48, 18.46	1.00-0.94 (18H, m)[f]
	4'	18.37×2, 18.35, 18.34, 18.19, 18.15	1.00-0.94 (18H, m)[f]
Hmp	1 *C*=O	169.88×2, 169.80	-
(3 units)	2	73.88, 73.77×2	5.32 (d, 7.0), 5.18 (2H, d, 7.5)
	3	37.79, 37.66×2	2.03 (m), 1.97-1.96 (2H, m)
	4	25.37, 25.35, 25.22	1.45-1.44 (3H, m), 1.19-1.18 (3H, m)
	5	14.85, 14.60, 14.36	0.93-0.88 (9H, m)[f]
	3-*CH*$_3$	14.85, 14.60, 14.36	1.00-0.94 (9H, m)[f]

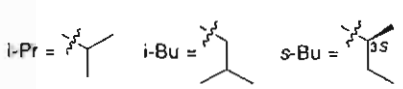
[a] Assignments of the carbonyl carbons can be interchanged between *N*MeVal and *N*MeLeu residues. [b] Assignments of the carbonyl carbons can be interchanged between Hiv and Hmp residues. [c] Assignments of the N-CH_3 carbons can be interchanged between the *N*MeVal and *N*MeLeu residues. [d] Assignments of the N-CH_3 protons can be interchanged between the *N*MeVal and *N*MeLeu residues. [e,f] ^1H signals are overlapping.

methylleucine, six 2-hydroxyisovaleric acid (Hiv), and three 2-hydroxy-3-methylpentanoic acid (Hmp) residues (Table 1). Considering also its HPLC retention time, eluted between enniatin G and enniatin I, the structures depicted as **8a**, **8b** and **8c** (Fig. 4), namely enniatins O_1, O_2 and O_3, respectively, were suggested. HMBC correlations from the *N*-methyl protons to the overlapping carbon signals at δ_C 169.3~169.9 indicated that these carbonyl carbons were assignable to those of the amides (Hiv and Hmp residues). The rest of the carbonyl carbons, situated at δ_C 170.4~170.9, were correlated from α-protons of both 2-amino acids and 2-hydroxyacids, and they were assigned to ester carbonyls (*N*MeVal and *N*MeLeu residues). Further structural analysis was conducted using the partial degradation procedure described by TOMODA *et al.* for the analysis of enniatin E.[6] $LiBH_4$ reduction of the mixture (THF, 0°C) followed by acetylation (Ac_2O, pyridine) gave a mixture of four amide fragments, **9**~**12**, in an approximate ratio of 4:2:2:1, and were separated by preparative HPLC. The authentic samples of **9**, **10** and **11** were obtained by the same reaction sequence from enniatins B (**1**), B_4 (**2**) and H (**5**), respectively. Structure of the fragment **12** was determined by NMR and mass spectral analysis.

Fig. 4. Structures of enniatins isolated from *V. hemipterigenum* BCC 1449.



	R^1	R^2	R^3	R^4	R^5	R^6
enniatin B (1)	i-Pr	i-Pr	i-Pr	i-Pr	i-Pr	i-Pr
enniatin B_4 (2)	i-Bu	i-Pr	i-Pr	i-Pr	i-Pr	i-Pr
enniatin G (3)	i-Bu	i-Bu	i-Pr	i-Pr	i-Pr	i-Pr
enniatin C (4)	i-Bu	i-Bu	i-Bu	i-Pr	i-Pr	i-Pr
enniatin H (5)	i-Pr	i-Pr	i-Pr	*s*-Bu	i-Pr	i-Pr
enniatin I (6)	i-Pr	i-Pr	i-Pr	*s*-Bu	*s*-Bu	i-Pr
MK1688 (7)	i-Pr	i-Pr	i-Pr	*s*-Bu	*s*-Bu	*s*-Bu
enniatin O_1 (8a)	i-Bu	i-Pr	i-Pr	*s*-Bu	i-Pr	i-Pr
enniatin O_2 (8b)	i-Bu	i-Pr	i-Pr	i-Pr	*s*-Bu	i-Pr
enniatin O_3 (8c)	i-Bu	i-Pr	i-Pr	i-Pr	i-Pr	*s*-Bu



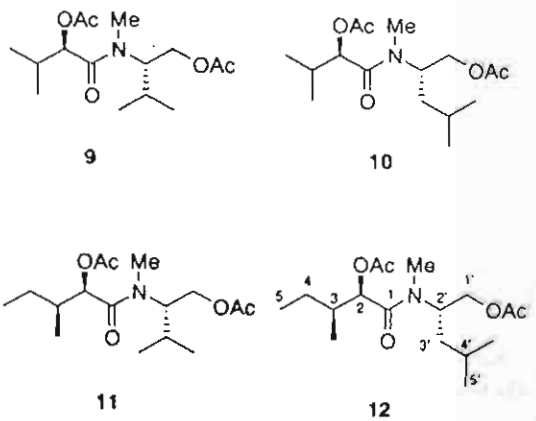
The ^1H and ^{13}C NMR data for the 2-hydroxy-3-methylpentanoic acid moiety of **12** were very similar to those of **11**, and the NMR data for the *N*-methylleucine-derived moiety of **12** were similar to those of **10**. Therefore, the absolute stereochemistry of **12** should be analogous to these analogs. These results are in good agreement with the proposed structures for the compounds **8a**, **8b** and **8c**.

Having established the fermentation condition for efficient enniatin production as well as the extracts profile for this standard fermentation, the fungus BCC 1449 is now well-suited for systematic studies on precursor-directed biosynthesis of unnatural enniatin analogs by feeding experiments with various amino acid precursors. It should also be reported that the established fermentation conditions have been shown to be efficient also for the production of diketopiperazines. While enniatins were obtained from the extracts from mycelia, large amounts of diketopiperazines, incluing several new analogs, have been found in the EtOAc extract from culture filtrate. Therefore, chemical investigation for such extracts are proceeding in our laboratory.

Biological Activities

The mixture of the three new enniatins O_1, O_2 and O_3, exhibited biological activities similar to those of enniatin B. Thus it inhibited the proliferation of the human malaria parasite (*Plasmodium falciparum* K1) with an IC_{50} value of 3.2 μg/ml (enniatin B: IC_{50} 3.4 μg/ml), and showed growth inhibitory activity against *Mycobacterium tuberculosis* $H_{37}Ra$ with a MIC value of 3.125 μg/ml (enniatin B: MIC 3.125 μg/ml). It was cytotoxic to cancer cell-lines, KB, BC-1 and NCI-185, with respective IC_{50} values of 2.4, 1.4 and 0.78 μg/ml.

Fig. 5. Structures of compounds **9**~**12**.



Experimental

A Mixture of Enniatins O_1 (**8a**), O_2 (**8b**) and O_3 (**8c**)

Colorless gum, $[\alpha]_D^{29}$ −93° (*c* 0.155, $CHCl_3$); UV leOH) λ_{max} (log ε) 206 (4.25) nm; IR (KBr) ν_{max} 2966, 48 (s), 1659 (s), 1471, 1189, 1012 cm^{-1}; HRMS (ESI-)F) *m/z* 668.4502 $[M+H]^+$ (calcd for $C_{35}H_{62}N_3O_9$ 8.4486); ^1H and ^{13}C NMR data, Table 1.

$LiBH_4$ Reduction of (**8a**, **8b**, **8c**)-Mixture and Acetylation th Ac_2O

To a solution of enniatins O_1 (**8a**), O_2 (**8b**) and O_3 (**8c**) xture (10.0 mg) in THF (0.5 ml), $LiBH_4$ (10 mg) was ded and stirred in an ice-water bath for 5 hours. The ction was terminated by addition of H_2O (3 ml) and tracted three times with EtOAc (3 ml). The combined ganic layer was concentrated *in vacuo*, the residue was solved in pyridine (0.3 ml), and Ac_2O (0.15 ml) was ded. After standing 20 hours, the reaction mixture was uted with EtOAc (10 ml) and washed with H_2O (4 ml). e organic layer was concentrated *in vacuo* to obtain a le yellow oil (10.8 mg), which was subjected to eparative HPLC (ODS, MeOH/H_2O=55:45) to obtain mpounds **9** (3.7 mg), **10** (1.8 mg), **11** (1.7 mg), and **12** 0 mg). Each compound was obtained as a mixture with C-3 epimer (4:1~9:1).

Compound **9**: Colorless oil; HRMS (ESI-TOF) *m/z* 2.1965 $[M+H]^+$ (calcd for $C_{15}H_{28}NO_5$ 302.1967); ^1H MR (400 MHz, $CDCl_3$) δ 5.02 (1H, d, *J*=6.5 Hz, H-2), 0 (1H, br, H-2=), 4.24~4.21 (2H, m, H-1′), 2.98 (3H, s, CH_3), 2.17 (1H, m, H-3), 2.12 (3H, s, acetyl), 2.03 (3H, cetyl), 1.88 (1H, m, H-3′), 1.01 (6H, d, *J*=6.8 Hz, H-4 d 3-CH_3), 1.00 (3H, d, *J*=6.4 Hz, H-4′), 0.87 (3H, d, 6.7 Hz, 3′-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.0 CH_3COO–), 170.8 (s, CH_3COO–), 170.4 (s, C-1), 75.3 C-2), 62.3 (t, C-1′), 59.5 (d, C-2′), 30.2 (q, N–CH_3), 6 (d, C-3), 27.0 (d, C-3′), 20.8 (q, CH_3COO–), 20.6 (q, $_3COO$–), 19.9×2 (q, C-4′ and 3′-CH_3), 18.8 (q, C-4), 5 (q, 3-CH_3).

Compound **10**: Colorless oil; HRMS (ESI-TOF) *m/z* 6.2140 $[M+H]^+$ (calcd for $C_{16}H_{30}NO_5$ 316.2124); ^1H MR (400 MHz, $CDCl_3$) δ 4.99 (1H, d, *J*=6.2 Hz, H-2), 7 (1H, m, H-2′), 4.12 (1H, dd, *J*=11.5, 8.9 Hz, Ha-1′), 1 (1H, dd, *J*=11.5, 4.01 Hz, Hb-1′), 2.93 (3H, s, CH_3), 2.15 (1H, m, H-3), 2.12 (3H, s, acetyl), 2.03 (3H, cetyl), 1.47 (1H, m, Ha-3′), 1.43 (m, H-4′), 1.18 (1H, Hb-3′), 1.01 (3H, d, *J*=6.7 Hz, 3-CH_3), 1.00 (3H, d, 6.8 Hz, H-4), 0.93 (3H, d, *J*=6.3 Hz, H-5′), 0.91 (3H, d, 6.4 Hz, 4′-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.0 CH_3COO–), 170.8 (s, CH_3COO–), 170.4 (s, C-1), 75.4 (d, C-2), 63.4 (t, C-1′), 49.9 (d, C-2′), 37.0 (t, C-3′), 29.7 (d, C-3), 29.2 (q, N-CH_3), 23.5 (q, C-5′), 21.6 (q, 4′-CH_3), 20.8 (q, CH_3COO–), 20.6 (q, CH_3COO–), 18.7 (q, C-4), 17.5 (q, 3-CH_3).

Compound **11**: Colorless oil; HRMS (ESI-TOF) *m/z* 316.2143 $[M+H]^+$ (calcd for $C_{16}H_{30}NO_5$ 316.2124); ^1H NMR (400 MHz, $CDCl_3$) δ 5.17 (1H, d, *J*=4.4 Hz, H-2), 4.42 (1H, br, H-2′), 4.24~4.21 (2H, m, H-1′), 2.94 (3H, s, N-CH_3), 2.12 (3H, s, acetyl), 2.04 (3H, s, acetyl), 1.86 (1H, m, H-3′), 1.84 (1H, m, H-3), 1.47 (1H, m, Ha-4), 1.30 (1H, m, Hb-4), 0.99 (3H, d, *J*=6.4 Hz, H-4′), 0.98 (3H, d, *J*=6.8 Hz, 3-CH_3), 0.96 (3H, t, *J*=7.4 Hz, H-5), 0.86 (3H, d, *J*=6.7 Hz, 3′-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.0 (s, CH_3COO–), 170.8 (s, CH_3COO–), 170.3 (s, C-1), 73.3 (d, C-2), 62.2 (t, C-1′), 58.7 (d, C-2′), 35.9 (d, C-3), 30.3 (q, N-CH_3), 27.0 (d, C-3′), 26.1 (t, C-4), 20.8 (q, CH_3COO–), 20.6 (q, CH_3COO–), 19.9 (q, C-4′), 19.7 (q, 3′-CH_3), 14.1 (q, 3-CH_3), 11.7 (C-5).

Compound **12**: Colorless oil; HRMS (ESI-TOF) *m/z* 330.2285 $[M+H]^+$ (calcd for $C_{17}H_{32}NO_5$ 330.2280); ^1H NMR (500 MHz, $CDCl_3$) δ 5.17 (1H, d, *J*=4.4 Hz, H-1), 4.99 (1H, br, H-2′), 4.14 (1H, dd, *J*=11.6, 9.2 Hz, Ha-1′), 4.01 (1H, dd, *J*=11.6, 4.6 Hz, Hb-1′), 2.91 (3H, s, N–CH_3), 2.14 (3H, s, acetyl), 2.05 (3H, s, acetyl), 1.84 (1H, m, H-3), 1.47 (2H, m, Ha-4 and Ha-3′), 1.43 (1H, m, H-4′), 1.31 (1H, m, Hb-4), 1.18 (1H, m, Hb-3′), 0.99 (3H, d, *J*=6.8 Hz, 3-CH_3), 0.97 (3H, t, *J*=7.4 Hz, H-5), 0.95 (3H, d, *J*=6.6 Hz, H-4′), 0.91 (3H, d, *J*=6.5 Hz, 3′-CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.0 (s, CH_3COO–), 170.8 (s, CH_3COO–), 170.3 (s, C-1), 73.4 (d, C-2), 63.3 (t, C-1′), 49.9 (d, C-2′), 36.9 (t, C-3′), 36.0 (d, C-3), 29.1 (q, N-CH_3), 26.1 (t, C-4), 24.9 (d, C-4′), 23.5 (q, C-5′), 21.6 (q, 4′-CH_3), 20.8 (q, CH_3COO–), 20.6 (q, CH_3COO–), 14.1 (q, 3-CH_3), 11.7 (C-5).

Acknowledgments

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Hirsutellones A–E, antimycobacterial alkaloids from the insect pathogenic fungus *Hirsutella nivea* BCC 2594

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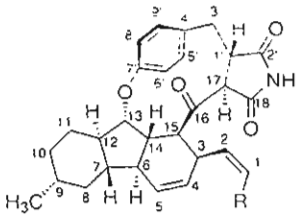
Abstract—Five new alkaloids, hirsutellones A–E, were isolated from the insect pathogenic fungus *Hirsutella nivea* BCC 2594. Their structures were elucidated by spectroscopic analysis and X-ray crystallography. Hirsutellones displayed significant growth inhibitory activity against *Mycobacterium tuberculosis* H37Ra.

1. Introduction

Tuberculosis, caused by *Mycobacterium tuberculosis*, has again become a serious endemic disease. It is estimated that one-third of the world's population is infected with the tubercule bacillus, causing each year eight million new cases of developing clinical tuberculosis and two million deaths.[1] Due to this burgeoning problem caused by the emergence of a multi-drug resistance strain, there is an urgent need to search for new chemical class of anti-tubercular drugs.[2] As a part of our ongoing research program on the identification of novel bioactive compounds from fungi in Thailand,[3] we have extensively screened extracts from fungal cultures for in vitro antimycobacterial activity. Herein, we report the isolation and structure elucidation of novel antitubercular alkaloids, hirsutellones A–E (1–5), from the insect pathogenic fungus *Hirsutella nivea* BCC 2594. Hirsutellones possess unique structural features: a highly strained 12- or 13-membered ring containing a γ-lactam or succinimide, a *para*-substituted phenyl ether, and a tricyclic polyketide moieties. The genus *Hirsutella* has rarely been chemically explored, only a toxic protein, hirsutellin A, from *H. thompsonii*,[4] and a cyclohexadepsipeptide, hirsutellide A, from *H. kobayasii* BCC 1660,[5] have been reported.



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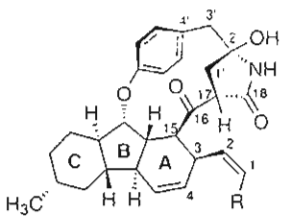
4 : R = CH_3



2 : R = H
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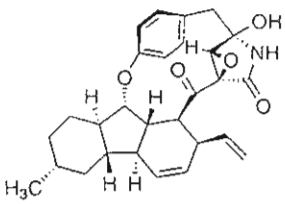

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2. Results and discussion

Mycelia from liquid fermentation in Erlenmeyer flasks (10 L) were extracted with MeOH, and the extract after concentration in vacuo was subjected to a combination of column chromatography, using Sephadex LH20, silica gel, and reversed-phase preparative HPLC, to obtain

Keywords: *Hirsutella nivea*; Antimycobacterial activity; Insect pathogenic fungi.
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Table 1. ^{1}H NMR data for compounds 1–5 in $CDCl_3$

Position	Hirsutellone A (1)	Hirsutellone B (2)	Hirsutellone C (3)	Hirsutellone D (4)	Hirsutellone E (5)
1	4.98 (d, 10.0)	4.84 (m)	4.91 (dd, 9.7, 1.6)	5.21 (ddq, 10.9, 0.9, 6.8)	5.30 (m)[a]
	4.83 (d, 17.0)	4.92 (dd, 16.7, 1.4)	4.94 (dd, 16.4, 1.6)	—	—
2	5.20 (ddd, 17.0, 10.3, 6.5)	5.22 (dt, 16.8, 9.6)	5.23 (dt, 16.9, 1.6)	4.88 (m)	4.82 (tq, 10.5, 1.0)
3	4.17 (ddd, 5.6, 5.4, 5.2)	3.50 (m)	3.82 (m)	4.37 (m)	4.00 (m)
4	5.49 (ddd, 9.7, 5.6, 2.4)	5.34 (ddd, 9.7, 4.7, 2.5)	5.39 (ddd, 9.8, 3.9, 2.9)	5.58 (ddd, 9.6, 5.6, 2.3)	5.30 (m)[a]
5	5.91 (d, 9.8)	5.81 (d, 9.8)	5.88 (d, 9.9)	5.74 (d, 9.6)	5.77 (d, 9.8)
6	2.23 (dt, 1.0, 11.2)	2.13 (m)	2.15 (m)	2.18 (m)	2.11 (m)
7	0.73 (dq, 2.3, 11.2)	0.82 (dq, 2.4, 11.1)	0.90 (m)	0.76 (dq, 2.3, 11.3)	0.82 (dq, 2.2, 11.3)
8	0.94 (m)	0.90 (m)	0.94 (m)	0.90 (m)	0.89 (m)
	1.98 (m)	1.96 (m)	2.01 (m)	1.94 (m)	1.97 (m)
9	1.43 (m)	1.43 (m)	1.44 (m)	1.40 (m)	1.45 (m)
10	1.12 (dq, 4.0, 12.3)	1.12 (dq, 3.5, 12.5)	1.11 (m)	1.12 (dq, 4.1, 12.5)	1.12 (dq, 4.0, 12.3)
	1.84 (m)	1.85 (m)	1.85 (m)	1.84 (m)	1.85 (m)
11	1.35 (dq, 3.5, 12.3)	1.41 (dq, 3.3, 12.5)	1.41 (dq, 3.7, 12.3)	1.36 (dq, 3.6, 12.4)	1.42 (m)
	2.13 (m)	2.16 (m)	2.12 (m)	2.15 (m)	2.17 (m)
12	1.68 (m)	1.64 (m)	1.65 (m)	1.68 (m)	1.66 (m)
13	4.46 (dd, 3.4, 3.3)	4.86 (m)	4.63 (dd, 3.8, 3.6)	4.55 (t, 3.6)	4.87 (t, 4.0)
14	1.48 (dt, 4.1, 11.3)	1.47 (dt, 4.6, 11.5)	1.71 (dt, 4.4, 11.8)	1.58 (m)	1.48 (m)
15	3.13 (dd, 10.9, 4.8)	3.45 (dd, 11.0, 5.9)	3.72 (dd, 12.0, 5.9)	3.17 (dd, 11.0, 5.8)	3.43 (dd, 11.5, 6.1)
17	3.52 (d, 6.4)	2.94 (dd, 12.3, 3.7)	—	3.44 (d, 6.1)	2.80 (dd, 12.3, 3.8)
1-CH_3	—	—	—	1.56 (dd, 6.7, 1.4)	1.6[b]
9-CH_3	0.96 (d, 6.6)	0.96 (d, 6.6)	0.97 (d, 6.4)	0.95 (d, 6.4)	0.95 (d, 6.5)
1′	3.36 (dt, 12.1, 6.0)	1.94 (dd, 14.8, 12.6)	3.69 (d, 2.4)	3.33 (dt, 12.1, 5.8)	1.91 (dd, 15.0, 12.0)
	—	2.70 (dd, 14.9, 3.7)	—	—	2.71 (dd, 15.0, 3.8)
3′	2.41 (t, 12.4)	2.86 (d, 13.0)	3.14 (d, 13.3)	2.38 (t, 12.3)	2.86 (d, 13.1)
	3.65 (dd, 12.7, 6.5)	3.00 (d, 13.0)	3.18 (d, 13.3)	3.64 (dd, 12.6, 5.6)	2.97 (d, 13.1)
5′	6.95 (dd, 8.1, 1.8)	6.91 (dd, 8.4, 1.5)	6.98 (s)[a]	6.94 (dd, 8.1, 1.9)	6.89 (dd, 8.4, 1.7)
6′	7.02 (dd, 8.1, 2.3)	6.86 (dd, 8.3, 2.2)	6.98 (s)[a]	7.03 (dd, 8.1, 2.4)	6.87 (dd, 8.3, 2.0)
8′	7.05 (dd, 8.6, 1.8)	7.05 (dd, 8.4, 2.3)	6.93 (d, 8.4)	7.06 (dd, 8.6, 2.4)	7.06 (dd, 8.4, 2.1)
9′	7.16 (dd, 8.6, 1.8)	7.10 (dd, 8.4, 1.7)	7.10 (d, 8.4)	7.08 (dd, 8.5, 1.9)	7.09 (dd, 8.4, 1.5)
NH	8.24 (br s)	6.15 (br s)	6.00 (d, 2.2)	7.53 (br s)	5.73 (br s)

[a] The proton signals were superimposed.
[b] The proton signal of this methyl group was overlapped with H_2O peak.

hirsutellones A–E. Structural elucidation of the new compounds was first, conducted for the most abundant constituent, hirsutellone B (2). The molecular formula of hirsutellone B (2) was determined as $C_{28}H_{33}NO_4$, by HRMS (ESI-TOF) and ^{13}C NMR spectroscopy. The IR spectrum of 2 showed a strong absorption band at ν_{max} 1683 (broad) cm^{-1} with shoulders at 1703 and 1670 cm^{-1}, consistent with the ^{13}C NMR signals assigned to a ketone (δ_C 200.9) and an amide (δ_C 172.1). The planar structure of 2 was deduced by analysis of ^{1}H and ^{13}C NMR, DEPT, COSY, HMQC and HMBC spectral data (Table 1). Thus, the tricyclic moiety of 2, from C-3 through C-15, was a perhydrofluorene attached to a vinyl group (C-1, C-2) at C-3 and a methyl substituent at C-9. This ring system was connected at C-15 to a γ-lactam moiety via a ketone (C-16), as indicated by the HMBC correlations from H-15, H-17 and two H-1′ protons to the carbonyl (C-16). The γ-lactam system was deduced from the chemical shifts data and HMBC correlations from an amide proton (δ_H 6.15, D_2O exchangeable) to C-17 and C-1′, and from H-17 and H-1′ to a carbonyl carbon at δ_C 172.1 (C-18). HMBC correlations from H-17 methine, H-1′ and H-3′ methylene protons to a quaternary carbon at δ_C 87.6 led to the assignment of this carbon for the γ-position of the lactam ring (C-2′), and its up-field shift indicated the attachment of a hydroxyl group. HMBC correlations also revealed the presence of a *para*-substituted benzene ring (C-4′ to C-9′) attached to the C-3′ methylene carbon on one side and an oxygen atom on the other (C-7′, δ_C 158.3). The non-symmetrical appearance of the four aromatic protons (H-5′, H-6′, H-8′ and H-9′) and corresponding carbons indicated the restricted rotation of this benzene ring. This question was solved by the formation of the 13-membered ring resulted from the connection of C-7′ (of the benzene ring) with C-13 (of B-ring) via an ether linkage. HMBC correlation from H-13 to C-7′ clearly indicated this unusual linkage system, and it was strongly supported by the NOESY correlation between H-13 and one of the aromatic protons, H-8′ (Table 2).

Relative stereochemistry of hirsutellone B (2) was elucidated by analysis of NOESY spectral data (Fig. 1) and vicinal *J*-values. The cyclohexane moiety (C-ring) of the tricyclic system adopts a chair conformation. The vicinal coupling constants of $J_{12,7}=J_{7,8ax}=11.1$ Hz, $J_{9,10ax}=J_{10ax,11ax}=J_{11ax,12}=12.5$ Hz demonstrated the antiperiplanar relationships (axial orientations) of these six protons. This assignment was strongly supported by the NOESY correlations: H-7 to H-9, and H-12 to H-8ax and H-10ax, which revealed the *trans*-junction between B- and C-rings, with a β-orientation of H-7 and an α-orientation of H-12. H-14 showed NOESY correlations with H-2, H-7 and H-13, indicated the β-orientations of H-13, H-14 and the vinyl group (C-1, C-2). On the other hand, H-6 showed NOESY cross signals with H-8ax, H-12 and H-15, which placed both H-6 and H-15 on the α-face. The vicinal coupling constants of $J_{7,6}=11.1$ Hz, $J_{6,14}=J_{14,15}=11.5$ Hz demonstrated the connectivity for these four protons, all with antiperiplanar relationships. This data reveals a *trans*-junction between A- and B-rings. Relative stereochemistry and the approximate conformation of the 13-membered ring moiety were further, addressed by NOESY spectral analysis. Correlations between H-13 to H-8′, and H-15 to H-6′ enabled the

Table 2. ^{13}C NMR data for hirsutellones A–D (1–4) in $CDCl_3$

Position	1	2	3	4	7 [lit.][a]	10 [lit.][b]
1	118.6 (t)	116.4 (t)	116.7 (t)	125.0 (d)	112.0 (t)	114.1 (t)
2	136.9 (d)	137.3 (d)	138.0 (d)	128.1 (d)	146.3 (d)	141.5 (d)
3	37.97 (d)	44.0 (d)	41.4 (d)	33.7 (d)	41.6 (s)	36.7 (d)
4	127.4 (d)	128.7 (d)	128.9 (d)	128.0 (d)	130.7 (d)	121.7 (d)
5	127.7 (d)	127.2 (d)	127.1 (d)	125.3 (d)	138.5 (s)	136.1 (s)
6	43.1 (d)	42.4 (d)	41.6 (d)	42.7 (d)	53.0 (d)	52.9 (d)
7	50.5 (d)	50.0 (d)	49.7 (d)	50.3 (d)	41.5 (s)	46.9 (s)
8	38.02 (t)	38.0 (t)	37.9 (t)	37.9 (t)	49.0 (t)	47.5 (t)
9	33.2 (d)	33.1 (d)	33.1 (d)	33.1 (d)	28.0 (d)	27.7 (d)
10	36.6 (t)	36.5 (t)	36.5 (t)	36.5 (t)	45.5 (t)	44.5 (t)
11	29.4 (t)	29.4 (t)	29.3 (t)	29.5 (t)	27.3 (d)	26.7 (d)
12	56.0 (d)	55.7 (d)	55.7 (d)	55.7 (d)	60.9 (d)	52.2 (d)
13	87.1 (d)	84.5 (d)	82.9 (d)	86.9 (d)	90.7 (d)	90.8 (d)
14	48.0 (d)	47.2 (d)	45.8 (d)	48.9 (d)[c]	50.6 (d)	43.5 (d)
15	54.2 (d)	48.9 (d)	49.8 (d)	53.2 (d)	56.7 (d)	49.7 (d)
16	198.6 (s)	200.9 (s)	199.5 (s)	198.6 (s)	200.2 (s)	209.6 (s)
17	59.9 (d)	54.0 (d)	58.5 (s)	59.7 (d)	56.7 (d)	53.3 (d)
18	170.2 (s)	172.1 (s)	167.8 (s)	169.1 (s)	171.8 (s)	172.3 (s)
1-CH_3	—	—	—	13.4 (q)	—	—
3-CH_3	—	—	—	—	25.7 (q)	—
5-CH_3	—	—	—	—	20.9 (q)	24.8 (q)
7-CH_3	—	—	—	—	16.0 (q)	22.9 (q)
9-CH_3	22.5 (q)	22.5 (q)	22.5 (q)	22.5 (q)	22.8 (q)	22.8 (q)
11-CH_3	—	—	—	—	19.8 (q)	19.8 (q)
1′	49.6 (d)	34.5 (t)	65.4 (d)	48.8 (d)[c]	33.4 (t)	36.7 (t)
2′	176.2 (s)	89.1 (s)	84.5 (s)	175.6 (s)	88.8 (s)	87.6 (s)
3′	35.6 (t)	46.7 (t)	44.0 (t)	35.8 (t)	47.0 (t)	44.6 (t)
4′	132.8 (s)	127.5 (s)	126.2 (s)	132.6 (s)	127.9 (s)	132.2 (s)
5′	129.9 (d)	131.4 (d)	131.1 (d)	130.3 (d)	133.3 (d)	133.1 (d)
6′	123.3 (d)	121.8 (d)	122.2 (d)	122.6 (d)	118.8 (d)	121.8 (d)
7′	158.6 (s)	158.3 (s)	158.6 (s)	158.9 (s)	159.8 (s)	157.3 (s)
8′	123.8 (d)	121.5 (d)	119.5 (d)	123.4 (d)	124.4 (d)	124.0 (d)
9′	132.7 (d)	131.7 (d)	131.9 (d)	130.8 (d)	131.5 (d)	131.7 (d)

[a] Literature data in $CDCl_3$, Ref. 10.
[b] Literature data in DMSO-d_6, Ref. 9.
[c] The assignment can be interchanged.

assignments of the aromatic protons. One of the C-1′ methylene protons situated at δ_H 1.94 showed an intense correlation to H-17. The large vicinal J-value (12.3 Hz) of these protons indicated the *cis*-relationship with a dihedral angle of approximately 0°. The NOESY correlations from another C-1′ methylene proton (δ_H 2.70) to H-9′ and Hβ-3′, and the correlations of the amide proton (NH) with Hα-3 (δ_H 3.00) and H-5′ strongly suggested that C-1′ methylene carbon of the γ-lactam situated on the β-face and the amide moiety on the opposite. On the basis of this spectral data, the relative stereochemistry of hirsutellone B (2) was proposed as depicted in the figure.

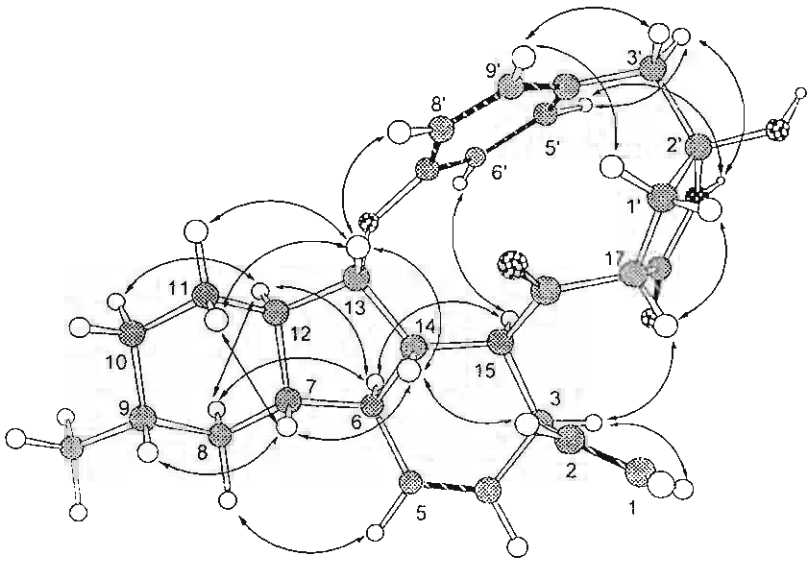


Figure 1. Plausible stereo structure of hirsutellone B (2). Selected NOESY correlations are illustrated with solid arrows.

The molecular formula of hirsutellone A (**1**) was determined as $C_{28}H_{31}NO_5$ by HRMS (ESI-TOF) and ^{13}C NMR data, possessing two hydrogen atoms less than hirsutellone B (**2**). Spectral analysis of **1** revealed that this compound contained a succinimide moiety instead of the γ-lactam, as in **2**. Thus, two amide carbonyl carbons were present (δ_C 176.2 and 170.2), which was consistent with the IR absorptions for carbonyls at ν_{max} 1769 and 1726 cm^{-1} in addition to another carbonyl (C-16, ketone) at 1702 cm^{-1}. Instead, the hemi-aminal carbon, as found in **2** (C-2′), was absent in hirsutellone A (**1**). Analysis of COSY and HMQC spectra of **1** indicated that benzylic methylene protons (H-3′) at δ_H 3.65 (dd, J=12.7, 6.5 Hz) and 2.41 (t, J=12.4 Hz) were vicinally coupled with a methine proton situated at δ_H 3.36 (dt, J=12.1, 6.0 Hz attached to C-1′, δ_C 49.6), which was connected to another methine (δ_H 3.52, d, J=6.4 Hz δ_C 59.9, C-17). The succinimide structure was confirmed by HMBC correlations from the imide proton (N*H*) to C-1′ and C-17. One of the imide carbonyl carbon, δ_C 176.2 (C-2′), was correlated from H-1′ and H-3′α (δ_H 2.41), while the other (δ_H 170.2, C-18) was correlated from H-17. The connectivity between the succinimide and the tricyclic ring moiety via a ketone (δ_C 198.6, C-16) was established by HMBC correlations to this carbonyl carbon from H-15, H-17 and H-1′. Stereochemistry of the succinimide moiety was revealed by the NOESY spectral data: H-17 showed correlation with H-3, H-3′α and H-5′, while H-1′ exhibited cross signals with H-3′β and H-9′. Finally, the proposed relative stereochemistry of hirsutellone A (**1**) was confirmed by X-ray crystallographic analysis (Fig. 2). The most interesting feature concerning the crystal structure of hirsutellone A is the 'bent' *para*-substituted benzene ring. This is due to the high ring strain, the aromatic nucleus can be viewed as part of an [8]paracyclophane system.[6]

Hirsutellone C (**3**), molecular formula $C_{28}H_{31}NO_5$ (HRMS, ^{13}C NMR), exhibited similar ^1H and ^{13}C NMR spectra to those of hirsutellone B (**2**), except for the part of γ-lactam moiety. Proton and carbon signals for methine of C-17 and methylene of C-1′ in **2** were lacking in **3**, instead, a quaternary carbon (δ_C 58.5, C-17) and a methine (δ_C 65.4, δ_H 3.69, C-1′) were present. In the HMBC spectrum of **3**, correlations from the amide proton (δ_H 6.00) to both of these carbons (C-17 and C-1′) were observed. Additional HMBC data, that is, benzylic protons (H-3′) to C-1′, and H-1 to C-18 (δ_C 167.8) and C-2′ (δ_C 84.5), indicated the presence of an epoxide in the γ-lactam moiety. Stereochemistry of the epoxide was addressed based on the NOESY correlations from H-1′ to H-3′β and H-9′, and the amide proton (N*H*) to H-3′α and H-5′.

The IR and UV spectra of hirsutellone D (**4**), a minor constituent, were very similar to those of hirsutellone A (**1**). The HRMS experiment and ^{13}C NMR data revealed its molecular formula as $C_{29}H_{33}NO_4$, having additional CH_2 unit to **1**. ^1H and ^{13}C NMR spectra of **4** indicated the presence of a *cis*-1-propenyl group replacing the vinyl group (C-1, C-2) observed in **1**. Thus, the signal attributable to the allylic methyl protons appeared as a doublet of doublets (δ_H 1.56, J=6.7, 1.4 Hz), and was vicinally coupled with H-1 (J=6.7 Hz). The *cis*-olefinic geometry was evident from the $J_{1,2}$-value of 10.9 Hz. NOESY correlations from the methyl protons to H-3, and H-2 to H-14, further, confirmed this partial structure. Other NOESY correlation data for **4** were similar to those of **1**. On the basis of these spectral data, hirsutellone D (**4**) was assigned as 1-methyl derivative of hirsutellone A (**1**).

Hirsutellone E (**5**) was obtained in very low quantity, 0.24 mg, from 10 L fermentation. UV spectrum of **5** in MeCN–H_2O, acquired during its purification by semi-preparative HPLC/UV, was similar to that of **2**. ^1H NMR, COSY and NOESY spectral data of **5** strongly suggested that this compound was the 1-methyl analog of hirsutellone B (**2**). The HRMS data for **5**, *m/z* 460.2483 $[M-H]^-$ (Calcd for $C_{29}H_{34}NO_4$ 460.2488), indicated its molecular formula as $C_{29}H_{35}NO_4$, which was consistent with the proposed structure.

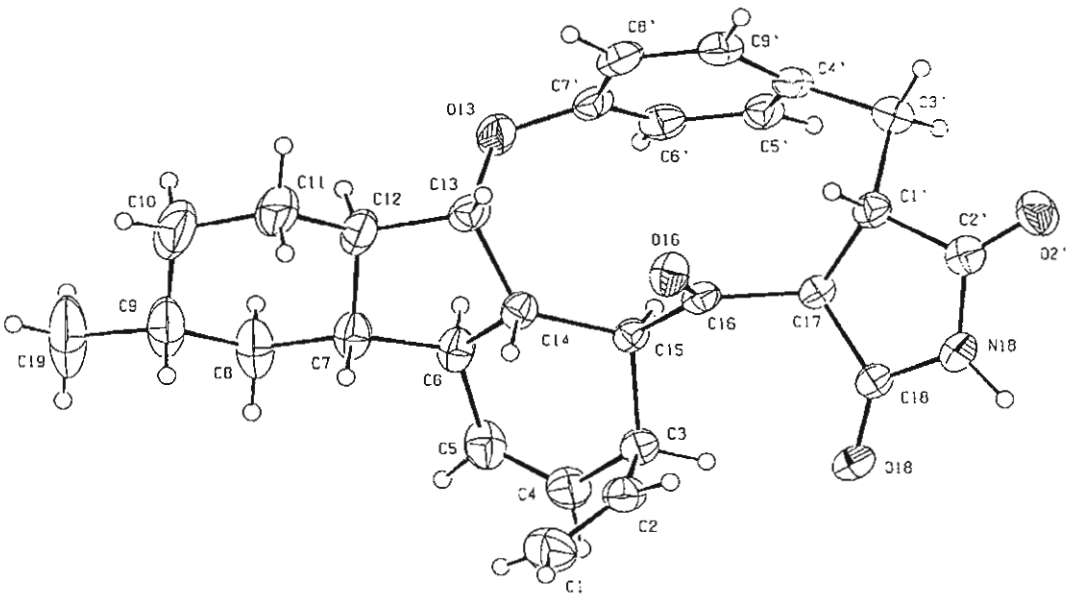
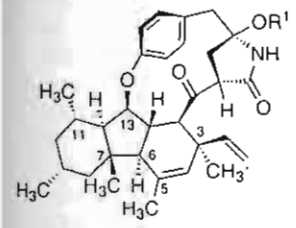
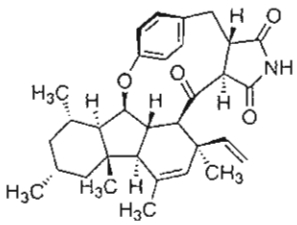


Figure 2. X-ray crystal structure of hirsutellone A (**1**). Thermal elipsoids given at 50% probability level.

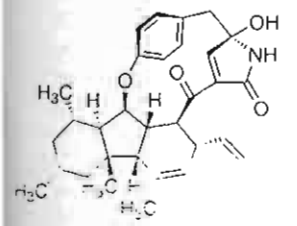
The structures of hirsutellones are related to those of GKK1032A$_1$ (**6**), GKK1032A$_2$ (**7**) and GKK1032B (**8**) isolated from *Penicillium* sp. GKK1032.[7,8] These compounds possess four additional methyl groups at C-3, C-5, C-7 and C-11. It is interesting to note that the relative stereochemistry of GKK1032s differs from that of hirsutellones at the C-13 position. Pyrrocidines A (**9**) and B (**10**), recently, isolated from an unidentified filamentous fungus *LL*-Cyan426,[9] also share a similar molecular framework, although their proposed relative stereochemistry on the tricyclic ring moiety differ from those of hirsutellones.



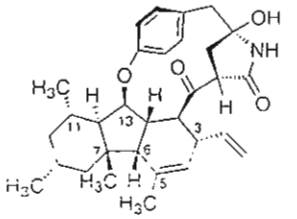
6: R^1 = CH$_3$ (GKK1032A$_1$)
7: R^1 = H (GKK1032A$_2$)



8 (GKK1032B)

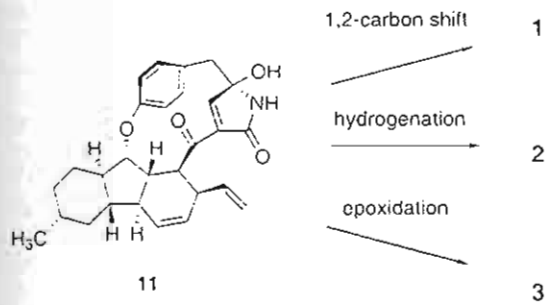


9 (pyrrocidine A)



10 (pyrrocidine B)

Biosynthesis of GKK 1032A$_2$ (**7**) has recently, been studied by administration of isotopically labeled (^{13}C and ^{2}H).



Scheme 1. Proposed biogenetic conversion from the plausible precursor 11 to 1, 2 and 3.

precursors to *Penicillium* sp. GKK1032.[10] Thus, the backbone of **7** is constructed from L-tylosine and a nonaketide chain flanked by five methyl groups, probably by a polyketide synthase and a nonribosomal peptide synthetase hybrid. It is not unreasonable to assume that the proposed biosynthetic pathway could be applied to hirsutellones production by *H. nivea* BCC 2595. Final transformations may also be similar to those for GKK1032s. Thus, a 1,2-carbon shift from C-1′ to C-2′ in the plausible intermediate **11** should give rise to hirsutellone A (**1**), while hirsutellone B (**2**) could be produced by hydrogenation of **11**. The occurrence of the epoxide derivative, hirsutellone C (**3**), can also be reasonably explained by the same precursor (Scheme 1).

Hirsutellones A–D exhibited potent antitubercular activity (*Mycobacterium tuberculosis* H$_{37}$Ra) with MIC values of 0.78–3.125 μg/mL, while they were not or only weakly cytotoxic to Vero cells and three cancer cell-lines (KB, BC, NCI-H187) (Table 3). Amongst these compounds, hirsutellone A displayed the best selectivity index. Compounds **1–3** were inactive in our antimalarial (*Plasmodium falciparum* K1) activity assay at a concentration of 20 μg/mL, and they were also inactive against *Candida albicans* at a concentration of 50 μg/mL. Related compounds, pyrrocidines A and B, are known to exhibit antibiotic activities against Gram-positive bacteria,[9] whilst weak anti-tumor activity (Hela S3) was reported for GKK01032s.[7] Although the antimycobacterial activities of hirsutellones were much weaker than standard drugs, example isoniazid (MIC 0.06 μg/mL, in our assay system), the unique and rare chemical skeleton and good selectivity index deserve further, studies on structural modification.

3. Experimental

3.1. General experimental procedures

Melting points were measured with an Electrothermal IA9100 digital melting point apparatus and were uncorrected. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. UV spectra were recorded on a Varian CARY 1E UV–Visible spectrophotometer. FT-IR spectra were taken on a Bruker VECTOR22 spectrometer. Mass spectra (ESI-TOF) were measured with a Micromass LCT mass spectrometer. ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), DEPT and 2D NMR spectra (COSY, NOESY, HMQC and HMBC) were taken on a Bruker DRX400 spectrometer.

Table 3. Antimycobacterial and cytotoxic activities of hirsutellones A–D

Compound	Anti-tuberculosis (MIC, μg/mL) *M. tuberculosis* H$_{37}$Ra	cytotoxicity (IC$_{50}$, μg/mL)			
		KB	BC	NCI-H187	Vero
Hirsutellone A (1)	0.78	>20	>20	>20	>50
Hirsutellone B (2)	0.78	>20	>20	6.0	>50
Hirsutellone C (3)	0.78	4.6	3.2	8.3	12
Hirsutellone D (4)	3.125	>20	>20	7.3	—[b]
Isoniazid[a]	0.06	—[b]	—[b]	—[b]	—[b]

[a] Standard antitubercular drug.
[b] Not tested.

3.2. Fungal material

Hirsutella nivea Hywel-Jones (Ascomycota, Mitosporic, Hypocreales, Clavicipitaceae)[11] was collected, identified and isolated from 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.

3.3. Fermentation, extraction and isolation

BCC 2594 was maintained on potato dextrose agar at 25 °C for 16 days, 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/L). 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/L), and static fermentation was carried out at 25 °C for 40 days. The cultures were filtered and the residue (mycelial cake) was extracted with MeOH (1000 mL, rt, 2 days). After filtration, H_2O (50 mL) was added to the filtrate, washed with hexane (400 mL), and the aqueous MeOH layer was concentrated under reduced pressure. The residual oil was dissolved in EtOAc (500 mL), washed with H_2O (150 mL), and concentrated to obtain a brown gum (1.57 g). This mycelial extract was passed through a Sephadex LH-20 column (3×25 cm; MeOH as eluent). Fractions containing hirsutellones were combined and subjected to column chromatography on silica gel (MeOH/CH_2Cl_2, step gradient elution) to obtain three fractions, Fr-A (a mixture of **1** and **4**, 140 mg), Fr-B (contained **3**, 35 mg), Fr-C (a mixture of **2**, **3** and **5**, 153 mg). Fr-A was subjected to preparative HPLC using a reversed-phase column (Prep Nova-Pak HR C_{18}, 6 μm, 40×100 mm) with MeCN/H_2O=75:25 as eluent at a flow rate of 20 mL/min to yield hirsutellones A (**1**; 76.1 mg, t_R 18 min) and D (**4**; 2.9 mg, t_R 23 min). Preparative HPLC (MeCN/H_2O =65:35) of Fr-B provided hirsutellone C (**3**, 13.6 mg, t_R 19 min). Trituration of Fr-C in MeOH (1 mL, r.t., 5 h) gave colorless solid of hirsutellone B (**2**, 92.0 mg). The filtrate was subjected to preparative HPLC (MeCN/H_2O=65:35) to obtain **2** (35.9 mg, t_R 16 min), **3** (1.6 mg, t_R 19 min) and hirsutellone E (**5**; 0.24 mg, t_R 21 min).

3.3.1. Hirsutellone A (1). Colorless solid; mp 155–157 °C; $[\alpha]^{29}_D$ +168° (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.42), 228 sh (3.72), 281 sh (2.99) nm; IR (KBr) ν_{max} 3473, 2920, 1769, 1726 (sh), 1702, 1505, 1359, 1239, 1192 cm^{-1}; HRMS (ESI-TOF, negative) *m/z* 444.2183 $[M-H]^-$ (Calcd for $C_{28}H_{30}NO_4$ 444.2175; Δ=1.8 ppm); ^1H and ^{13}C NMR data in $CDCl_3$, Tables 1 and 2.

3.3.2. Hirsutellone B (2). Colorless solid; mp 261–263 °C (dec); $[\alpha]^{27}_D$ +256° (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.37), 227 sh (3.78), 276 sh (2.99) nm; IR (KBr) ν_{max} 3405, 3647, 2917, 1708 (sh), 1683, 1670 (sh), 1508, 1241, 1093 cm^{-1}; HRMS (ESI-TOF, negative) *m/z* 446.2336 $[M-H]^-$ (Calcd for $C_{28}H_{32}NO_4$ 446.2332; Δ= 0.9 ppm); ^1H and ^{13}C NMR data in $CDCl_3$, Tables 1 and 2.

3.3.3. Hirsutellone C (3). Colorless solid; mp 234–235 °C (dec); $[\alpha]^{29}_D$ +129° (*c* 0.20, MeOH); UV (MeOH) λ_{max} (MeOH) 203 (4.42), 230 (3.93), 277 (3.12) nm; IR (KBr) ν_{max} 3396, 3264, 2921, 1713, 1698 (sh), 1687 (sh), 1507, 1241, 1134, 923 cm^{-1}; HRMS (ESI-TOF, negative) *m/z* 460.2121 $[M-H]^-$ (Calcd for $C_{28}H_{30}NO_5$ 460.2124; Δ= 0.7 ppm); ^1H and ^{13}C NMR data in $CDCl_3$, Tables 1 and 2.

3.3.4. Hirsutellone D (4). Colorless amorphous solid; mp 106–109 °C; $[\alpha]^{29}_D$ +214° (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.46), 229 sh (3.78), 283 sh (3.07) nm; IR (KBr) ν_{max} 3226, 2914, 1780, 1723, 1707, 1504, 1353, 1246, 1183 cm^{-1}; HRMS (ESI-TOF, negative) *m/z* 458.2325 $[M-H]^-$ (Calcd for $C_{29}H_{32}NO_4$ 458.2332; Δ=1.5 ppm); ^1H and ^{13}C NMR data in $CDCl_3$, Tables 1 and 2.

3.3.5. Hirsutellone E (5). Colorless amorphous solid; UV (MeCN/H_2O) λ_{max} 227 (sh), 276 nm; HRMS (ESI-TOF, negative) *m/z* 460.2483 $[M-H]^-$ (Calcd for $C_{29}H_{34}NO_4$ 460.2488; Δ=1.1 ppm); ^1H NMR data in $CDCl_3$, Table 1.

3.4. X-ray crystallographic analysis of hirsutellone A (1)

Crystal data for compound **1** at 298 (2) K: $C_{28}H_{34}NO_4 \cdot H_2O$, M_r=463.57, orthorhombic, space group $P2_12_12_1$ (No. 19) with a=9.3530 (3) Å, b=15.8705 (7) Å, c=34.1922 (14) Å, V=5075.3 (3) Å^3, Z=8, D_{calc}=1.169 Mg/m^3, F_{000}=1912, λ (Mo Kα)=0.71073 Å, μ=0.077 mm^{-1}. Data collection and reduction: crystal size 0.10×0.15×0.20 mm, θ range 1.02–21.49°, 17033 reflection collected, 3318 independent reflections (R_{int}=0.053), final *R* indices ($I>2\sigma(I)$: 0.0433, wR_2=0.1054 for 615 parameters, GOF=1.091. Intensity data were measured on a Bruker–Nonius kappa CCD diffractometer. Crystallographic data for the structure **1** in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 257861. Copies of the data can be obtained, free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [Fax: +44 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk].

3.5. Biological assays

Growth inhibitory activity against *Mycobacterium tuberculosis* $H_{37}Ra$ was performed using the Microplate Alamar Blue Assay (MABA) described by Collins and Franzblau.[12] Cytotoxic activities of the purified compound to Vero cells (African green monkey kidney fibroblast) and three cancer cell-lines, KB (human epidermoid carcinoma on the mouth), BC (human breast cancer) and NCI-H187 (human small cell lung cancer), were evaluated using the colorimetric method.[13]

Acknowledgements

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A Cytotoxic Xanthone Dimer from the Entomopathogenic Fungus *Aschersonia* sp. BCC 8401

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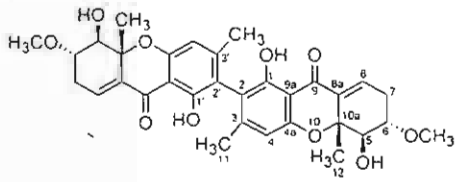
Ascherxanthone A (1), a novel symmetrical tetrahydroxanthone dimer, was isolated from the entomopathogenic fungus *Aschersonia* sp. BCC 8401. The structure of 1 was elucidated by spectroscopic analysis, especially 2D-NMR. Compound 1 exhibited activity against *Plasmodium falciparum* K1 with an IC_{50} value of 0.20 μg/mL, but it also showed cytotoxic activities against Vero cells and three tumor cell lines.

In our search for novel bioactive compounds from insect pathogenic fungi,[1,2] we observed significant antimalarial activity (IC_{50} 0.30 μg/mL) in the extract from a culture of *Aschersonia* sp. BCC 8401, which was collected on a Homoptera-scale insect. Investigation of the chemical constituents was undertaken, which led to the isolation of a novel tetrahydroxanthone dimer, ascherxanthone A (1). There have been few reports on secondary metabolites from the genus *Aschersonia*: 3β,15α,22-trihydroxyhopane (a triterpene) from *A. aleyroidis*,[3] its 3β-*O*-acetate from *A. tubulata* BCC 1785,[4] and destruxins A_4 and A_5 (cyclodepsipeptides) from an *Aschersonia* species.[5]



1

Ascherxanthone A (1) was isolated as a yellow powder from the MeOH extract of mycelia of *Aschersonia* sp. BCC 8401. The molecular formula of this compound was estimated to be $C_{32}H_{34}O_{10}$ by HRMS. The presence of only 16 carbon signals in the ^{13}C NMR spectrum indicated a symmetric, homodimer structure. Analysis of ^1H and ^{13}C NMR, DEPT, and HMQC spectra revealed that half of the molecule, $C_{16}H_{17}O_5$, possessed a conjugated ketone carbonyl (δ_C 185.3), seven quaternary carbons (δ_C 160.5, 158.3, 150.8, 135.6, 115.9, 105.3, and 82.3), two sp^2 methines (δ_C 133.3, δ_H 6.87; δ_C 109.3, δ_H 6.61), two oxymethines (δ_C 75.9, δ_H 4.13; δ_C 76.9, δ_H 3.48), one methylene (δ_C 30.9, δ_H 2.97 and 2.33), two methyl groups attached to quaternary carbons (δ_C 21.0, δ_H 2.10; δ_C 20.0, δ_H 1.59), and one methoxyl (δ_C 57.3, δ_H 3.52), and also observed was a proton signal of a chelated phenolic hydroxyl (δ_H 12.40). COSY correlations revealed the connectivity of C-5 to C-8, and the tetrahydroxanthone structure was established on the basis of the HMBC correlations. Intense correlation from the chelated phenolic proton (*OH*-1; δ_H 12.40) to the quaternary carbon at δ_C 115.9 placed this carbon at C-2; hence, this compound was a C-2−C-2′ dimer. In addition to the standard two- and three-bond correlations, a weak four-bond correlation from an aromatic proton (H-4, δ_H 6.61) to a ketone carbonyl (C-9, δ_C 185.3) was observed.

The relative configuration at C-6, C-5, and C-10a in ascherxanthone A (1) was deduced from the ^1H NMR and NOESY data. The ring fusion necessitated the pseudoaxial orientation of the methyl group adjacent to C-10a. In the NOESY spectrum of 1, a correlation between the C-12 methyl protons (δ_H 1.59) and H-6 was observed. The large coupling constant between H-5 and H-6, J = 10.2 Hz, indicated the antiperiplanar relationship of these protons (both pseudoaxial); therefore the hydroxyl group (on C-5) and the methoxyl group (on C-6) should be pseudoequatorial. Unfortunately, attempts to prepare α-methoxy-α-trifluoromethylphenylacetic acid (MTPA) esters of ascherxanthone A (1) were not successful, and the absolute configuration of 1 remains questionable.

The structure of ascherxanthone A (1), the C-2−C-2′ dimer of 5,6,8a,10a-tetrahydroxanthone, is related to the secalonic acids[6] and substances TMC315A1 and TMC315A2, which were recently isolated from *Ceuthospora* sp. TMC1678 and claimed in a Japanese patent as RANKL (receptor activator of NF-κB ligand) antagonists.[7] A remarkable structural difference between 1 and other secalonic acid derivatives is the lack of hydroxyl groups at C-8 and C-8′ in 1.

Ascherxanthone A (1) exhibited significant activity against *Plasmodium falciparum* K1 with an IC_{50} value of 0.20 μg/mL; however, it also showed cytotoxicity to Vero cells (IC_{50} 0.80 μg/mL) and three cancer cell lines, KB, BC, and NCI-H187, with respective IC_{50} values of 1.7, 1.7, and 0.16 μg/mL. Related compounds, secalonic acids, are reported to exhibit various biological activities such as cytostatic activity (mouse leukemia L1210 cells),[8] phlogistic activity,[9] inhibition of protein kinase C and cyclic AMP-dependent protein kinase,[10] and as toxicity to mice.[11]

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 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. *Aschersonia* sp. was collected on a Homoptera-scale insect, at Khao Yai National Park, central

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Thailand. This fungus was deposited at the BIOTEC Culture Collection (BCC) as BCC 8401.

Fermentation, Extraction, and Isolation. The fungus BCC 8401 was maintained on potato dextrose agar at 25 °C, after which the mycelium was cut into pieces (1 × 1 cm) and inoculated in two 250 mL Erlenmeyer flasks, each containing 25 mL of Difco potato dextrose broth (PDB). After incubation at 25 °C for 8 days on a rotary shaker (200 rpm), these primary seed cultures were transferred into two 1000 mL Erlenmeyer flasks, each containing 250 mL of PDB, and incubated at 25 °C for 8 days on a rotary shaker (200 rpm). Each 25 mL portion of the secondary seed cultures was transferred into twenty 1000 mL Erlenmeyer flasks, each containing 250 mL of M102 medium (composition: sucrose 30.0 g, malt extract 20.0 g, bacto-peptone 2.0 g, yeast extract 1.0 g, KCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, and KH_2PO_4 0.5 g, in 1000 mL of distilled water), and fermentation was carried out at 25 °C for 28 days on rotary shakers. Then, the cultures were filtered, and the residual mycelial cakes were extracted at rt with MeOH (1000 mL) for 2 days and filtered. To the filtrate was added H_2O (25 mL), and the mixture was washed with hexane (800 mL). The aqueous MeOH layer was concentrated under reduced pressure. The residue was dissolved in EtOAc (1000 mL) and washed with H_2O (100 mL), and the organic layer was concentrated under reduced pressure, leaving a deep yellow solid (3.1 g). This extract was triturated in MeOH (5 mL) and filtered by suction. The residual solid (1.52 g) was mainly composed of zeorin (known hopane triterpene). The filtrate (dry weight, 1.35 g) was subjected to a Sephadex LH20 column (4.0 × 30 cm; elution with MeOH). Fractions containing yellow pigment were combined (296 mg) and subjected to silica gel column chromatography (2.5 × 20 cm; EtOAc/CH_2Cl_2, gradient elution from 0:100 to 30:70) to obtain a yellow solid (**1**; 108 mg; R_f 0.35, MeOH/CH_2Cl_2, 5:95). Ascherxanthone A (**1**) was further purified by recrystallization in CH_2Cl_2/hexane.

Ascherxanthone A (1): yellow solid; mp 240−244 °C; $[\alpha]^{24}_D$ −227° (c 0.20, CHCl$_3$); UV (MeOH) λ_{max} (log ε) 264 (4.76), 221 sh (4.69), 255 sh (4.34), 308 (4.61), 351 (3.92) nm; IR (KBr) ν_{max} 3482, 2925, 1632, 1604, 1407, 1264, 1091, 802 cm^{-1}; ^{1}H NMR (CDCl$_3$, 500 MHz) δ 12.40 (2H, s, 1-O*H* and 1′-O*H*), 6.87 (2H, dd, J = 5.0, 2.8 Hz, H-8 and H-8′), 6.61 (2H, s, H-4 and H-4′), 4.13 (2H, d, J = 10.3 Hz, H-5 and H-5′), 3.52 (6H, s, 6-OC*H*$_3$ and 6′-OC*H*$_3$), 3.48 (2H, ddd, J = 10.3, 9.2, 6.2 Hz, H-6 and H-6′), 2.97 (2H, ddd, J = 19.4, 6.2, 5.0 Hz, H-7a and H-7′a), 2.33 (2H, ddd, J = 19.4, 9.2, 2.8 Hz, H-7b and H-7′b), 2.10 (6H, s, H-11 and H-11′), 1.59 (6H, s, H-12 and H-12′); ^{13}C NMR (CDCl$_3$, 125 MHz) δ 185.3 (C, C-9 and C-9′), 160.5 (C, C-1 and C-1′), 158.3 (C, C-4a and C-4a′), 150.8 (C, C-3 and C-3′), 135.6 (C, C-8a and C-8a′), 133.3 (CH, C-8 and C-8′), 115.9 (C, C-2 and C-2′), 109.3 (CH, C-4 and C-4′), 105.3 (C, C-9a and C-9a′), 82.3 (C, C-10a and C-10a′), 76.9 (CH, C-6 and C-6′), 75.9 (C-5 and C-5′), 57.3 (CH$_3$, 6-OCH$_3$ and 6′−OCH$_3$), 30.9 (CH$_2$, C-7 and C-7′), 21.0 (CH$_3$, C-11 and C-11′), 20.0 (CH$_3$, C-12 and C-12′); HRMS (ESI-TOF) *m/z* 579.2228 (calcd for $C_{32}H_{35}O_{10}$, 579.2230) [M + H]$^+$.

Biological Assay. Assay for activity against *P. falciparum* (K1, multidrug-resistant strain) was performed using the microculture radioisotope technique described by Desjardins.[12] IC_{50} represents the concentration that causes 50% reduction of parasite growth as indicated by the in vitro uptake of [^{3}H]-hypoxanthine by *P. falciparum*. A standard antimalarial compound, dihydroartemisinin, showed an IC_{50} value of 1.8 ng/mL in the same assay system. Cytotoxicity of the purified compounds against African green monkey kidney fibroblast (Vero), human epidermoid carcinoma cells (KB), human breast cancer cells (BC), and human lung cancer cells (NCI-H187) was evaluated using the colorimetric method.[13]

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

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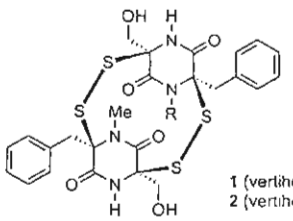
ABSTRACT



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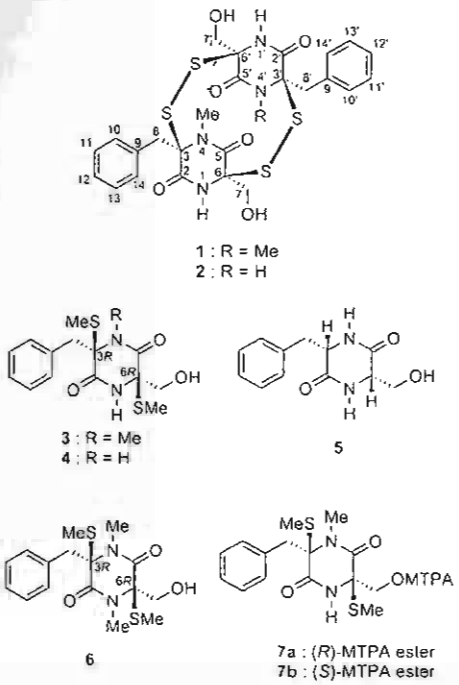
extract of the culture filtrate (10 L) by Sephadex LH20 column (MeOH), repeated silica gel column chromatography ($EtOAc/CH_2Cl_2$, and $MeOH/CH_2Cl_2$), followed by preparative HPLC (reverse-phase column, $MeOH/H_2O$).[6]



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; NCH_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

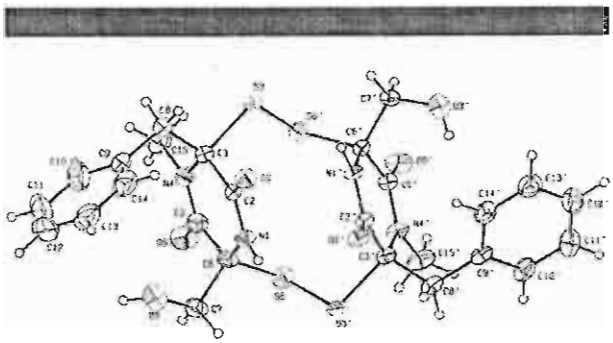


Figure 1. Crystal structure of vertihemiptellide A (**1**).

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,[5] 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: $C_{26}H_{28}N_4O_6S_4{\cdot}C_4H_8O_2$, 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} (log *ε*) 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]	

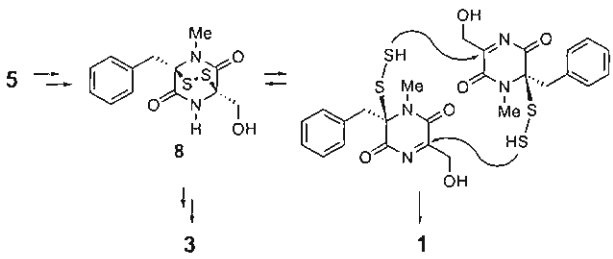
[a] The proton signals of the phenyl group(s) are overlapping. [b−e] Assignments of carbons are interchangeable.

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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