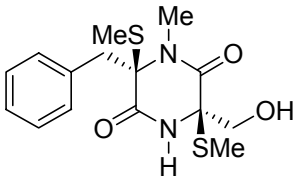
3. RESULTS AND DISCUSSION

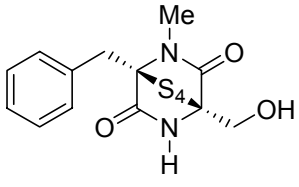
Chemical investigation of the fungus *Verticillium hemipterigenum* BCC 1449 is divided into three parts. The first part involves the compounds in the extract from culture filtrate (broth extract), and the second part implies the compounds in the extract from mycelia (cell extract). The last part deals with compounds derived from the precursor-directed biosynthesis using BCC 1449. The structures of the compounds were elucidated by the analysis of 1D and 2D NMR data together with MS, UV and IR spectral data. For known compounds, their ^{1}H and ^{13}C NMR data were compared with those reported in the literature.

3.1 Chemical constituents from broth extract of *Verticillium hemipterigenum* BCC 1449

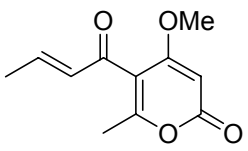
Chemical investigation of the broth extract of the insect pathogenic fungus *Verticillium hemipterigenum* BCC 1449 was conducted (Nilanonta, *et al*. 2003a). Activity-guided chromatographic fractionation of the broth extract led to the isolation of two new diketopiperazines, **51** and **52**, together with four known compounds, pyrenocine A (**53**), pyrenocine B (**54**), enniatin B (**55**), and enniatin B_4 (**56**).



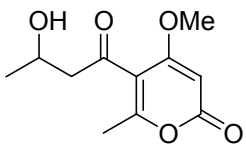
51



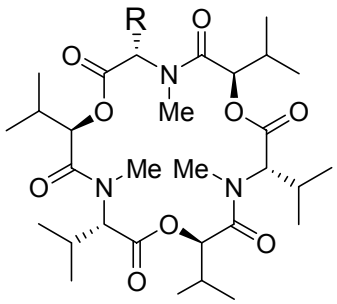
52



53

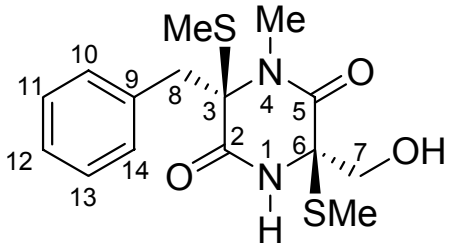


54



55 : R = i-Pr
56 : R = i-Bu

3.1.1 Structure elucidation of compound 51



Compound **51** was obtained as colorless crystals; mp 154-157 °C, $[\alpha]^{26}_{D}$ – 70 (*c* 0.21, $CHCl_3$). The UV spectrum of **51** showed the absorption at λ_{max} (log ε) 205 (4.35) and 258 (2.94) nm. The IR spectrum of **51** exhibited two strong absorption bands of amide carbonyls at ν_{max} 1693 and 1634 cm^{-1}, hydroxyl and amide NH absorptions at ν_{max} 3399 and 3205 cm^{-1}, and C-H deformation (out of plane) of a monosubstituted benzene at ν_{max} 701 cm^{-1}.

The ^{1}H NMR spectrum of compound **51** (in $CDCl_3$, 400 MHz) indicated that this molecule possesses twenty protons. Four doublet signals at δ_H 3.54 (1H, d, *J* = 13.9 Hz, Ha-8), 3.42 (1H, d, *J* = 11.8 Hz, Ha-7), 3.15 (1H, d, *J* = 13.8 Hz, Hb-8), and 2.74 (1H, brd, *J* = 11.6 Hz, Hb-7) indicated the presence of two non-equivalent methylenes. Multiplet signals at δ_H 7.11-7.15 (2H, H-11 and H-13) and 7.26-7.32 (3H, H-10, H-12 and H-14) indicated five protons of a mono-substituted benzene. The ^{1}H NMR spectrum of **51** also exhibited three singlet signals of methyl groups at δ_H 3.28 (amide N-C*H*$_3$), 2.23 (-SC*H*$_3$), and 2.20 (-SC*H*$_3$). Two D_2O exchangeable broad singlets at δ_H 6.39 (1H) and 1.85 (1H) were assigned respectively to a secondary amide and a hydroxyl group.

The ^{13}C NMR spectrum of compound **51** (in $CDCl_3$, 100 MHz) showed twelve carbon signals where two carbonyl carbons were superimposed at δ_C 164.9 ppm. This was evident from the detection of two separate signals at δ_C 167.3 and 167.4 ppm in the spectrum acquired in MeOH-d_4 (total 13 signals). Analysis of DEPTs and HMQC spectral data of compound **51** led to the categorization of each carbon into three methyl, two methylene, five methine (aromatic region) and five quaternary carbons. Since this compound possesses a mono-substituted benzene

(benzyl group), it should consist of fifteen carbons. The molecular formula of **51**, $C_{15}H_{20}S_2N_2O_3$, was determined by HRMS (ESI-TOF) analysis, showing a $[M–H]^-$ ion peak at *m/z* 339.0841 (Δ = 0.4 mmu). Therefore, this compound has IHD (index of hydrogen deficiency) = 7 indicating seven unsaturation points in its molecule.

The combined analysis of ^1H, ^{13}C, DEPTs, COSY and HMQC spectral data revealed that compound **51** possesses a benzyl group, a hydroxymethyl group, two methylthio groups (δ_C 13.5, δ_H 2.23; and δ_C 14.4, δ_H 2.20), a tertiary amide with a N-methyl (δ_C 30.3, δ_H 3.28), a secondary amide (N-*H*; δ_H 6.39, exchangeable with D_2O), and two quaternary carbons at δ_C 64.8 and 75.7.

HMBC correlations (in MeOH-d_4) demonstrated that the benzyl group, one of the methylthio groups (δ_H 2.27), one carbonyl (δ_C 167.3), and the methylated amide nitrogen were attached to the δ_C 76.5 quaternary carbon. Another quaternary carbon at δ_C 66.6 was attached with a hydroxymethyl group, an amide carbonyl and a secondary amide nitrogen. Considering also other HMBC correlations shown in Figure1, and its seven degree of unsaturation, this compound should form a cyclodipeptide ring, therefore, the gross structure of **51** was elucidated as depicted.

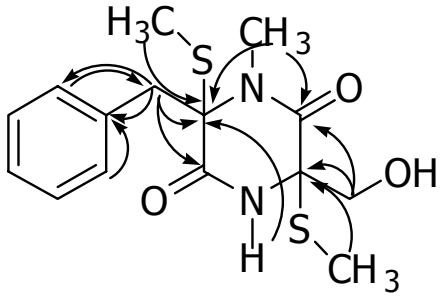


Figure 8. Selected HMBC correlations for **51**

The structure of **51** was confirmed by X-ray crystallographic analysis and it revealed that the two methylthio groups are attached to the same side of the six-membered ring (Figure 2). The absolute configuration of **51** was initially presented as (3*S*,6*S*) (Nilanonta, *et al*., 2003a), however, later it was revised to be (3*R*,6*R*) (Figure 2; Isaka and Kongsaeree, unpublished).

Crystal Data

$C_{15}H_{20}N_2O_3S_2$

$M_r = 340.464$

Monoclinic

$P2_1$

a = 10.9060(5) Å

b = 8.0074(2) Å

c = 19.0249(8) Å

$\alpha = 90.00°$

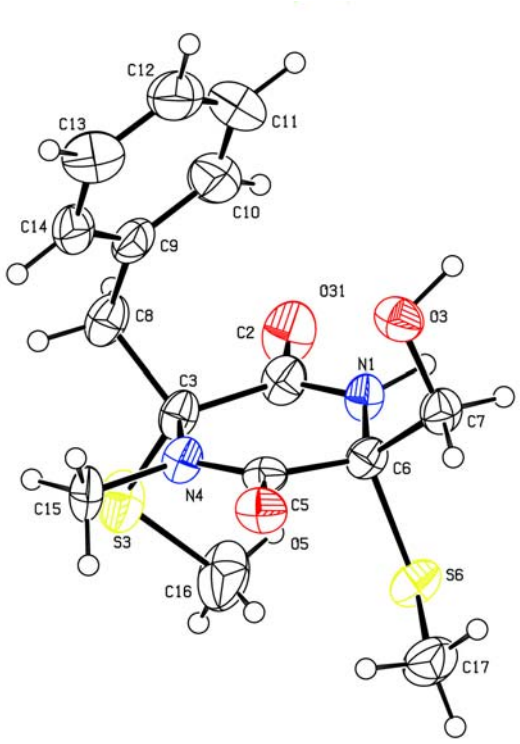
$\beta = 94.790(2)°$

$\gamma = 90.00°$

$V = 1655.62(11)$ Å^3

$Z = 4$

$D_x = 1.360$ Mg m^{-3}



Density measured by: not measured

fine-focus sealed tube

Mo *K*α radiation

$\lambda = 0.71073$

Cell parameters from 2951 reflections

$\theta = 0.998 - 24.72°$

$\mu = 0.333$ mm^{-1}

T = 298 K

Rod

Colourless

Source: *V. hemipterigenum* BCC1449

Figure 9. X-ray crystal structure of **51**, and crystallographic data

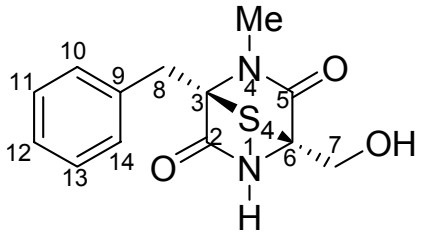
Table 4. ^{1}H and ^{13}C NMR data of compound **51**

position	δ_H (mult., *J* in Hz) in $CDCl_3$	δ_C (mult.) in $CDCl_3$	δ_C (mult.) in MeOH-d_4
2		164.9[a](s)	167.3[b](s)
3		75.7 (s)	76.5 (s)
5		164.9[a](s)	167.4[b](s)
6		64.8 (s)	66.6 (s)
7	2.74 (brd, 11.6)	65.2 (t)	66.3 (t)
	3.42 (d, 11.8)		
8	3.15 (d, 13.8)	42.3 (t)	43.1 (t)
	3.54 (d, 13.9)		
9		133.7 (s)	135.6 (s)
10, 14	7.26-7.32 (m)	128.8 (d)	129.7 (d)
11, 13	7.11-7.15 (m)	130.0 (d)	131.1 (d)
12	7.26-7.32 (m)	128.0 (d)	128.7 (d)
N(4)-*CH*$_3$	3.28 (s)	30.3 (q)	30.9 (q)
N(1)-*H*	6.39 (brs)		
3-S*CH*$_3$	2.20 (s)	14.4 (q)	14.1 (q)
6-S*CH*$_3$	2.23 (s)	13.5 (q)	13.7 (q)
7-O*H*	1.85 (brs)		

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

Compound **51** is a new 1-desmethyl analog of the known bisdethiodi (methylthio)hyalodendrin (Strunz, *et al*., 1974; Michel, *et al*., 1974; DeVault and Rosenbrook, 1973). (3*S*,6*S*)-bisdethiodi(methylthio)hyalodendrin was previously isolated from *Hyalodendron* sp. (Strunz, *et al*., 1974), and its (3*R*,6*R*)-isomer, A26771E, was isolated from *Penicillium turbatum* (Michel, *et al*., 1974). Prior to these reports, the same compound is isolated from an unidentified fungus NRRL 3888 (DeVault and Rosenbrook, 1973), but its stereochemistry was not presented and lacked full spectral data. Thus, compound **51** could be designated as (3*R*,6*R*)-bisdethiodi(methylthio)-1-demethylhyalodendrin.

3.1.2 Structure elucidation of compound 52



Compound **52** was obtained as colorless crystals. The UV spectrum of **52** showed absorption bands at λ_{max} (log ε) 204 (4.35) and 299 (3.59) nm. The IR spectrum of **52** showed two absorption bands at ν_{max} 1694 and 1634 cm^{-1} indicating the C=O stretching of secondary amide and tertiary amide. IR spectrum also showed OH stretching peak at ν_{max} 3290 cm^{-1}, associated with N-H stretching of the amide at ν_{max} 3102 cm^{-1}.

The ^{1}H NMR spectrum of compound **52** (in $CDCl_3$, 400 MHz) suggested that this compound should have fourteen protons. The spectrum showed two doublet of doublets at δ_H 3.94 (1H, dd, *J* = 12.2, 4.7 Hz, H-7) and 3.79 (1H, dd, *J* = 12.2, 8.7 Hz, H-7), two doublet at δ_H 3.87 (1H, d, *J* = 14.6 Hz, H-8) and 3.30 (1H, d, *J* = 14.6 Hz, H-8), which indicated the presence of two nonequivalent methylenes. Two multiplet at δ_H 7.16-7.19 (2H, m, H-11, H-13) and 7.25-7.31 (3H, m, H-10, H-12, H-14) were five protons of benzene ring. A broad singlet at δ_H 6.60 (1H, brs) and a multiplet at δ_H 2.71 (1H, m) were respectively assignable to a secondary amide proton, and a hydroxyl. A singlet at δ_H 3.17 (3H, s) was that of an amide N-methyl group.

The ^{13}C NMR spectrum of compound **52** (in $CDCl_3$, 100 MHz) showed ten carbon signals, where two carbonyl carbon signals were superimposed at δ_C 168.0 ppm. Analysis of ^{13}C NMR, DEPTs and HMQC spectral data of **52** led to the categorization of carbons: one methyl (δ_C 30.3, –CO-N-*C*H_3), two methylene, five methine (aromatic region) and five quaternary carbons. The presence of two downfield carbon signals at δ_C 168.0 (inseparable in $CDCl_3$) indicated the appearance of two amide carbonyls in this molecule. The quaternary carbon at δ_C 133.4 is an

aromatic carbon and two quaternary carbons at δ_C 71.0 and 78.1 indicated R_3C-N partial structure in this molecule. From ^{13}C NMR, DEPTs and HMQC data it was concluded that this compound should have thirteen carbons.

Combined analyses of ^{1}H, ^{13}C, DEPTs, COSY and HMQC spectra revealed that this compound possesses a benzyl group, a hydroxymethyl group, a methyl group attached to an amide nitrogen (δ_C 30.3, δ_H 3.17 ppm), a secondary amide proton (δ_H 6.60 ppm), two quaternary carbons at δ_C 71.0 and 78.1 ppm, and two carbonyls.

The molecular formula of **52**, $C_{13}H_{14}S_4N_2O_3$, was determined by HRMS (ESI-TOF) analysis and data from ^{1}H NMR and ^{13}C NMR spectra. The ion peak of $[M - H]^-$ was found at *m/z* 372.9820 (Δ = 1.2 mmu). Thus, this compound has IHD value = 8, indicating eight unsaturation points in its molecule.

The NMR, IR and UV spectra of compound **52** were close to those of **51** except for the lack of the two NMR signals of sulfur-connected methyl groups both in ^{1}H and ^{13}C spectra. The molecular formula of $C_{13}H_{14}S_4N_2O_3$, established by HRMS, requested a structure bearing –SSSS– bridge depicted as **52**, instead of the two methylthio groups in **51**. Taken together with the HMBC correlation data (Figure 3), the structure of **52** was elucidated as depicted.

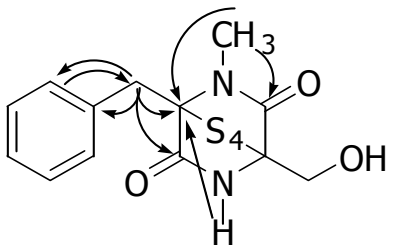


Figure 10. Selected HMBC correlations for **52**

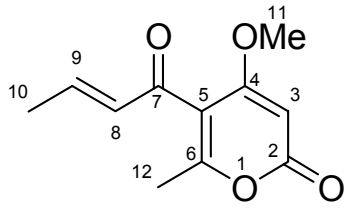
Table 5. ^{1}H and ^{13}C NMR data of compound **52** in $CDCl_3$

position	δ_H (mult.; *J* in Hz)	δ_C (mult.)
2	-	168.0[a](s)
3	-	78.1 (s)
5	-	168.0[a](s)
6	-	71.0 (s)
7	3.79 (dd, 12.2, 8.7)	65.4 (t)
	3.94 (dd, 12.2, 4.7)	
8	3.30 (d, 14.6)	39.4 (t)
	3.87 (d, 14.6)	
9		133.4 (s)
10, 14	7.25-7.31 (m)	128.9 (d)
11, 13	7.16-7.19 (m)	129.4 (d)
12	7.25-7.31 (m)	127.9 (d)
N(4)-*CH*$_3$	3.17 (s)	30.3 (q)
N(1)-*H*	6.60 (brs)	
7-O*H*	2.71 (m)	

[a] Two ^{13}C signals are overlapping.

By analogy to the co-metabolite **51**, compound **52** should possess (3*R*,6*R*)-configuration. Compound **52** is a new 1-desmethyl analog of the known hyalodendrin tetrasulfide (Michel, *et al*., 1974; Strunz, *et al*., 1975). (3*S*,6*S*)-hyalodendrin tetrasulfide has previously been isolated from *Hyalodendron* sp. (Strunz, *et al*., 1975), while the (3*R*,6*R*)-isomer, A26771C, has been isolated from *Penicillium turbatum* (Michel, *et al*., 1974). Therefore, compound **52** could be designated as (3*R*,6*R*)-1-demethylhyalodendrin tetrasulfide.

3.1.3 Structure elucidation of compound 53 (pyrenocine A)



Compound **53** was obtained as a colorless solid. The UV spectrum of **53** displayed λ_{max} (log ε) at 205 (4.41), 228 (4.20) and 273 (3.96) nm, indicating conjugated carbonyl chromophore in this molecule. The IR spectrum of **53** exhibited two strong absorption bands at ν_{max} 1728 and 1674 cm^{-1}, indicating the presence of a conjugated ester and a conjugated ketone, respectively. Also absorption bands at ν_{max} 1629, 1603, 1558 and 1448 cm^{-1} suggested aromatic-like nucleus in this molecule. The EIMS spectrum of **53** gave the molecular ion peak at *m*/*z* 208. The ^{1}H NMR spectrum of **53** indicated twelve protons, while its ^{13}C NMR spectrum suggested eleven carbons. Therefore, the molecular formula of **53** was established as $C_{11}H_{12}O_4$.

Analysis of ^{13}C NMR (in $CDCl_3$, 100 MHz) and DEPTs spectral data of compound **53** led to the categorization of the carbons: three methyls (including one -OCH_3 at δ_C 56.3), three methines, and five quaternary carbons. The carbon signal at δ_C 190.6 indicated the presence of a conjugated ketone.

The ^{1}H NMR spectrum of compound **53** (in $CDCl_3$, 400 MHz) showed signals of a *trans* olefin (*J* = 15.7 Hz) at δ_H 6.32 (1H, dq, *J* = 15.7, 1.5 Hz, H-8) and 6.81 (1H, dq, *J* = 15.6, 6.9 Hz, H-9). A doublet signal of a methyl group at δ_H 1.98 (3H, dd, *J* = 6.9, 1.3 Hz, H-10) was coupled with one of the *trans* olefinic protons (H-9) with a *J*-value of 6.9 Hz, also an allylic coupling, $J_{8,10}$ = 1.3 Hz, was observed. The downfield shift of H-9 (δ_H 6.81) suggested that the other side of the olefin is attached to a carbonyl, hence, the partial structure should be a crotonyl group. In addition to these proton signals, a ring proton at δ_H 5.48 (1H, s, H-3), a methoxyl signal at δ_H 3.81 (3H, s, H-11), and methyl protons attached to the lactone ring at δ_H 2.18 (3H, s, H-12) were present.

Analysis of the HMQC and HMBC spectral data revealed the gross structure of compound **53**. Thus, HMBC correlations from β-proton of α, β-unsaturated ketone at δ_H 6.81 (1H) to ketone carbonyl carbon at δ_C 190.6 (C-7), and to methyl carbon at δ_C 18.5 (C-10) were observed. The α-proton of α, β-unsaturated ketone at δ_H 6.32 (1H) correlated to ketone carbonyl carbon at δ_C 190.6 (C-7), to aromatic carbon at δ_C 113.9 (C-5), and to methyl carbon at δ_C 18.5 (C-10). The α-proton of α, β-unsaturated ester at δ_H 5.48 (1H) showed correlation to the ester carbonyl carbon at δ_C 163.1 (C-2), and to C-5 at δ_C 113.9, 161.4 (C-6), and 168.7 (C-4). The methoxy protons at δ_H 3.81 (3H) showed correlation to the ring carbons at δ_C 113.9 (C-5), and 168.7 (C-4). The H-12 methyl protons at δ_H 2.18 (3H) showed correlation to ester carbonyl carbon at δ_C 163.1 (C-2), and to ring carbons at δ_C 161.4 (C-6), 113.9 (C-5), while The H-10 methyl protons at δ_H 1.98 (3H) was correlated to α and β carbons of α, β-unsaturated ketone at δ_C 133.0 (C-8), and 147.4 (C-9), respectively.

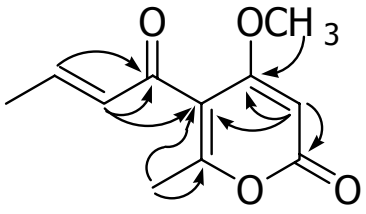


Figure 11. Selected HMBC correlations for **53**

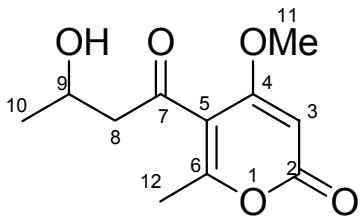
Compound **53** is identical to pyrenocine A, which was previously isolated from an onion pink root fungus *Pyrenochaeta terrestris* (Sato, *et al*., 1979), and soon later from *Penicillium citreo-viride* (Niwa, *et al*., 1980). The X-ray crystal structure of pyrenocine A was reported by Sato *et al.* (1981). Spectral data of **53**, isolated from BCC 1449, were identical to those reported in the literature (Sato, *et al*., 1981) in all respects.

Table 6. ^{1}H and ^{13}C NMR data of compound **53** in $CDCl_3$

	compound **53**		pyrenocine A [literature][a]	
position	δ_H (mult., *J* in Hz)	δ_C (mult.)	δ_H (mult., *J* in Hz)	δ_C (mult.)
2	-	163.1 (s)	-	163.0 (s)
3	5.48 (s)	87.6 (d)	5.44 (s)	87.7 (d)
4	-	168.7 (s)	-	168.7 (s)
5	-	113.9 (s)	-	114.4 (s)
6	-	161.4 (s)	-	161.4 (s)
7	-	190.6 (s)	-	190.5 (s)
8	6.32 (dq, 15.7, 1.5)	133.0 (d)	6.25 (dq, 16, 1)	133.1 (d)
9	6.81 (dq, 15.6, 6.9)	147.4 (d)	6.76 (m)	147.3 (d)
10	1.98 (dd, 6.9, 1.3)	18.5 (q)	1.97 (dd, 7, 1)	18.5 (q)
11	3.81 (s)	56.3 (q)	3.80 (s)	56.4 (q)
12	2.18 (s)	18.2 (q)	2.18 (s)	18.2 (q)

[a] Literature data (Sato, *et al*., 1981)

3.1.4 Structure elucidation of compound 54 (pyrenocine B)



Compound **54** was obtained as a pale yellow solid. The UV spectrum of **54** showed λ_{max} (log ε) at 204 (4.34), 220 (4.08), 258 (3.98) and 284 (3.86) nm, indicating conjugated carbonyl chromophore and aromatic-like nucleus in this molecule. The IR spectrum of **54** showed a hydroxyl absorption at ν_{max} 3455 cm^{-1} and two absorptions at ν_{max} 1713 and 1684 cm^{-1} indicated the -C=O stretching of conjugated ester and -C=O stretching of conjugated ketone. IR spectrum also showed characteristic peak of C=C stretching (skeletal) of aromatic compound at ν_{max} 1604, 1547, 1449 cm^{-1}.

The ^{1}H NMR spectrum of compound **54** (in $CDCl_3$, 400 MHz) was similar to that of compound **53**. The proton and carbon signals of *trans* olefin in **53** were absent in **54**, instead, an oxymethine at δ_H 4.31 (1H, m, H-9), a pair of methylene protons at δ_H 2.92 (1H, dd, *J* = 17.5, 3.1 Hz, Ha-8) and 2.82 (1H, dd, *J* = 17.5, 8.8 Hz, Hb-8), and a hydroxyl proton at δ_H 2.73 (1H, brd, *J* = 3.2 Hz, O*H*) were present in **54**. The doublet at δ_H 1.25 (3H, d, *J* = 6.4 Hz H-10) lacked allylic coupling and downfield shifted when compared to H-10 methyl in **53**. A ring proton at δ_H 5.49 (1H, s, H-3), methoxyl protons at δ_H 3.87 (3H, s, H-11), and methyl protons at δ_H 2.28 (3H, s, H-12), similar to those of **53**, were observed.

Analysis of ^{13}C NMR ($CDCl_3$) and HMQC spectral data of compound **54** led to the categorization of the eleven carbons: three methyl (one -O*C*H_3 at δ_C 56.5), one methylene, two methine and five quaternary carbons. The presence of one downfield carbon signal at δ_C 202.5 indicated the presence of C=O of a ketone.

The EIMS spectrum of **54** gave the molecular ion peak at *m*/*z* 226, which is 18 unit mass more than that of **53**. Taken together with the ^{1}H and ^{13}C NMR data, the molecular formula of this compound was determined to be $C_{11}H_{14}O_5$. These data

indicated that compound **54** should possess a 3-hydroxybutanoyl group, instead of the crotonyl group in **53**.

The gross structure of **54** was confirmed by HMBC correlation data (Figure 5). Thus, HMBC correlations from α-proton of the α, β-unsaturated ester at δ_H 5.49 (1H) to ring carbons at δ_C 115.4 (C-5), and 168.2 (C-4) were observed. The methoxy protons at δ_H 3.87 (3H) showed correlation to ring carbon at δ_C 168.2 (C-4). Two methylene protons at δ_H 2.82 (1H) and δ_H 2.92 (1H) correlated to the ketone carbonyl carbon at δ_C 202.5 (C-7), and methine carbon at δ_C 64.3 (C-9). The H-12 methyl protons at δ_H 2.28 (3H) showed correlation to ester carbonyl carbon at δ_C 163.9 (C-2), and to ring carbons at δ_C 162.5 (C-6), and 115.4 (C-5), while the H-10 methyl protons at δ_H 1.25 (3H) correlated to methylene carbon at δ_C 52.8 (C-8), and methine carbon at δ_C 64.3 (C-9).

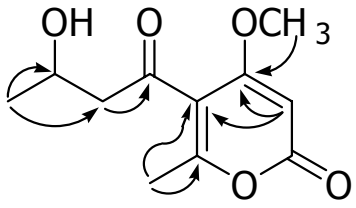


Figure 12. Selected HMBC correlations for **54**

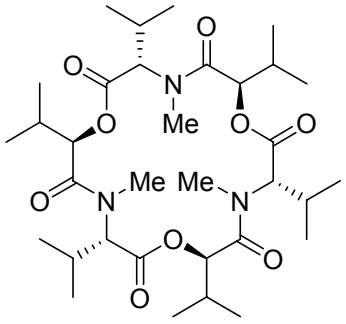
This compound is identical to pyrenocine B (Sato, *et al*., 1979; 1981). It is argued, in the original report, that pyrenocine B might be an isolation artifact derived from hydration of pyrenocine A. Indeed, compound **54**, isolated from *V. hemipterigenum* BCC 1449, exhibited very poor optical rotation which strongly suggested that it is a racemate.

Table 7. ^{1}H and ^{13}C NMR data of compound **54** in $CDCl_3$

	compound **54**		pyrenocine B [literature][a]
position	δ_H (mult., *J* in Hz)	δ_C (mult.)	δ_C (mult.)
2	-	163.9 (s)	163.8 (s)
3	5.49 (s)	87.8 (d)	87.8 (d)
4	-	168.2 (s)	168.3 (s)
5	-	115.4 (s)	115.4 (s)
6	-	162.5 (s)	162.4 (s)
7	-	202.5 (s)	201.0 (s)
8	2.92 (dd, 17.5, 3.1)	52.8 (t)	52.9 (t)
	2.82 (dd, 17.5, 8.8)		
9	4.31 (m)	64.3 (d)	64.3 (d)
10	1.25 (d, 6.4)	22.7 (q)	22.8 (q)
11	3.87 (s)	56.3 (q)	56.5 (q)
12	2.28 (s)	18.2 (q)	18.6 (q)
9-O*H*	2.73 (brd, 3.2)	-	-

[a] Literature data (Sato, *et al*., 1981)

3.1.5 Structure elucidation of compound 55 (enniatin B)



Compound **55** was obtained as a colorless solid; mp 173-175 °C. It displayed negative sign of optical rotation; $[\alpha]^{29}_{D}$ –96 (*c* 1.04, $CHCl_3$). The UV spectrum of **55** displayed λ_{max} (log ε) at 206 (4.57) and 291 (3.16) nm. The IR spectrum of **55** showed strong absorption bands at ν_{max} 1741 and 1664 cm^{-1} which suggested the presence of alkyl ester(s) and amide(s).

The ^1H NMR spectrum of compound **55** (in $CDCl_3$, 400 MHz) showed signals of nineteen protons: two downfield doublets at δ_H 5.14 (1H, d, *J* = 8.6 Hz) and 4.52 (1H, d, *J* = 9.7 Hz), one singlet at δ_H 3.13 (3H, s, N-C*H*$_3$), two multiplets at δ_H 2.28 (1H, m) and δ_H 2.31 (1H, m), and four doublets of methyl at δ_H 1.06 (3H, d, *J* = 6.3 Hz), 0.99 (3H, d, *J* = 6.8 Hz), 0.96 (3H, d, *J* = 6.9 Hz) and 0.89 (3H, d, *J* = 6.7 Hz). The ^{13}C NMR spectrum of **55** (in $CDCl_3$, 100 MHz) exhibited eleven carbon signals. The DEPT and HMQC experiment revealed the type of carbons: five methyl (four C-*C*H_3 at δ_C 18.5, 18.7, 19.3 and 20.4, and one N-*C*H_3 at δ_C 33.2), four methine (δ_C 75.7, 63.2, 29.9 and 27.9) and two quaternary carbons (δ_C 169.2 and 170.2, carbonyl).

Analysis of COSY and HMQC spectral data revealed that compound **55** consists of a *N*-methylvaline (*N*MeVal) and a 2-hydroxyisovaleric acid (Hiv) residues. Partial structures and their connectivity were established by analysis of HMBC data. Correlations from proton at δ_H 4.52 (1H) to carbon of N-CH_3, to methine carbon at δ_C 27.9 and to ester carbonyl carbon at δ_C 170.2 were observed. The proton at δ_H 5.14 (1H) correlated to the ester carbonyl carbon at δ_C 170.2, and methine protons at δ_H 2.31 (2H, overlapping) showed correlations to four methyl carbons at δ_C 18.5, 18.7,

19.3 and 20.4, and two methine carbons at δ_C 63.2 and 75.7. The methyl protons at δ_H 1.06 (3H) showed correlations to methine carbon at δ_C 63.2, methyl carbon at δ_C 19.3, and methine carbon at δ_C 27.9, while the methyl protons at δ_H 0.96 (3H) correlated to methine carbon at δ_C 75.7. On the basis of these data, a 2,5-diketomorpholine structure was initially proposed (Figure 6).

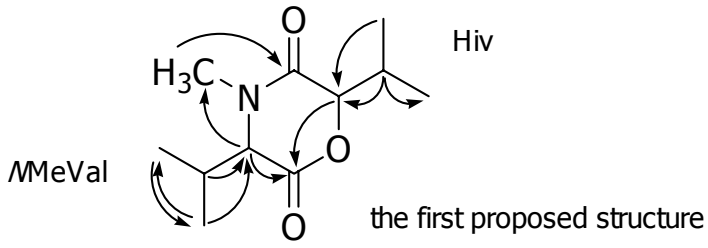


Figure 13. Selected HMBC correlations

The first proposed structure has molecular formula $C_{11}H_{19}NO_3$ (MW 213), however, it is not in accordance with the molecular ion peak at *m/z* 639 of the EIMS spectrum of **55**. The ESI-TOF Mass spectrum also showed *pseudo* molecular ion peaks at *m/z* 640 $[M + H]^+$ and 662 $[M + Na]^+$. These data indicated that compound **55** must possess a trimeric structure of the first proposed compound, consisting of three units of *N*MeVal and three units of Hiv. Considering the C_3-symmetry shown in NMR spectra, the only possible structure is the cyclohexadepsipeptide where each 3 units of *N*MeVal and Hiv are connected alternately (Figure 7).

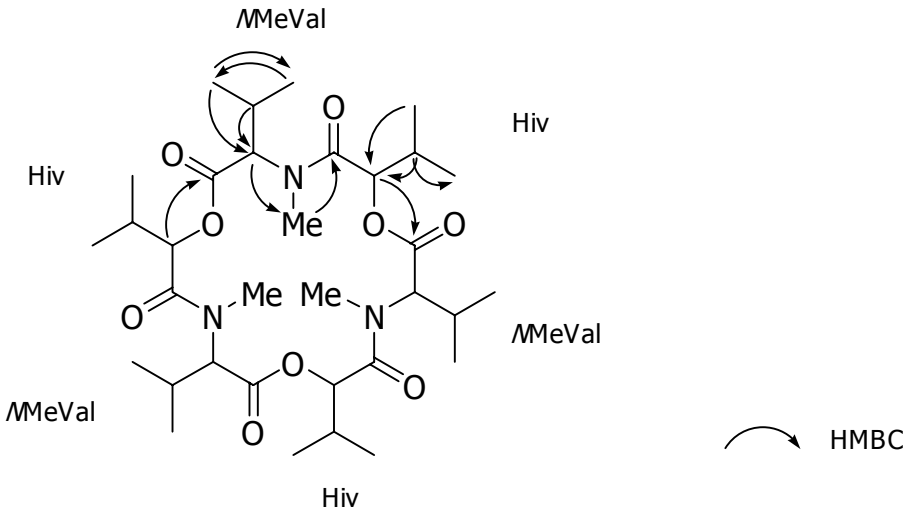


Figure 14. The gross structure of compound **55**.

Based on literature search, it was found that compound **55** is identical to enniatin B (Plattner, *et al*., 1948). The optical rotation data of **55** (isolated from BCC 1449) was consistent with the literature data, therefore, the α-position (C-2) of all three *N*MeVal residues possess (2*S*)-configuration, and the α-position (C-2) of all Hiv residues have (2*R*)-configuration.

Table 8. ^{1}H and ^{13}C NMR data of compound **55**

	compound **55**		enniatin B [literature][a]	
position	^{13}C	^{1}H (mult, *J* in Hz)	^{13}C	^{1}H
***N*MeVal**	(3 units, symmetrical)			
1 *C*=O	170.2	-	170.3	-
2	63.2	4.52 (3H, d, 9.7)	63.2	4.42
3	27.9	2.31 (3H, m)	27.9	2.27
4	20.4	1.06 (9H, d, 6.3)	20.4	1.03
4′	19.3	0.89 (9H, d, 6.7)	19.3	0.86
N-CH_3	33.2	3.13 (9H, s)	33.2	3.09
Hiv	(3 units, symmetrical)			
1 *C*=O	169.2	-	169.3	-
2	75.7	5.14 (3H, d, 8.6)	75.7	5.11
3	29.9	2.28 (3H, m)	29.9	2.27
4	18.5	0.96 (9H, d, 6.9)	18.5	0.93
4′	18.7	0.99 (9H, d, 6.8)	18.6	0.96

[a] Literature data (Visconti, *et al*., 1992)

Enniatins are well-known antibiotics produced by various *Fusarium* species. This class of compounds have been known to exhibit antibiotic (Tsantrizos, *et al*., 1993; Tomoda, *et al*., 1992; Tirunarayanan and Sirsi, 1957), insecticidal (Strongman, *et al*., 1988; Grove and Pople, 1980), and phytotoxic (Burmeister and Plattner, 1987; Gauman, *et al*., 1960) activities. They also inhibit acyl-CoA: cholesterol acyltransferase (ACAT) (Tomoda, *et al*., 1992a; Tomoda, *et al*., 1992b).

Enniatin B is the most common analog in this class. All naturally-occurring enniatins possess (2*S*)-configuration at the *N*-methylamino acid residues, and (2*R*)-configuration at the Hiv residues.

During spectroscopic analysis of compound **55** (enniatin B) and other enniatins (as described in later sections), it was found that NOESY spectral data (in $CDCl_3$) provide useful information to confirm the relative stereochemistry of the six residues and also the conformation of the macrocyclic ring. An example using the known compound, **55**, is shown in Figure 8. Intense NOESY correlations from *N*-methyl protons of *N*MeVal residues to α-protons (H-2) of Hiv residues revealed that these protons are both directed to the same side and vertically oriented to the depsipeptide ring. In contrast, the NOESY cross signal between the *N*-methyl protons and the α-protons (H-2) of *N*MeVal is much weaker, suggesting that these are placed opposite to the macrocyclic ring. The large vicinal *J*-value (9.7 Hz) between α- and β-protons of *N*MeVal and the weak NOE for these protons indicated an antiperiplanar relationship. This partial conformation was strongly supported by the observation of intense NOESY correlation from α-protons (H-2) to both of the methyl protons of the isopropyl, H-4 and H-4′. Similarly, the large coupling constant ($J_{2,3}$ = 8.6 Hz) and weak NOESY correlation between H-2 and H-3 of the Hiv residues indicated their antiperiplanar relationship. NOESY correlation from H-2 of Hiv to both the methyl groups of the isopropyl side chain of Hiv was observed. These data suggested the relative configuration of the six residues as well as an approximate conformation of the cyclohexadepsipeptide in $CDCl_3$, and they are consistent with the known conformation of enniatin B in solution (Ovchinnikov, 1974) and crystal structures.

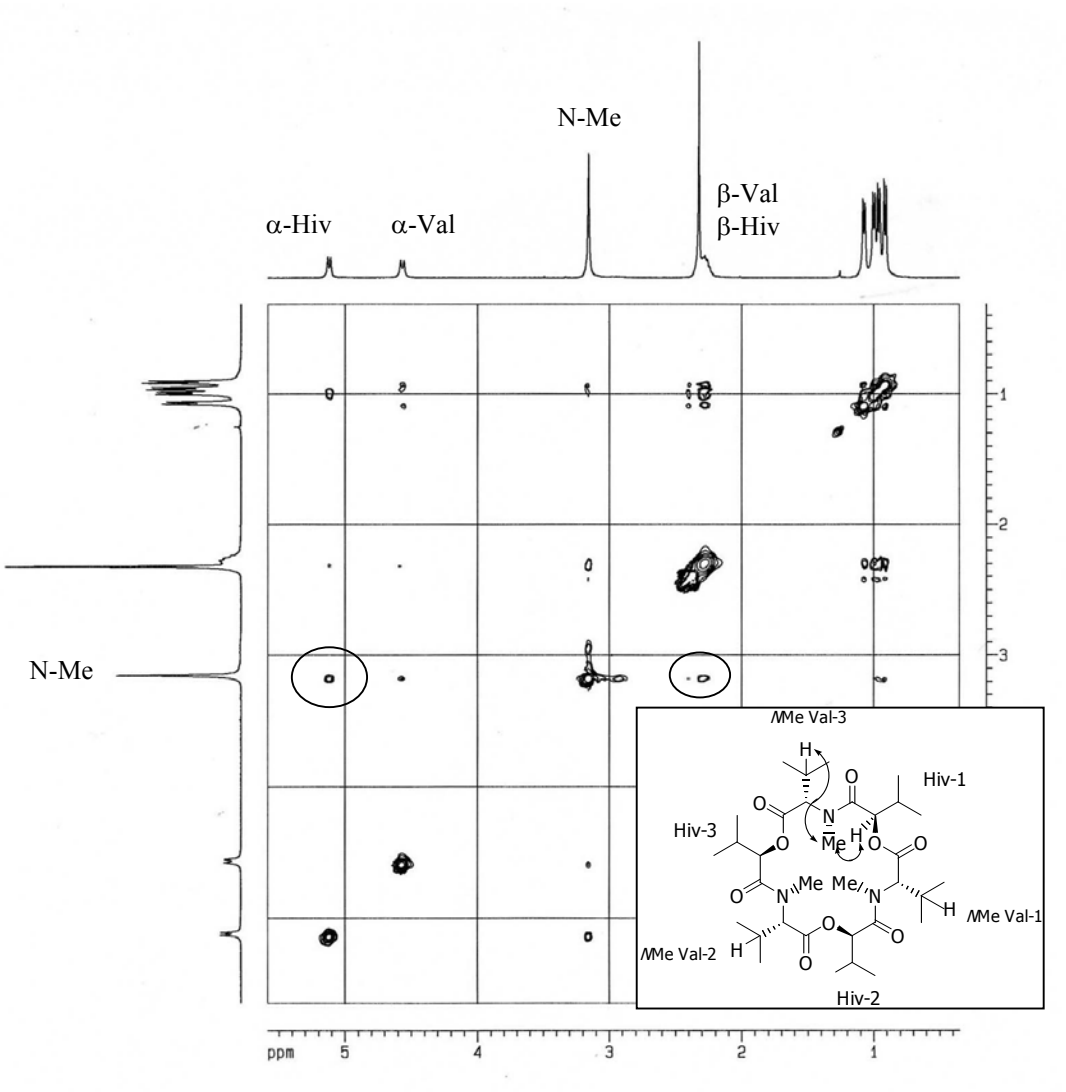
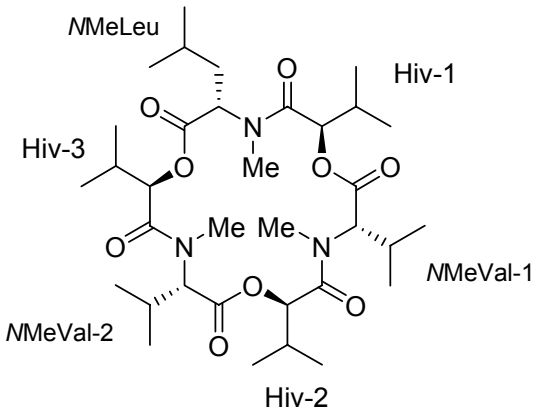


Figure 15. NOESY spectrum of compound **55** (enniatin B) in $CDCl_3$

3.1.6 Structure elucidation of compound 56 (enniatin B_4)



Compound **56** was obtained as a colorless amorphous solid. It displayed negative sign of optical rotation; $[\alpha]^{27}_{D}$ –57 (*c* 0.09, $CHCl_3$). The UV spectrum of **56** displayed λ_{max} (log ε) at 207 (4.30) and 279 (2.88) nm. The IR spectrum of **56** showed two strong absorption peaks at ν_{max} 1733 and 1662 cm^{-1}, suggested the presence of alkyl ester(s) and amide(s).

The 400 MHz ^1H NMR and 100 MHz ^{13}C NMR spectral data of **56** (in $CDCl_3$) were similar to those of **55**, but lacked symmetry. These spectra also indicated that compound **56** is an enniatin analog composing of three *N*-methyl amino acid and three Hiv residues. The EIMS spectrum of **56** exhibited molecular ion peak at *m*/*z* 653, which was 14 unit mass more than that of enniatin B (**55**).

The ^1H NMR spectrum of compound **56** showed six proton signals of α-protons (H-2) for each residue: δ_H 5.19 (1H, d, *J* = 8.4 Hz, Hiv), 5.00 (1H, d, *J* = 8.3 Hz, Hiv), 4.99 (1H, d, *J* = 9.0 Hz, Hiv), 4.93 (1H, d, *J* = 10.0 Hz), 4.49 (1H, d, *J* = 10.1 Hz) and 4.74 (1H, brs). Three amide *N*-methyls were present at δ_H 3.15 (s, 3H), 3.14 (s, 3H) and 3.11 (s, 3H). Three multiplets were observed at δ_H 2.33-2.21 (5H, m, H-3), 1.77-1.69 (2H, m) and 1.53 (1H, m) and many doublets of methyl at δ_H 1.05-0.88 (total 36H).

Analysis of ^{13}C NMR and DEPTs spectral data of compound **56** led to the categorization of carbons: fifteen methyl (twelve C-CH_3 and three *N*-CH_3), a methylene (δ_C 37.9), twelve methine and six quaternary carbons. The downfield

carbon signals at δ_C 169.1, 169.3 and 169.7 confirmed the appearance of amides and the signals at δ_C 170.3, 170.4 and 170.7 indicated the presence of esters. Three methine carbons at δ_C 57.2, 61.3, 63.1 were assignable to those at α-position (C-2) of the *N*-methylamino acid residues, and three methine carbons at δ_C 75.0, 75.3 and 75.3 were the α-position (C-2) of the Hiv residues.

The COSY correlations, from δ_H 4.74 proton (1H, brs) to δ_H 1.77-1.69 methylene (2H, m), and that of these methylene protons to δ_H 1.53 methine proton (1H, m), and the correlation from this methine proton to δ_H 0.93 methyl (2 × 3H, d) indicated the presence of one *N*-methylleucine (*N*MeLeu) residue. COSY correlations from five α-protons at δ_H 5.19 (1H, d), 5.00 (1H, d), 4.99 (1H, d), 4.93 (1H, d) and 4.49 (1H, d) to δ_H 2.33-2.21 methines (5H, m) together with the correlations from these methine protons to methyl protons situated δ_H 0.88-1.05 indicated the presence of two *N*MeVal and three Hiv residues.

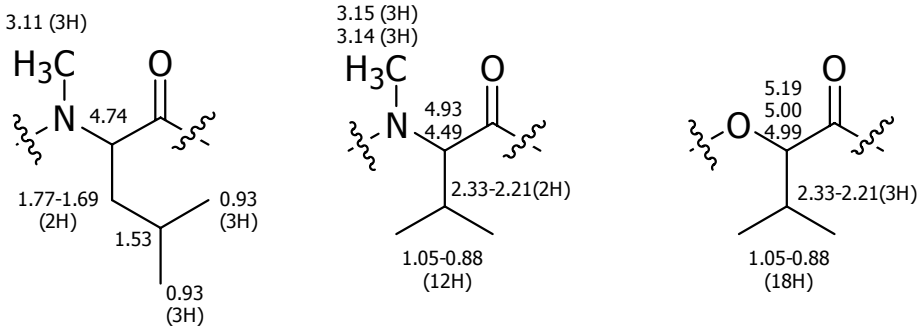


Figure 16. Proton assignments for six residue

On the basis of these spectroscopic data, it was concluded that compound **56** composed of two units of *N*MeVal, one unit of *N*Methylleucine (*N*MeLeu) and three units of Hiv. Three *N*-methylamino acid units and three Hiv units are cyclized alternately, in the same pattern as enniatin B, to form cyclohexadepsipeptide structure as depicted in the top of this subsection.

Compound **56** is identical to enniatin B_4, whose absolute stereochemistry has been established (Visconti, *et al*., 1992). The same compound is reported almost at the same time as enniatin D (Tomoda, *et al*., 1992a). The optical rotation data of compound **56**, isolated from BCC 1449, was consistent with those of enniatin B_4 and enniatin D.

Table 9. ^{1}H and ^{13}C NMR data of compound **56**

	compound **56**		enniatin B_4 [literature][g]	
position	^{13}C	^{1}H (mult, *J* in Hz)	^{13}C	^{1}H
Hiv	(3 units)			
1 *C*=O	169.7	-	169.6	-
	169.3	-	169.3	-
	169.1	-	169.0	-
2	75.3	5.19 (1H, d, 8.4)	75.6	5.18 (1H, d, 8.4)
	75.3	5.00 (1H, d, 8.3)	75.2	5.09 (1H, d, 8.5)
	75.0	4.99 (1H, d, 9.0)	74.9	4.98 (1H, d, 9.0)
3	30.2[a]	2.33-2.21 (3H, m) [b]	30.2	2.27 (1H, m)
	29.8[a]		29.8	2.22 (1H, m)
	29.7[a]		29.6	2.24 (1H, m)
4/1	19.0	1.00 (3H, d, 7.8)	18.3-18.9	0.93-0.97 (3H, m)
4′/1	18.7	0.94 (3H, d, 7.0)[c]	18.3-18.9	0.93-0.97 (3H, m)
4/2	18.6	0.98 (3H, d, 7.2)	18.3-18.9	0.93-0.97 (3H, m)
4′/2	18.4	0.94 (3H, d, 7.0)[c]	18.3-18.9	0.93-0.97 (3H, m)
4/3	18.3[f]	0.96 (3H, d, 7.4)	18.3-18.9	0.93-0.97 (3H, m)
4′/3	18.3[f]	0.93 (3H, d, 7.0)[d]	18.3-18.9	0.93-0.97 (3H, m)
***N*MeVal**	(2 units)			
1 *C*=O	170.4	-	170.4	-
	170.3	-	170.3	-
2	63.1	4.49 (1H, d, 10.1)	63.2	4.44 (1H, d, 10.0)

Table 9. ^{1}H and ^{13}C NMR data of compound **56** (continued)

	compound **56**		enniatin B_4 [literature][g]	
position	^{13}C	^{1}H (mult, *J* in Hz)	^{13}C	^{1}H
	61.3	4.93 (1H, d, 10.0)	61.3	4.89 (1H, d, 10.2)
3	27.8	2.33-2.21 (2H, m)[b]	27.8	2.16 (1H, m)
	27.6		27.5	2.23 (1H, m)
4	20.3 ×2	1.05 (3H, d, 6.4)	20.3 ×2	1.02 (3H, d, 6.2)
		1.03 (3H, d, 6.4)		1.02 (3H, d, 6.2)
4′	19.9 ×2	0.91 (3H, d, 6.8)	19.9 ×2	0.85 (3H, d, 6.8)
		0.88 (3H, d, 6.8)		0.85 (3H, d, 6.8)
N-*CH*$_3$ /1	33.7	3.15 (3H, s)	33.8	3.10 (3H, s)
N-*CH*$_3$ /2	32.9	3.14 (3H, s)	33.0	3.07 (3H, s)
***N*MeLeu**	(1 unit)			
1 *C*=O	170.7	-	170.7	-
2	57.2	4.74 (1H, brs)	57.2	4.66
3	37.9	1.77-1.69 (2H, m)[e]	37.9	1.73 (1H, d, 13.5)
				1.81 (1H, dd, 10.1, 5.1)
4	25.3	1.53 (1H, m)	25.2	1.55 (1H, m)
5	23.3	0.93 (3H, d, 7.0)[d]	23.3	0.92 (3H, d, 6.6)
5′	21.5	0.93 (3H, d, 7.0)[d]	21.5	0.91 (3H, d, 6.6)
N-*CH*$_3$	31.7	3.11 (3H, s)	31.6	3.04 (3H, s)

[a] Assignments can be interchanged.

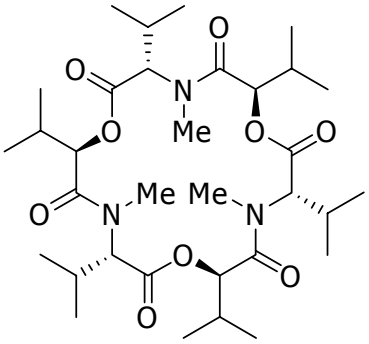
[b-e] The ^{1}H signals are overlapping.

[f] The ^{13}C signals are superimposed.

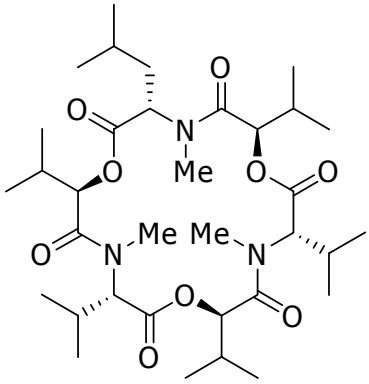
[g] Literature data (Visconti, *et al*., 1992)

3.2 Chemical constituents from the cell extract of *Verticillium hemipterigenum* BCC 1449

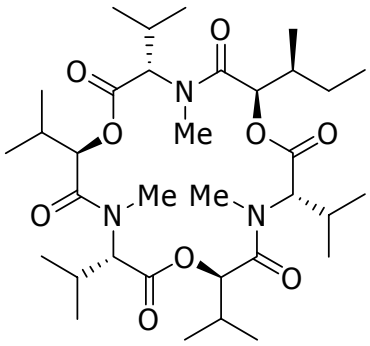
Chemical investigation of the cell extract of the insect pathogenic fungus *Verticillium hemipterigenum* BCC1449 was conducted (Nilanonta, *et al*., 2003b). Activity-guided chromatographic fractionation of the mycelial extract led to the isolation of two new enniatins, **57** and **58**, together with two known enniatins B (**55**) and B_4 (**56**).



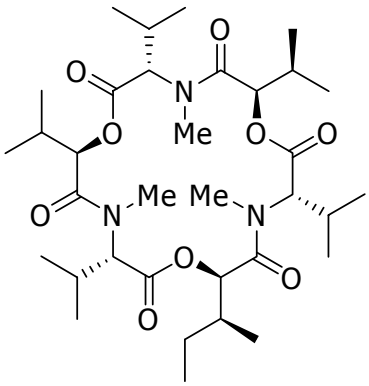
55



56

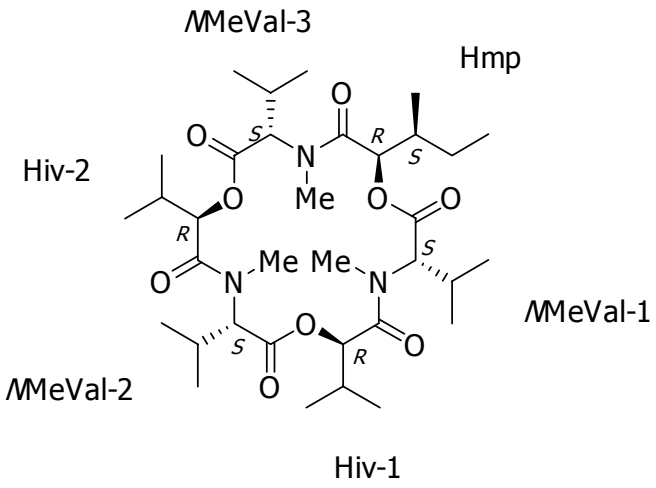


57



58

3.2.1 Structure elucidation of compound 57 (enniatin H)



Compound **57** was obtained as a colorless solid; mp 105-106 °C, $[\alpha]^{29}_{D}$ -102 (*c* 0.22, $CHCl_3$). The UV spectrum of **57** displayed absorption at λ_{max} (log ε) 206 (4.23) nm, similar to those of known enniatins. The IR spectrum of **57** was very similar to those of **55** and **56,** showing strong absorption bands at ν_{max} 1743 and 1663 cm^{-1}, indicated the C=O stretching of alkyl ester and amide. The ESI-TOF mass spectrum of **57** showed the $[M + Na]^+$ ion peak at *m/z* 676.4121 (calcd for $C_{34}H_{59}N_3O_9Na$ 676.4149, $\Delta = 2.8$ mmu), which indicated the molecular formula of this compound as $C_{34}H_{59}N_3O_9$.

The ^1H NMR spectrum of compound **57** (in $CDCl_3$, 400 MHz) presented the peak pattern of enniatins. It showed: a doublet at δ_H 5.27 (1H, d, J = 6.8 Hz), six multiplets at δ_H 5.15-5.13 (2H, m), 4.57-4.55 (3H, m), 2.29-2.28 (5H, m), 2.00 (1H, m), 1.46 (1H, m), and 1.19 (1H, m). Three singlets at δ_H 3.14 (3H, s), 3.13 (3H, s) and 3.11 (3H, s) indicated amide N-CH_3. A set of multiplets at δ_H 1.06-0.89 (36H) suggested twelve methyl groups in this molecule.

Analysis of ^{13}C NMR (in $CDCl_3$), DEPTs, and HMQC spectral data of compound **57** led to the categorization of carbons: fifteen methyl (twelve C-CH_3 and three N-CH_3), one methylene, twelve methine and two quaternary carbons. Two downfield carbon signals at δ_C 169.3 (three carbons were superimposed) and 170.3 (three carbons were superimposed) confirmed the presence of amides and esters. Six

methine carbons situated at δ_C 63.1, 63.2, 63.3, 74.3, 75.6, and 75.9 were assignable to α-protons (H-2) of *N*-methylamino acid and 2-hydroxycarboxylic acid residues.

NMR analysis (^{1}H, ^{13}C, DEPTs, COSY, HMQC and HMBC; in $CDCl_3$) revealed that this compound consists of three *N*-methylvaline (*N*MeVal), two 2-hydroxyisovaleric acid (Hiv) and one 2-hydroxy-3-methylpentanoic acid (Hmp) residues. Thus, in the ^{1}H NMR spectrum of **57**, protons of three *N*MeVal residues and two Hiv residues appeared as superimposed signals with the chemical shifts very close to those of enniatin B (**55**). In addition to these, signals assignable to another 2-hydroxycarboxylic acid residue were present. Signal at δ_H 5.27 (1H, d, J = 6.8 Hz) assigned to the proton situated at the α-position (H-2; attached to C-2, δ_C 74.3) showed vicinal coupling (COSY) to a multiplet signal at δ_H 2.00 (1H, H-3; attached to C-3, δ_C 36.1). This methine (C-3) which, in turn, was connected to a methyl group (δ_H 0.96, overlapping signal; δ_C 14.6) and a methylene (δ_H 1.46 and 1.19, 2H, H-4; δ_C 25.4, C-4). The C-4 methylene was attached to a terminal methyl (δ_H 0.92, overlapping signal, H-5; δ_C 11.3, C-5) as indicated by the COSY cross signal. Therefore, the 2-hydroxycarboxylic acid residue was assigned to 2-hydroxy-3-methylpentanoic acid (Hmp), and this was consistent with HMBC correlations: H-2 to a carbonyl (δ_C 170.3), C-3 and 3-*C*H$_3$; and H-4 to C-2, C-5 and 3-*C*H$_3$.

^{1}H and ^{13}C NMR assignments of the three *N*MeVal residues for **57** could not be distinguished due to the very close signals overlap, however, this partial structure was confirmed by 2D-NMR analyses (COSY and HMBC) as a set of signals. Important HMBC correlations for *N*MeVal residues are H-2 to C-3, C-4, C-4′, N-*C*H$_3$ and two carbonyl signals at δ_C 169.3 and 170.3, and from both H-4 and H-4’ to C-2. Two Hiv residues were also assigned as a set of signals (Table 10).

Analysis of NOESY spectral data revealed the connectivity of six residues, three *N*MeVal and three 2-hydroxycarboxylic acid. Thus, intense correlations were observed for the three *N*-methyl singlet signals at δ_H 3.11, 3.13 and 3.14 respectively with the α-protons (H-2) of the 2-hydroxycarboxylic acid residues at δ_H 5.27 (Hmp), 5.13 (Hiv) and 5.15 (Hiv), which clearly indicated that three *N*MeVal residues are linked alternately with the three 2-hydroxycarboxylic acid residues.

The connectivity of six residues was also established by analysis of HMBC correlations from α-protons of Hiv (δ_H 5.15-5.13) and Hmp (δ_H 5.27) to carbonyl carbons of *N*MeVal units (δ_C 170.3).

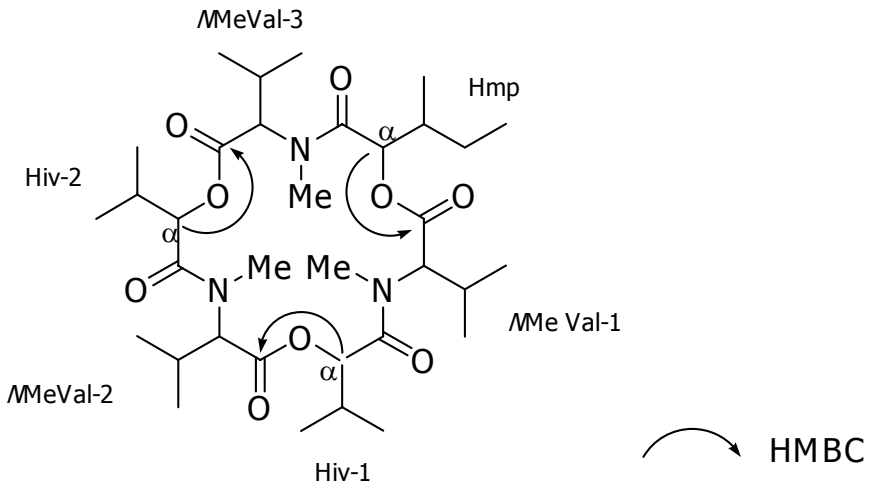


Figure 17. The gross structure of compound **57**

Finally, ^{13}C NMR assignment of the carbonyl carbons, which appeared as only two signals at δ_C 169.3 and 170.3, was achieved based on the HMBC correlations from the three *N*-methyl proton signals to the δ_C 169.3 peak, not to δ_C 170.3. Therefore, the δ_C 169.3 signal was assigned to that of amide carbonyls (C-1 for two Hiv and a Hmp), and δ_C 170.3 signal to ester carbonyls (C-1 for three *N*MeVal). Another possibility of compound **57** structure, bearing one *N*MeIle instead of *N*MeVal in **55**, enniatin B_1, was clearly ruled out by these spectroscopic analysis. Furthermore, ^1H and ^{13}C NMR spectral data of **57** (in $CDCl_3$) were apparently different from those reported for enniatin B_1 (Blais, 1992; Tomoda, 1992).

The relative stereochemistries at α-carbons of *N*MeVal, Hmp, and Hiv residues were confirmed by NOESY spectral analysis (Figure 11). NOESY correlations from *N*-methyl protons of *N*MeVal residues to α-protons of Hmp and Hiv residues revealed that these protons are both directed to the same side and vertically oriented to the depsipeptide ring. On the other hand, weak NOE between the N-methyl protons and the α-protons of *N*MeVal residues indicated that they are placed opposite to the macrocyclic ring. The absolute configuration of the β-carbon (C-3) of

Hmp unit could not be addressed by spectroscopic means, however, it was determined to be (3*S*)-configuration by precursor-directed biosynthesis, as described in the later section.

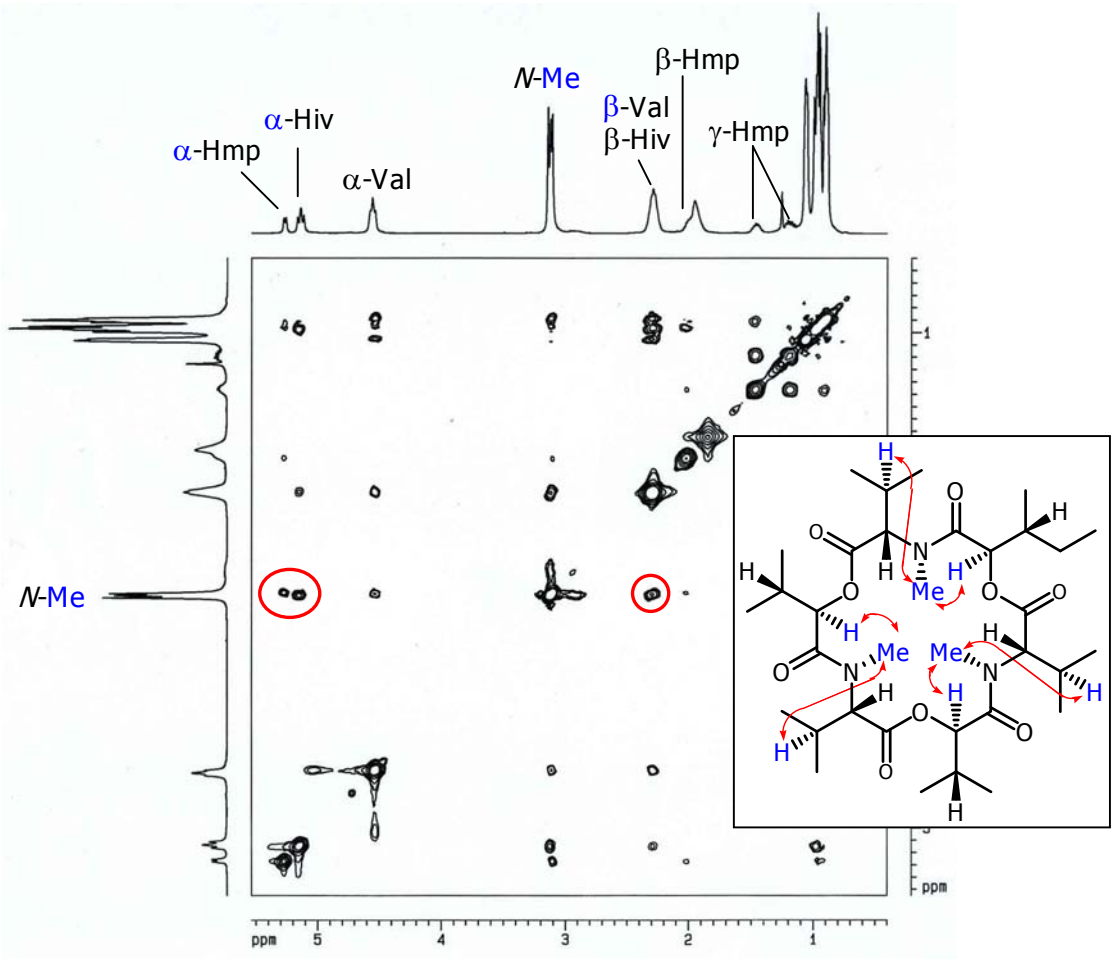


Figure 18. NOESY spectrum of compound **57**

Compound **57**, named enniatin H, is an unusual new enniatin analog possessing a Hmp unit instead of Hiv. All known naturally-occurring enniatins possess three Hiv units, while their structures differ to each other at the three *N*-methylamino acid moieties.

Table 10. NMR data for compound **57** in $CDCl_3$

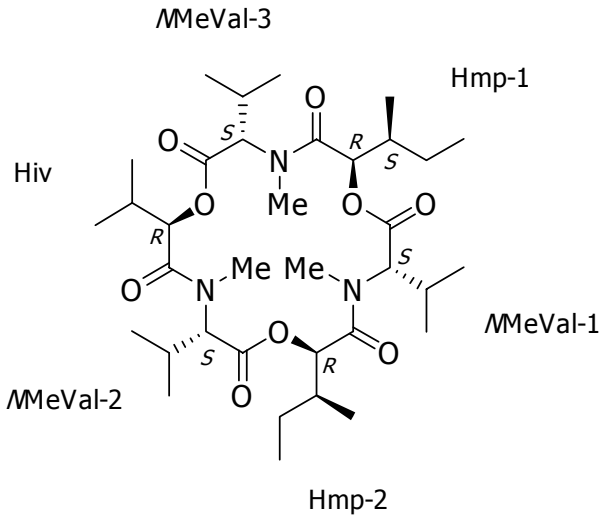
position	δ_C	δ_H (mult., *J* in Hz)	HMBC (H to C)
***N*MeVal**	(3 units)		
1 *C*=O	170.3×3	-	-
2	63.3, 63.2, 63.1	4.57-4.55 (3H, m)	1, 3, 4, 4′, δ_C 169.3, N-*C*H_3
3	28.0, 27.9, 27.8	2.29-2.28 (3H, m)[a]	-
4	20.4, 20.3×2	1.06 (9H, m)	2, 3, 4′
4′	19.5, 19.4, 19.3	0.90-0.89 (9H, m)	2, 3, 4
N-*C*H_3	33.1, 32.9×2	3.14 (3H, s)	1, δ_C 169.3
		3.13 (3H, s)	1, δ_C 169.3
		3.11 (3H, s)	1, δ_C 169.3
Hiv	(2 units)		
1 *C*=O	169.3×2[c]	-	-
2	75.9, 75.6	5.15-5.13 (2H, m)	3, 4, 4′
3	29.9×2	2.28 (2H, m) [a]	2, 4, 4′
4	18.7 [d], 18.6 [d]	0.98 (6H, m) [b]	2, 3, 4′
4′	18.5×2 [d]	0.96 (6H, m)[b]	2, 3, 4
Hmp	(1 unit)		
1 *C*=O	169.3[c]	-	-
2	74.3	5.27 (1H, d, 6.8)	3, 3-*C*H_3, δ_C 170.3
3	36.1	2.00 (1H, m)	-
4	25.4	1.46 (1H, m)	2, 5, 3-*C*H_3
		1.19 (1H, m)	3
5	11.3	0.92 (3H, m)	3
3-*C*H_3	14.6	0.96 (3H, m) [b]	3, 4

[a,b] The ^{1}H signals are overlapping.

[c] The carbon signals are superimposed.

[d] Assignments can be interchanged.

3.2.2 Structure elucidation of compound 58 (enniatin I)



Compound **58** was obtained as a colorless gum; $[\alpha]^{29}_{D}$ -87 (*c* 0.12, $CHCl_3$). The UV spectrum of **58** displayed absorption at λ_{max} (log ε) 207(4.23) nm similar to those of enniatins **55**, **56** and **57**. The IR spectrum of **58** also resembled those of other enniatins, showing ν_{max} 2965, 1745, 1665, 1468, 1383, 1281, 1192, and 1012 cm^{-1}. The ESI-TOF mass spectrum of **58** showed the $[M + Na]^+$ ion peak at *m/z* 690.4277 (calcd for $C_{35}H_{61}N_3O_9Na$, 690.4306; Δ = 2.9 mmu), which indicated the molecular formula of this compound as $C_{35}H_{61}N_3O_9$.

The ^1H NMR spectrum of compound **58** (in $CDCl_3$, 400 MHz) showed signals of a doublet at δ_H 5.15 (1H, d, *J* = 8.2 Hz), and six multiplets at δ_H 5.28-5.27 (2H, m), 4.56-4.55 (3H, m), 2.30-2.28 (4H, m), 2.02 (2H, m), 1.46 (2H, m) and 1.19-1.18 (2H, m). Three singlets at δ_H 3.12 (3H, s), 3.11 (3H, s) and 3.09 (3H, s) indicated the presence of three N-CH_3 groups. A set of multiplets at δ_H 1.06-0.89 (36H) suggested twelve methyl groups in this molecule.

Analysis of ^{13}C NMR (in $CDCl_3$), DEPTs and HMQC spectral data of compound **58** led to the categorization of carbons: fifteen methyl (twelve C-*C*H_3 and three N-*C*H_3), two methylene, twelve methine and two quaternary carbons. The appearance of two carbonyl carbon signals at δ_C 169.2 (three carbon signals are

overlapped) and 170.3 (three carbon signals are overlapped) confirmed the presence of amides and esters. Six methine carbons situated at δ_C 63.1 (three carbons), 74.2, 74.4, and 75.7 were assigned to α-carbons (C-2) for *N*-methylamino acid and 2-hydroxycarboxylic acid residues..

^{1}H and ^{13}C NMR spectra of **58** were similar to those of **57** where chemical shifts of the protons and carbons in each residue were superimposed but with different composition. Compound **58** composed of three *N*MeVal, one Hiv, and two Hmp as described below.

Vicinal coupling (COSY) from δ_H 2.30-2.28 methine protons (3H, m, H-3, *N*MeVal) to δ_H 1.06 methyls (9H, m, H-4, *N*MeVal) and 0.89 methyls (9H, m, H-4′, *N*MeVal) revealed the assignments of the isopropyl groups in three *N*MeVal. COSY correlations from δ_H 2.02 (2H, m, H-3, Hmp) to δ_H 0.96 (6H, m, 3-C*H*$_3$, Hmp) indicated methyl group on Hmp unit at position 3, while the correlations from δ_H 1.46 (2H, m, H-4, Hmp) and 1.19-1.18 (2H, m, H-4, Hmp) methylene protons to δ_H 0.92 (6H, t, *J* = 7.5 Hz, H-5, Hmp) indicated ethyl group on Hmp unit. The COSY spectrum also exhibited the correlations from H-2 to H-3 of each residue: from δ_H 5.28-5.27 (2H, m, H-2, Hmp) to δ_H 2.02 (2H, m, H-3, Hmp), from δ_H 5.15 (1H, d, *J* = 8.2 Hz, H-2, Hiv) to δ_H 2.29 (1H, m, H-3, Hiv), and from δ_H 4.56-4.55 (3H, m, H-2, *N*MeVal) to δ_H 2.30-2.28 (3H, m, H-3, *N*MeVal).

HMBC correlations for *N*MeVal residues are H-2 (δ_H 4.56-4.55) to C-3 (δ_C 27.9 × 2, 27.8), C-4 (δ_C 20.3 × 3), C-4′ (δ_C 19.2, 19.3, 19.4), N-*C*H$_3$ (δ_C 32.7, 32.9 × 2 and two carbonyl signals at δ_C 169.2 and 170.3, from protons in N-C*H*$_3$ group (δ_H 3.09, 3.11, 3.12) to C-2 (δ_C 63.1 × 3), and carbonyl signal at δ_C 169.2, from H-3 (δ_H 2.30-2.28) to C-2 (δ_C 63.1 × 3), C-4 (δ_C 20.3 × 3), and C-4′ (δ_C 19.2, 19.3, 19.4), and from both H-4 (δ_H 1.06) and H-4′ (δ_H 0.89) to C-2 (δ_C 63.1 × 3), and C-3 (δ_C 27.9 × 2, 27.8). The NOESY spectral data further supported these assignments for the three *N*MeVal residues: from H-2 (δ_H 4.56-4.55) to H-3 (δ_H 2.30-2.28), H-4 (δ_H 1.06), H-4′ (δ_H 0.89), N-C*H*$_3$ (δ_H 3.12, 3.11, 3.09), from H-3 (δ_H 2.30-2.28) to H-4 (δ_H 1.06), H-4′ (δ_H 0.89), from N-C*H*$_3$ (δ_H 3.12, 3.11, 3.09) to H-4 (δ_H 1.06), H-4′ (δ_H 0.89).

HMBC correlations for Hiv residues are H-4 (δ_H 0.98) to C-2 (δ_C 75.7), C-3 (δ_C 29.9), C-4′ (δ_C 18.5), and from H-4′ (δ_H 0.95) to C-2 (δ_C 75.7), C-3 (δ_C 29.9), C-4 (δ_C 18.6). The NOESY spectral data further supported these assignments for the Hiv residue: from H-2 (δ_H 5.15) to H-3 (δ_H 2.29), H-4 (δ_H 0.98), H-4′ (δ_H 0.95), N-CH_3 (δ_H 3.12, 3.11, 3.09), from H-3 (δ_H 2.29) to H-4 (δ_H 0.98), H-4′ (δ_H 0.95).

HMBC correlations for Hmp residues are H-3 (δ_H 2.02) to 3-CH$_3$ (δ_C 14.6 × 2), from H-4a (δ_H 1.46) to C-2 (δ_C 74.4, 74.2), C-3 (δ_C 36.1 × 2), C-5 (δ_C 11.3 × 2), 3-CH$_3$ (δ_C 14.6 × 2), from H-4b (δ_H 1.19-1.18) to C-3 (δ_C 36.1 × 2), C-5 (δ_C 11.3 × 2), 3-CH$_3$ (δ_C 14.6 × 2), and from protons of 3-CH_3 (δ_H 0.96) to C-2 (δ_C 74.4, 74.2), C-3 (δ_C 36.1 × 2), C-4 (δ_C 25.3 × 2), and from H-5 (δ_H 0.92) to C-3 (δ_C 36.1 × 2), C-4 (δ_C 25.3 × 2). The NOESY spectral data further supported these assignments for the two Hmp residues: from H-2 (δ_H 5.28-5.27) to H-3 (δ_H 2.02), H-4a (δ_H 1.46), H-4b (δ_H 1.19-1.18), H-5 (δ_H 0.92), 3-CH_3 (δ_H 0.96), N-CH_3 (δ_H 3.12, 3.11, 3.09), from H-3 (δ_H 2.02) to H-4a (δ_H 1.46), H-5 (δ_H 0.92), 3-CH_3 (δ_H 0.96), from H-4a (δ_H 1.46) to H-4b (δ_H 1.19-1.18), H-5 (δ_H 0.92), 3-CH_3 (δ_H 0.96), from H-4b (δ_H 1.19-1.18) to H-4a (δ_H 1.46), H-5 (δ_H 0.92), 3-CH_3 (δ_H 0.96).

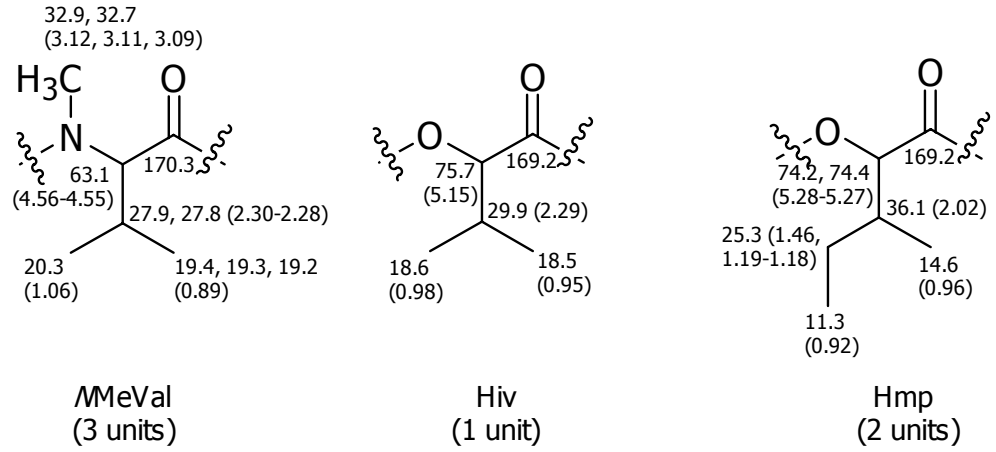


Figure 19. Partial structures and NMR assignments for compound **58**

Analysis of NOESY spectral data also revealed the connectivity of six residues, three *N*MeVal and three 2-hydroxycarboxylic acid. Thus, intense

correlations were observed for the three *N*-methyl singlet signals at δ_H 3.12, 3.11 and 3.09 respectively with the α-protons (H-2) of the 2-hydroxycarboxylic acid residues at δ_H 5.28-5.27 (Hmp), and 5.15 (Hiv), which clearly indicated that three *N*MeVal residues are linked alternately with the three 2-hydroxycarboxylic acid residues.

The relative stereochemistries at α-carbons of *N*MeVal, Hmp, and Hiv residues were confirmed by NOESY spectral analysis (Figure 13).

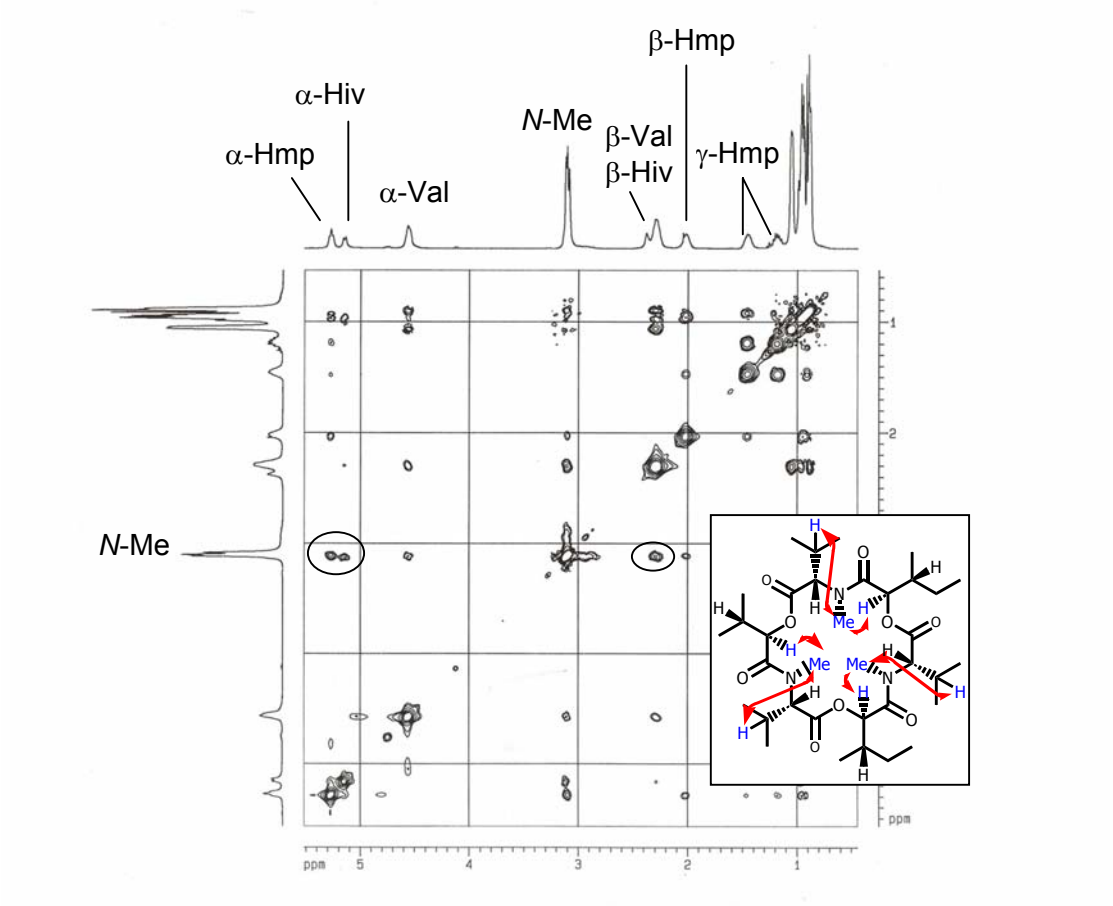


Figure 20. NOESY spectrum of compound **58**

NOESY correlations from *N*-methyl protons of *N*MeVal residues to α-protons of Hmp and Hiv residues revealed that these protons are both directed to the same side and vertically oriented to the depsipeptide ring. On the other hand, weak NOE between the N-methyl protons and the α-protons of *N*MeVal residues indicated that they are placed opposite to the macrocyclic ring. Therefore, the structure of compound **58** was established as depicted on the top of this subsection. The absolute

configuration of the β-carbon (C-3) of the two Hmp units was determined to be (3*S*) by precursor-directed biosynthesis, as described in the following section.

Compound **58**, named enniatin I, is also an unusual new enniatin analog.

Table 11. NMR data for compound **58** in $CDCl_3$

position	δ_C	δ_H (mult., *J* in Hz)	HMBC (H to C)
***N*MeVal**	(3 units)		
1 *C*=O	170.3×3	-	-
2	63.1×3	4.56-4.55 (3H, m)	1, 3, 4, 4′, δ_C 169.2, N-*C*H_3
3	27.9×2, 27.8	2.30-2.28 (3H, m)[a]	2, 4, 4′
4	20.3×3	1.06 (9H, m)	2, 3, 4′
4′	19.4, 19.3, 19.2	0.89 (9H, m)	2, 3, 4
N-*C*H_3	32.9×2, 32.7	3.12 (3H, s)	1, δ_C 169.2
		3.11 (3H, s)	1, δ_C 169.2
		3.09 (3H, s)	1, δ_C 169.2
Hiv	(1 unit)		
1 *C*=O	169.2 [c]	-	-
2	75.7	5.15 (1H, d, 8.2)	-
3	29.9	2.29 (1H, m) [a]	4, 4′
4	18.6 [d]	0.98 (3H, m) [b]	2, 3, 4′
4′	18.5 [d]	0.95 (3H, m) [b]	2, 3, 4
Hmp	(2 units)		
1 *C*=O	169.2 ×2 [c]	-	-
2	74.4, 74.2	5.28-5.27 (2H, m)	-
3	36.1×2	2.02 (2H, m)	-
4	25.3×2	1.46 (2H, m)	3, 5, 3-*C*H_3
		1.19-1.18 (2H, m)	-
5	11.3×2	0.92 (6H, t, 7.5)	3, 4

3-CH_3	14.6×2	0.96 (6H, m) [b]	2, 3, 4

[a,b] ^{1}H signals are overlapping.

[c] The carbon signals are superimposed.

[d] Assignments can be interchanged.

3.3 Studies on precursor-directed biosynthesis using *Verticillium hemipterigenum* BCC 1449

Enniatin synthetase, a multifunctional enzyme catalyzing enniatin biosynthesis, has previously been isolated from *Fusarium oxysporum* (Zocher *et at*., 1982) and the cell-free synthesis of enniatins has also been reported (Zocher *et al*., 1976, 1978, 1982; Pieper *et al*., 1992). It is known that the biosynthetic precursor for L-*N*MeVal residues of the major metabolite enniatin B (compound **51**) is L-valine, which was elucidated by the uptake of radio-active substrate as reported in the literature (Lee *et al*., 1992). It is also claimed that the D-2-hydroxyisovaleric acid (D-Hiv) residues are derived from L-valine via 2-ketoisovalerate (Figure 14).

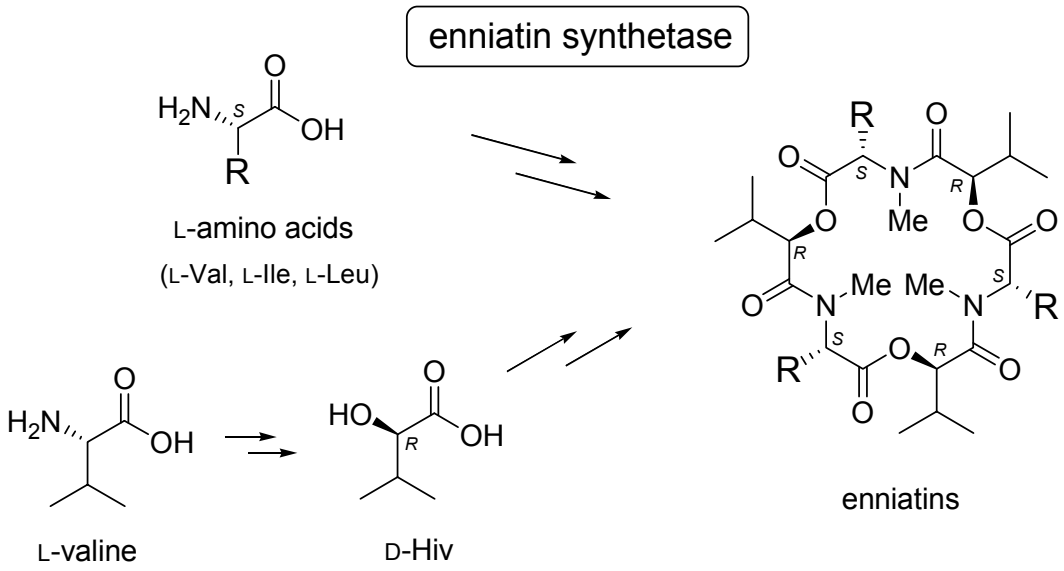


Figure 21. Biosynthesis of enniatins

Enniatin H (**57**) and enniatin I (**58**), found in this study, possess one and two 2-hydroxy-3-methylpentanoic acid (Hmp) moiety instead of Hiv. It suggested that the nature of enniatin synthetase of the fungus *V. hemipterigenum* BCC 1449 might be different from those of previously investigated enniatins-producing *Fusarium* species, especially at the region associated with Hiv substrate recognition. Because the NMR data did not provide enough information to determine the stereochemistry at C-3 of Hmp unit of two new enniatins, it was planed to undertake directed biosynthesis using L-leucine and L-isoleucine as precursors. By this method, the feeding of the substrate analog might provide the incorporation of these mimics at L-*N*-methylamino acid or D-2-hydroxycarboxylic acid residues in the enniatin molecules.

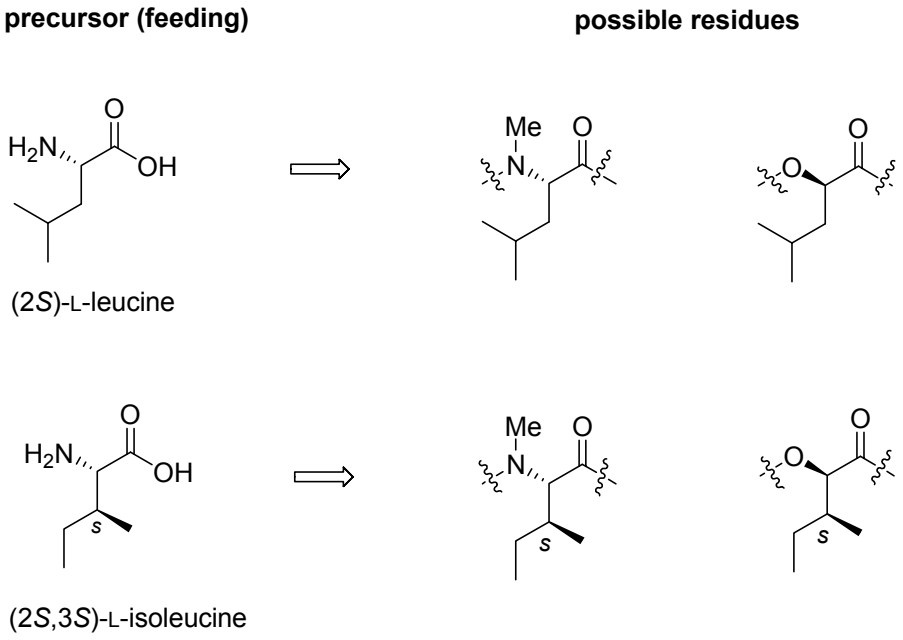


Figure 22. Precursors and possible residues in enniatins

3.3.1 Precursor-directed biosynthesis

A feeding experiment with 20 mM of L-leucine (fermentation: 4 × 1L Erlenmeyer flasks, each containing 250 mL of potato dextrose broth) led to the enhanced production of enniatins as compared to controlled fermentation (no additive) (Figure 16). HPLC/UV analysis (ODS column: MeCN/H_2O = 70:30; detection at 210 nm) of the extract from culture filtrate showed that enniatin B_4 (**56**) and a new analog (**59**), corresponding to the peak at t_R 20 min, were produced in higher amounts relative to enniatin B (**55**). In addition, HPLC peaks due to several other minor isomers were observed. It should also be noted that the total amount of enniatins in the L-leucine-fed culture, 16 mg of total enniatins per 1 L culture broth (calculated using an internal standard), was higher than that of the control (5 mg per 1 L culture broth). Similar results were observed for the analysis of the extract from mycelia (Figure 17).

The extracts from filtrate and mycelia were combined and subjected to chromatographic separation. Compounds **55**, **56**, **59** and a minor product corresponding to the HPLC peak at t_R 25 min (**60**) were isolated (see experimental section). Although the HPLC retention time (in MeCN/H_2O) of compound **59** was very close to **57**, the preparative HPLC fraction corresponding to this peak contained mainly compound **59** and a trace amount of **57** which was removed by subsequent re-chromatography employing MeOH/H_2O as the solvent system. Spectral data for enniatins B (**55**) and B_4 (**56**) obtained from this feeding experiment were identical to those obtained from non-additive fermentation.

NMR analysis of another major product, **59**, having a molecular formula of $C_{35}H_{61}N_3O_9$ (HRMS, ^{13}C NMR), revealed that this molecule consists of one *N*MeVal, two *N*MeLeu and three Hiv residues, hence, the structure was identical to enniatin G which was isolated from the mangrove fungus *Halosarpheia* sp. (strain 732) (Lin *et al*., 2002). The minor product, **60**, exhibited a C_3-symmetric structure as indicated by its molecular formula ($C_{36}H_{63}N_3O_9$, HRMS) and ^1H and ^{13}C NMR spectra. Analysis of the 2D-NMR spectral data revealed that this compound consists of *N*MeLeu and Hiv residues, therefore, it is identical to enniatin C which is a

synthetically known compound but not a naturally occurring enniatin analog (Ovchinnikov, 1964)

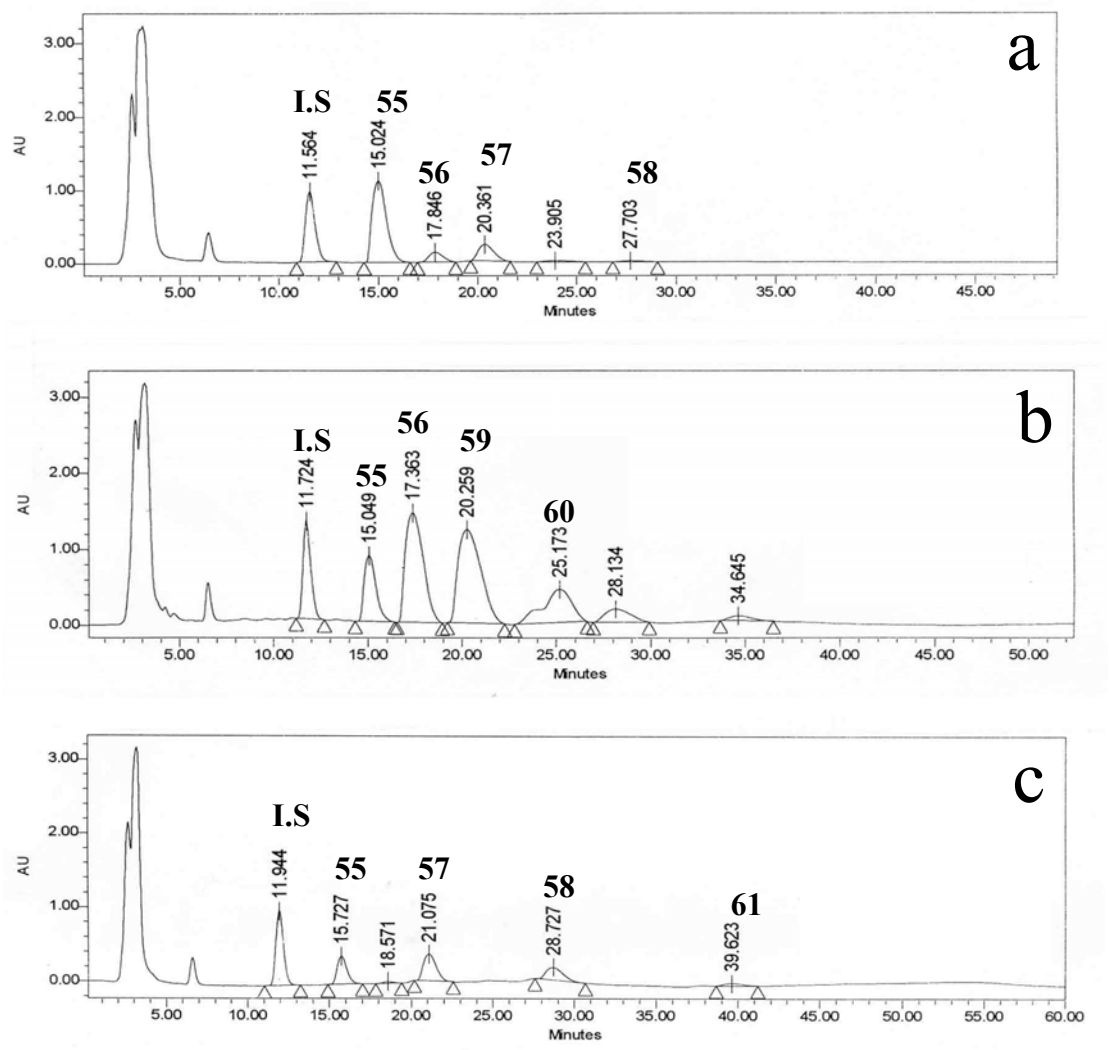


Figure 23. HPLC chromatogram of the EtOAc extracts from culture filtrate (detection at 210 nm): (**a**) control (non-additive); (**b**) L-leucine fed (20 mM); (**c**) L-Isoleucine fed (20 mM). Internal standard (I.S.): ethyl 4-phenylbenzoate (0.50 mg)

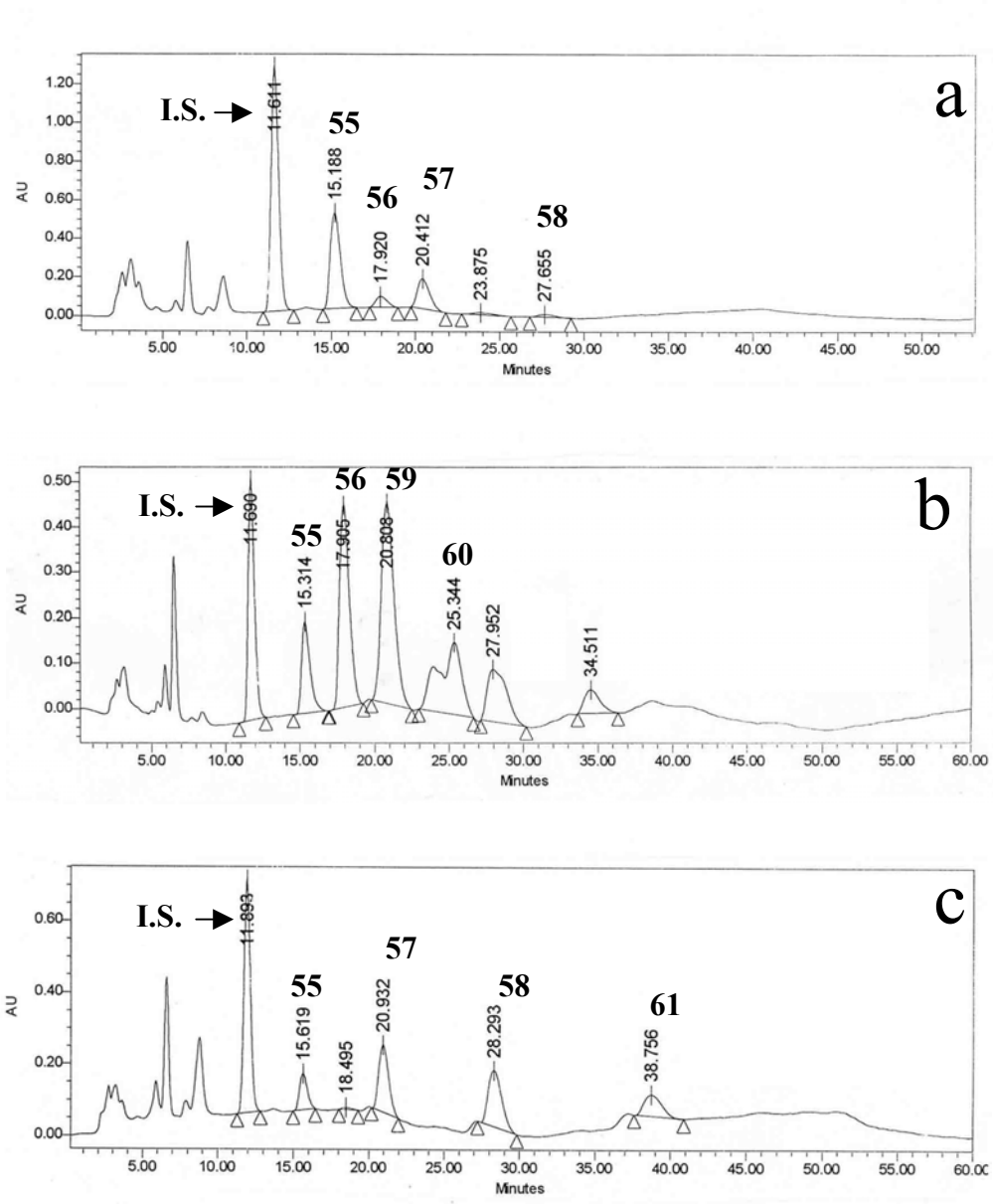
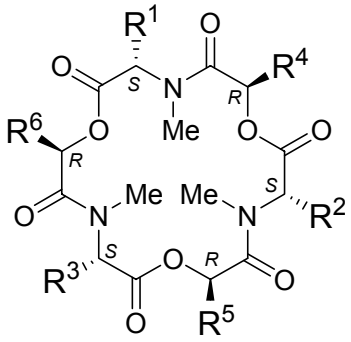


Figure 24. HPLC chromatogram of the MeOH extracts from mycelia (detection at 210 nm): (**a**) control (non-additive); (**b**) L-leucine fed (20 mM); (**c**) L-Isoleucine fed (20 mM). Internal standard (I.S.): ethyl 4-phenylbenzoate (0.50 mg)

A feeding experiment with L-isoleucine (20 mM) gave dramatically different results in which enhanced production of enniatins H (**57**) and I (**58**), and the appearance of another derivative, **61**, at t_R 40 min, were observed by HPLC/UV analysis (Figure 16). Due to the small amounts of enniatin products, a 10 Litre fermentation (250 mL × 40 flasks) was conducted from which four compounds, **55**,

57, **58**, and **61**, were isolated. Spectral data of **55**, **57** and **58**, obtained from the L-isoleucine-fed culture were identical in all respects to those from non-additive fermentation. The newly produced analog, **61**, molecular formula $C_{36}H_{63}N_3O_9$ (HRMS), possessed a C_3-symmetric structure as indicated by its NMR spectra. NMR analyses also revealed that this compound bears three *N*MeVal and three Hmp residues. Results from L-isoleucine-feeding experiments also confirmed the (3*S*)-configuration at the β-position of Hmp residues in the naturally occurring enniatins H (**57**) and I (**58**), and the missing analog, **61**. A related compound MK1688, obtained from *Fusarium oxysporum* D338, was claimed as an antifungal substance in a Japanese patent (Mikawa *et al*., 1991) although its stereochemistries at the Hmp residues have not been presented. By comparison of ^{1}H NMR (taken in methanol-d_6) and IR spectrum, and optical rotation data of **61**, with those of MK1688 in the patent, it was concluded that they are the same compound. Therefore, MK1688 (**61**) possesses (2*R*,3*S*)-configuration at the Hmp residues.



	R^1	R^2	R^3	R^4	R^5	R^6
enniatin B (**55**)	i-Pr	i-Pr	i-Pr	i-Pr	i-Pr	i-Pr
enniatin B_4 (**56**)	**i-Bu**	i-Pr	i-Pr	i-Pr	i-Pr	i-Pr
enniatin G (**59**)	**i-Bu**	**i-Bu**	i-Pr	i-Pr	i-Pr	i-Pr
enniatin C (**60**)	**i-Bu**	**i-Bu**	**i-Bu**	i-Pr	i-Pr	i-Pr
enniatin H (**57**)	i-Pr	i-Pr	i-Pr	***s*-Bu**	i-Pr	i-Pr
enniatin I (**58**)	i-Pr	i-Pr	i-Pr	***s*-Bu**	***s*-Bu**	i-Pr
MK1688 (**61**)	i-Pr	i-Pr	i-Pr	***s*-Bu**	***s*-Bu**	***s*-Bu**

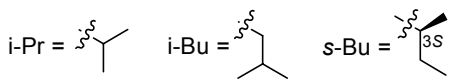


Figure 25. Enniatins from precursor-directed biosynthesis

Table 12. Enniatin composition in extracts from precursor-feeding experiments

feeding	extract	enniatin composition: mg per (1 Litre fermentation)[a]								**Total**
		B	**B_4**	**G**	**C**	**H**	**I**	**MK**	others[b]	(mg)
control	filtrate	3.25	0.38	-	-	0.71	0.05	-	0.08	4.5
	mycelia	0.99	0.13	-	-	0.37	0.05	-	0.04	1.8
+ L-Leu	filtrate	1.93	4.86	5.30	1.97	[c]	[d]	-	1.55	15.6
	mycelia	0.96	2.48	3.21	1.32	[c]	[d]	-	2.46	10.4
+ L-Ile	filtrate	0.89	0.06	-	-	1.11	0.71	0.14	0.00	2.9
	mycelia	0.36	0.03	-	-	0.83	0.94	0.47	0.00	2.6

[a] Amount of each compound was determined by HPLC analysis using an internal standard (ethyl 4-phenylbenzoate, 0.50 mg).

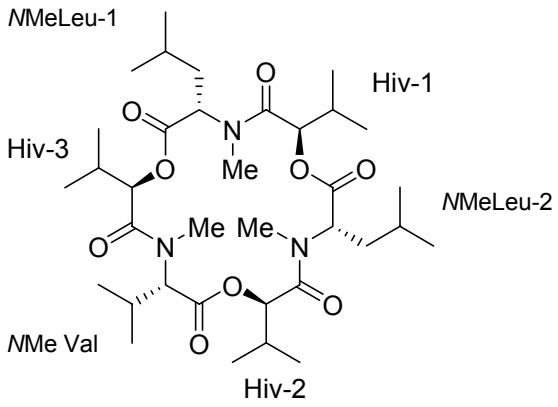
[b] Other unidentified minor enniatin isomers (combined amount).

[c] Peak of enniatin H (minor) was overlapped with that of enniatin C (major).

[d] Peak of enniatin I was overlapped with those of unidentified minor isomers.

The present results are of particular interest concerning specificity of the substrate recognition domain of the enzyme enniatin synthetase in strain *V. hemipterigenum* BCC 1449. It is evident that the enzyme favors L-leucine over L-isoleucine as a substrate of the L-*N*-methylamino acid residue in enniatin biosynthesis. In contrast, the domain which recognizes 2-hydroxycarboxylic acid substrates readily accepts Hmp, derived from L-isoleucine, as indicated by the enhancement of production of enniatins H (**57**), I (**58**) and MK1688 (**61**) in the feeding experiment as well as by the production of **57** and **58** even in the standard fermentation. It should be noted that this observation is of marked contrast to the study of substrate specificity in the precursor-directed biosynthesis of *Fusarium* spp, recently reported by Zocher's group (Krause *et al*., 2001).

3.3.2 Structure elucidation of compound 59 (enniatin G)



Compound **59** was obtained as a colorless solid; mp 143-145 °C, $[\alpha]^{26}_{D}$ – 75 (*c* 0.21, $CHCl_3$). It possesses the molecular formula $C_{35}H_{61}N_3O_9$, as determined by the HRMS (ESI-TOF) *m/z* 690.4301 $[M + Na]^+$ (calcd for $C_{35}H_{61}N_3O_9Na$, 690.4306, Δ = 0.5 mmu). The UV spectrum of **59** showed an intense absorption at λ_{max} (log ε) 206(4.28) nm and its IR spectrum showed the absorption bands at ν_{max} 2964, 1749, 1740, 1655, 1471, 1269, 1204 and 1016 cm^{-1}, which revealed that compound **59** is also an enniatin analog.

The ^1H NMR spectrum of compound **59** (in $CDCl_3$, 400 MHz) was similar to that of **56** (enniatin B_4) which showed: signals of two double doublet at δ_H 5.26 (1H, dd, *J* = 10.8, 4.4 Hz, H–2, *N*MeLeu), 4.84 (1H, dd, *J* = 10.3, 5.1 Hz, H–2, *N*MeLeu), four doublet signals at δ_H 5.05 (1H, d, *J* = 8.8 Hz, H–2, Hiv), 5.02 (1H, d, *J* = 8.8 Hz, H–2, Hiv), 4.97 (1H, d, *J* = 8.8 Hz, H–2, Hiv), 4.92 (1H, d, *J* = 10.1 Hz, H–2, *N*MeVal), two singlets at δ_H 3.15 (3H, s, N-C*H*$_3$), 3.13 (6H, s, 2 × N-C*H*$_3$), six multiplets at δ_H 2.31-2.18 (3H, m, H–3, Hiv), 2.20 (1H, m, H-3, *N*MeVal), 1.79-1.74 (2H, m, H-3a, *N*MeLeu), 1.73-1.65 (2H, m, H-3b, *N*MeLeu), 1.53 (1H, m, H-4, *N*MeLeu), 1.47 (1H, m, H-4, *N*MeLeu), a set of doublets and multiplets at δ_H 0.90-1.04 (total 36H, 12 methyl groups).

Analysis of ^{13}C NMR, DEPTs and HMQC spectral data of compound **59** led to the categorization of carbons: fifteen methyl (twelve C-*C*H$_3$ and three N-*C*H$_3$), two methylene (δ_C 37.6 and 37.8), twelve methine and six quaternary carbons. The downfield carbon signals at δ_C 169.6, 169.8 and 169.9 confirmed the presence of

amides and the signals at δ_C 170.6, and 171.0 (two carbons) indicated the esters moieties. Three methine carbons at δ_C 54.6, 56.0 and 61.3 were assignable to α-position (C-2) of the *N*-methylamino acid residues, while three methine carbons at δ_C 75.0 and 75.8 (two carbons) were α-position (C-2) of the Hiv residues.

The COSY spectrum showed correlations from δ_H 5.26 (1H, dd) and 4.84 (1H, dd) protons to δ_H 1.79-1.74 methylene (2H, m), and δ_H 1.73-1.65 methylene (2H, m). These methylene protons coupled with δ_H 1.53 (1H, m) and 1.47 (1H, m) methine protons, respectively, and these two methine protons correlated with two methyls, each, situated at δ_H 0.93-0.91 and 0.95-0.94. These data indicated the presence of two *N*-methylleucine (*N*MeLeu) residues. COSY correlations from three α-protons at δ_H 5.05 (1H, d), 5.02 (1H, d) and 4.97 (1H, d) to δ_H 2.33-2.21 methines (3H, m) and one α-proton at δ_H 4.92 (1H, d) to δ_H 2.20 methine (1H, m) were observed. These four methine protons showed COSY cross signals to methyl protons situated δ_H 0.90-1.04 which indicated the presence of three Hiv and one *N*MeVal residues.

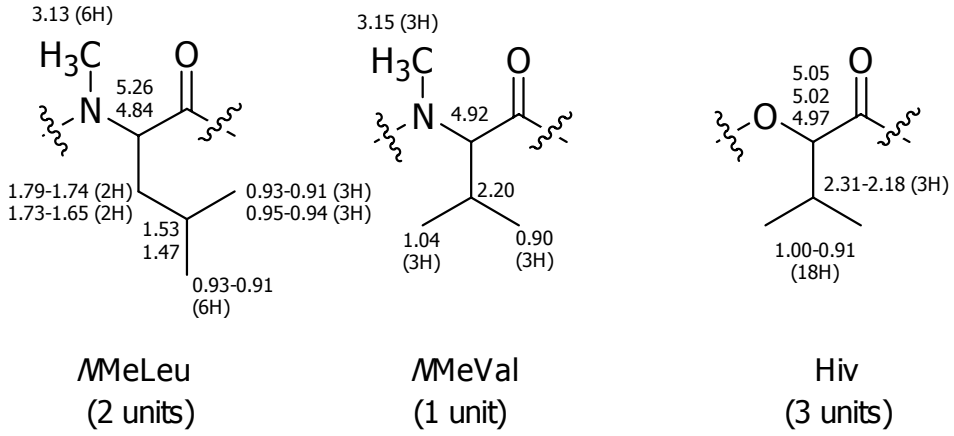


Figure 26. Proton assignments for six residues

On the basis of these spectroscopic data, it was concluded that compound **59** composed of two units of *N*MeVal, one unit of *N*MeLeu and three units of Hiv. Three *N*-methylamino acid units and three Hiv units alternately linked, in the same pattern as other enniatins, to form cyclohexadepsipeptide structure as depicted previously in this subsection.

Compound **59** is identical to enniatin G, which was previously isolated from the mangrove fungus *Halosarpheia* sp. (strain 732) collected from the South China Sea (Lin, *et al*., 2002).

Table 13. NMR data for compound **59** (enniatin G) in $CDCl_3$

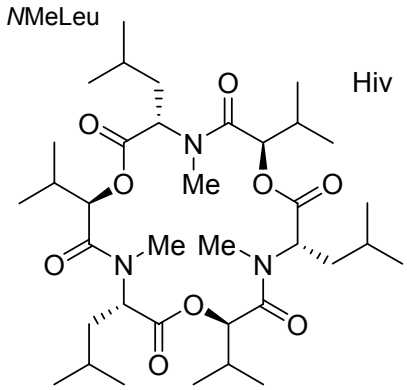
position	^{13}C	^{1}H (mult, *J* in Hz)	HMBC (H to C)
Hiv	(3 units)		
1 *C*=O	169.6, 169.8, 169.9	-	-
2	75.0	5.05 (1H, d, 8.8)	1, 3, 4, 4′, δ_c 171.0
	75.8	5.02 (1H, d, 8.8)	1, 3, 4, 4′, δ_c 170.6
	75.8	4.97 (1H, d, 8.8)	1, 3, 4, 4′, δ_c 171.0
3	29.7, 30.0, 30.1	2.31-2.18 (3H, m) [a]	1, 2, 4, 4′
4	18.1	0.93-0.91 (3H, m) [b]	2, 3
	18.3	0.95-0.94 (3H, m) [c]	3
	18.2	0.95-0.94 (3H, m) [c]	3
4′	18.55	0.98 (3H, d, 6.6)	2, 3
	18.8	1.00 (3H, m)	2, 3
	18.6	0.996 (3H, d, 6.5)	2, 3
***N*MeVal**	(1 unit)		
1 *C*=O	170.6	-	-
2	61.3	4.92 (1H, d, 10.1)	1, 3, 4, 4′, δ_c 169.9
3	27.8	2.20 (1H, m)[a]	1, 2, 4, 4′
4	19.5	0.90 (3H, m)	2, 3, 4′
4′	20.0	1.04 (3H, d, 6.6)	2, 3, 4
N-CH_3	32.0	3.15 (3H, s)	1, 2, δ_c 169.9

Table 13. NMR data for compound **59** (enniatin G) in $CDCl_3$ (continued)

position	^{13}C	^{1}H (mult, *J* in Hz)	HMBC (H to C)
***N*MeLeu**	(2 units)		
1 *C*=O	171.0×2	-	-
2	54.6	5.26 (1H, dd, 10.8, 4.4)	3, δ_c 169.8
	56.0	4.84 (1H, dd, 10.3, 5.1)	3, δ_c 169.6
3	37.6	1.73-1.65 (2H, m)	1, 2, 4, 5, 5′
	37.8	1.79-1.74 (2H, m)	1, 2, 4, 5, 5′
4	25.1	1.53 (1H, m)	3, 5, 5′
	25.2	1.47 (1H, m)	3, 5, 5′
5	21.3×2	0.93-0.91 (6H, m)[b]	3, 4
5′	23.30	0.93-0.91 (3H, m)[b]	3, 4, 5′
	23.35	0.95-0.94 (3H, m)[c]	3, 4
N-CH_3	31.8, 32.0	3.13 (6H, s)	1, 2, δ_c 169.8

[a-c] The ^{1}H signals are overlapping.

3.3.3 Structure elucidation of compound 60 (enniatin C)



Compound **60** was obtained as a colorless solid; mp 159-160 °C, $[\alpha]^{27}_{D}$ – 47 (*c* 0.11, $CHCl_3$). It possesses the molecular formula $C_{36}H_{63}N_3O_9$, as determined by the HRMS (ESI-TOF) *m/z* 704.4443 $[M + Na]^+$ (calcd for $C_{36}H_{63}N_3O_9Na$, 704.4462, Δ = 1.9 mmu), which is 42 unit mass more than enniatin B (**55**). The UV spectrum of **60** showed an intense absorption at λ_{max} (log ε) 205 (4.23) nm and its IR spectrum showed the absorption bands at ν_{max} 2964, 1748, 1659, 1471, 1268, 1204 and 1014 cm^{-1}, which revealed that compound **60** was also an enniatin analog.

The ^1H NMR spectrum of compound **60** (in $CDCl_3$, 400 MHz) also indicated that it was a C_3-symmetric enniatin analog, showing signals of twenty-one protons: a broad doublet at δ_H 5.33 (1H, brd, *J* = 7.1 Hz), a doublet at δ_H 4.91 (1H, d, *J* = 8.2 Hz, H–2), one singlet at δ_H 3.10 (3H, s, N-CH_3), four multiplet at δ_H 2.20 (1H, m), 1.73 (1H, m), 1.66 (1H, m) and 1.45 (1H, m), and four doublets at δ_H 1.01 (3H, d, *J* = 6.4 Hz), 0.95 (3H, d, *J* = 6.5 Hz), 0.94 (3H, d, *J* = 6.7 Hz), and 0.91 (3H, d, *J* = 6.5 Hz). The ^{13}C NMR spectrum of **60** (in $CDCl_3$, 100 MHz) exhibited 12 signals. The HMQC spectral data revealed categorization of carbons: five methyl, one methylene, four methine and two quaternary carbons.

The COSY correlations from δ_H 4.91 proton (1H, d) to δ_H 2.20 methine (1H, m), and the correlation from this methine proton to methyl protons at δ_H 1.01 (3H, d) and 0.94 (3H, d), indicated the Hiv structure. Correlations from δ_H 5.33 proton (1H, brd) to δ_H 1.73 (1H, m) and 1.66 (1H, m) methylene, and from this methylene protons to δ_H 1.45 methine (1H, m) were observed. This methine proton attached to

two methyl groups situated at δ_H 0.95 (3H, d) and 0.91 (3H, d). Therefore, the other unit was assigned to *N*MeLeu.

HMBC correlations from α-proton at δ_H 4.91 (1H) to ester carbonyl carbon at δ_C 171.1, to methine carbon at δ_C 30.0, and to two methyl carbons at δ_C 18.6 and 18.0 were observed. Methine proton at δ_H 2.20 (1H) correlated to the methine carbon at δ_C 75.7 and two methyl carbons at δ_C 18.6 and 18.0. Methyl protons at δ_H 1.01 (3H) showed correlations to two methine carbons at δ_C 75.7, and 30.0, and to the methyl carbon at δ_C 18.0, while methyl protons at δ_H 0.94 (3H) correlated to methine carbons at δ_C 75.7, and 30.0, and to the methyl carbon at δ_C 18.6. These HMBC data confirmed the partial structural assignments for the Hiv residue.

Another set of HMBC correlation data confirmed the *N*MeLeu residue. Correlations from the amide *N*-methyl at δ_H 3.10 (3H, s) to methine carbon at δ_C 54.2 (C-2) and amide carbonyl carbon at δ_H 170.3, and to ester carbonyl carbon at δ_H 171.1 were observed. The methylene protons at δ_H 1.73 and 1.66 (H-3) correlated to the ester carbonyl (δ_C 171.1), methine carbons at δ_C 54.2 (C-2) and 25.3 (C-4), and methyl carbons at δ_C 23.4 and 20.9. The methine proton at δ_H 1.45 (H-4) showed correlations to methyl carbon at δ_C 23.4 (C-5). Methyl protons at δ_H 0.95 (3H, H-5) exhibited correlations to methylene carbon at δ_C 37.3 (C-3), methine carbon (δ_C 25.3, C-4) and another methyl (δ_C 20.9, C-5′), while the methyl proton at δ_H 0.91 (3H, H-5′) correlated to C-3 (δ_C 37.3), C-4 (δ_C 25.3) and C-5 (δ_C 23.4).

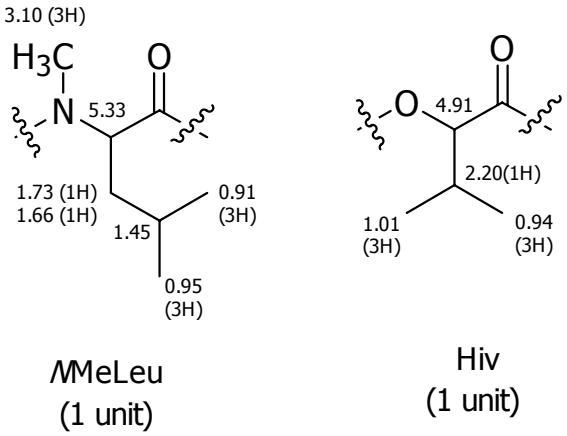


Figure 27. Proton assignments for two residues

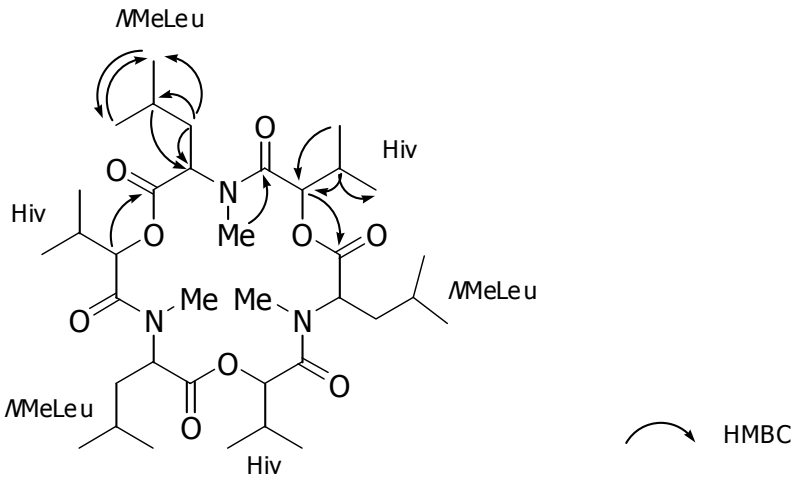


Figure 28. The gross structure of compound **60**

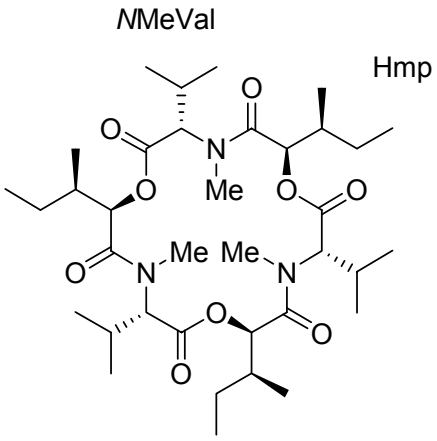
The relative stereochemistry at α-carbons of *N*MeLeu and Hiv residues was confirmed by NOESY correlations. Intense NOESY correlations from *N*-methyl protons of *N*MeLeu residues to α-protons of Hiv residues were observed, which strongly suggested that these protons are both situated on the same side and vertically oriented to the depsipeptide ring. In contrast, the NOESY cross signal from the N-methyl to α-protons of *N*MeLeu was much weaker, hence, the opposite orientation on the macrocyclic ring. These data were similar to other enniatins. By comparison with the co-metabolites (e.g., enniatin B), the absolute configuration of compound **60** should be 2*R* for Hiv, and 2*S* for *N*MeLeu.

The structure of compound **60** is identical to enniatin C, which was previously reported as a synthetic compound (Ovchinnikov, 1964) but not known as a naturally occurring enniatin analog.

Table 14. NMR data for compound **60** (enniatin C) in $CDCl_3$

position	^{13}C	^{1}H (mult, *J* in Hz)	HMBC (H to C)
***N*MeLeu**	(3 units, symmetrical)		
1 *C*=O	171.1	-	-
2	54.2	5.33 (3H, brd, 7.1)	-
3	37.8	1.73 (3H, m)	1, 2, 4, 5, 5′
		1.66 (3H, m)	1, 2, 4, 5, 5′
4	25.3	1.45 (3H, m)	5
5	23.4	0.95 (9H, d, 6.5)	3, 4, 5′
5′	20.9	0.91 (9H, d, 6.5)	3, 4, 5
N-*CH*$_3$	31.4	3.10 (9H, s)	1, 2, δ_c170.3
Hiv	(3 units, symmetrical)		
1 *C*=O	170.3	-	-
2	75.7	4.91 (3H, d, 8.2)	3, 4, 4′, δ_c 171.1
3	30.0	2.20 (3H, m)	2, 4, 4′
4	18.6	1.01 (9H, d, 6.4)	2, 3, 4′
4′	18.0	0.94 (9H, d, 6.7)	2, 3, 4

3.3.4 Structure elucidation of compound 61 (MK1688)



Compound **61** was obtained as a colorless gum; $[\alpha]^{26}_{D}$ –89 (*c* 0.25, $CHCl_3$). It possesses the molecular formula $C_{36}H_{63}N_3O_9$, as determined by the HRMS *m/z* 704.4458 $[M + Na]^+$ (calcd for $C_{36}H_{63}N_3O_9Na$, 704.4462), which is the same mass as enniatin C (**60**) but 42 unit mass more than enniatin B (**55**). The UV spectrum of **61** showed an intense absorption at λ_{max} (log ε) 207 (4.17) nm, and its IR spectrum showed the absorption bands at ν_{max} 2970, 1737, 1662, 1465, 1191, and 1007 cm^{-1}, which revealed that compound **61** is also an enniatin analog.

The ^1H NMR spectrum of compound **61** (in $CDCl_3$, 400 MHz) showed signals of twenty-one protons, and the ^{13}C NMR spectrum (in $CDCl_3$, 100 MHz) exhibited 12 signals, indicated that this compound is C_3-symmetric. The ^1H NMR spectrum exhibited two downfield doublets at δ_H 5.28 (1H, brd, *J* = 5.6 Hz) and 4.59 (1H, brd, *J* = 9.4 Hz), one singlet at δ_H 3.10 (3H, s, N-CH_3), four multiplets at δ_H 2.29 (1H, m), 2.02 (1H, m), 1.45 (1H, m), 1.19 (1H, m), three doublets at δ_H 1.06 (3H, d, *J* = 6.3 Hz), 0.96 (3H, d, *J* = 6.4 Hz), 0.89 (3H, d, *J* = 6.7 Hz) and one triplet at δ_H 0.92 (3H, t, *J* = 7.4 Hz). Analysis of DEPT135 and HMQC spectra disclosed that five methyl, one methylene, four methine and two quarternary carbons were the same types as those of all carbons in compound **61** and also confirmed the existence of a methylene carbon of Hmp residue.

The COSY showed correlation from the δ_H 5.28 methine proton to δ_H 2.02 methine (1H, m, H-3), and that of this methine proton (H-3) to δ_H 0.96 methyl (3H, d,

3-CH_3), and δ_H 1.45 and 1.19 methylene protons (H-4). The data also indicated that the methylene (H-4) was attached to δ_H 0.92 methyl (3H, t, H-5). Therefore, the 2-hydroxycarboxylic acid unit was assigned as Hmp. The *N*-methylamino acid residue of **61** was found to be *N*MeVal based on following COSY correlation data: δ_H 4.59 methine proton (1H, brd, H-2) to methine proton at δ_H 2.29 (1H, m, H-3), H-3 to two methyl groups at δ_H 1.06 (3H, d, H-4), and 0.89 (3H, d, H-4′).

Analysis of HMBC correlation data confirmed Hmp and *N*MeVal partial structures. Correlations from proton at δ_H 1.45 (1H) to methine carbons at δ_C 74.3, and 36.2, to methyl carbons at δ_C 14.6, and 11.3 were observed. The methyl protons at δ_H 0.96 (3H) correlated to methine carbons at δ_C 74.3, and 36.2, and to methylene carbon at δ_C 25.4, while methyl protons at δ_H 0.92 (3H) correlated to the amide carbonyl (δ_C 169.2), methine carbon at δ_C 36.2 (C-3), and methylene carbon at δ_C 25.4. These HMBC data confirmed the presence of Hmp residue. HMBC correlations from *N*-methyl (δ_H 3.10) to the amide carbonyl (δ_C 169.2) and ester carbonyl (δ_C 170.4) were observed. The methyl protons at δ_H 1.06 (H-4) showed correlations to methine carbons at δ_C 63.1 (C-2) and 27.8 (C-3), and methyl proton at δ_C 19.3, while the methyl protons at δ_H 0.89 (H-4′) correlated to methine carbons at δ_C 63.1 (C-2) and 27.8 (C-3), and to methyl carbon at δ_C 20.3 (C-4). These HMBC data confirmed the assignment of the *N*MeVal residue.

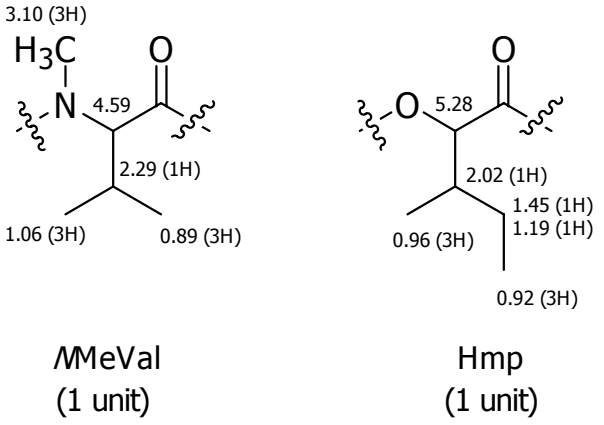


Figure 29. Proton assignments for two residues

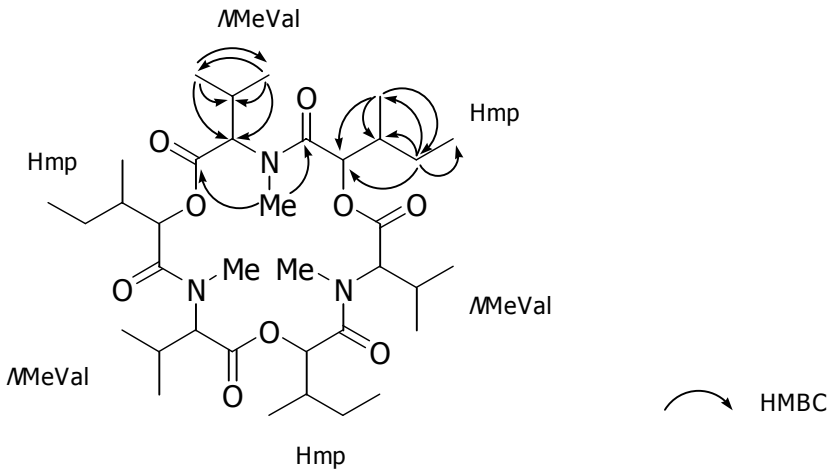


Figure 30. The gross structure of compound **61**.

The relative stereochemistry at α-carbons of *N*MeVal and Hiv residues was confirmed by NOESY correlation data. Intense NOESY correlations from *N*-methyl protons of *N*MeVal residues to α-protons (H-2) of Hiv residues were observed, which strongly suggested that these protons are both directed to the same side and vertically oriented to the depsipeptide ring. In contrast, the NOESY cross signal from the N-methyl to α-protons (H-2) of *N*MeVal was much weaker, hence, the opposite orientation on the macrocyclic ring. These data were similar to other enniatins. By comparison with the co-metabolites (e.g., enniatin B), the absolute configuration of compound **61** should be 2*R* for Hiv, and 2*S* for *N*MeVal. As described earlier, the configuration at C-3 of Hmp should be identical to that of the precursor, L-isoleucine, therefore, 3*S*-configuration.

Spectral data of compound **61** were identical, in all respects, to those of the antifungal substance, MK1688, which is claimed in a Japanese patent (Mikawa *et al*., 1991).

Table 15. NMR data for compound **61** (MK 1688) in $CDCl_3$

position	^{13}C	^{1}H (mult, *J* in Hz)	HMBC (H to C)
***N*MeVal**	(3 units, symmetrical)		
1 *C*=O	170.4	-	-
2	63.1	4.59 (3H, brd, 9.4)	-
3	27.8	2.29 (3H, m)	-
4	20.3	1.06 (9H, d, 6.1)	2, 3, 4′
4′	19.3	0.89 (9H, d, 6.9)	2, 3, 4
N-CH_3	32.7	3.10 (9H, s)	1, δ_C169.2
Hmp	(3 units, symmetrical)		
1 *C*=O	169.2	-	-
2	74.3	5.28 (3H, brd, 5.6)	-
3	36.2	2.02 (3H, m)	-
4	25.4	1.45 (3H, m)	2, 3, 5, 3-CH_3
		1.19 (3H, m)	2, 3, 5, 3-CH_3
5	11.3	0.92 (9H, t, 7.4)	1, 3, 4
3-CH_3	14.6	0.96 (9H, d, 6.4)	2, 3, 4

3.4 Biological activities

Compounds **51**-**58**, isolated from the fungus *V. hemipterigenum* BCC 1449, and compounds **59**-**61**, obtained by precursor-directed biosynthesis, were tested for their activities against *Plasmodium falciparum* K1, and cytotoxic activity towards two cancer cell-lines (KB, BC-1) and Vero cells (Table 16). For enniatins, antitubercular activity against *Mycobacterium tuberculosis* H_{37}Ra were also tested.

The new diketopiperazine **51** was inactive against *P. falciparum*, and it was non-cytotoxic. In contrast, the tetrathio analog, **52**, exhibited cytotoxic activities. Pyrenosines A (**53**) and B (**54**) showed moderate antimalarial and cytotoxic activities. Enniatins **55-61** strongly inhibited the proliferation of the human malaria parasite (*P. falciparum* K1), they also exhibited inhibitory activity against the growth of mycobacteria (*M. tuberculosis* H37Ra). This is the first report on the in vitro activities of enniatins against *P. falciparum* and *M. tuberculosis*, although enniatin B was previously reported to be active against *M. paratuberculosis* and *M. phlei* (Plattner, et. al., 1948). It should also be commented that these enniatins also exhibited cytotoxic activities, but these were rather weak when compared to their antimalarial activities.

Table 16. Antiplasmodial, antimycobacterial, and cytotoxic activities of enniatins **55-61**

	anti-malaria[a]	anti-TB[b]	cytotoxicity[c] (IC_{50}, μg/mL)		
compound	(IC_{50}, μg/mL)	(MIC, μg/mL)	KB	BC-1	Vero
compound **51**	>20	n.t.[d]	>20	>20	>50
compound **52**	2.5	n.t.[d]	15	3.9	8.9
pyrenocine A (**53**)	7.1	n.t.[d]	3.2	1.2	1.3
pyrenocine B (**54**)	22	n.t.[d]	>20	4.3	7.2
enniatin B (**55**)	0.27	3.12	16	18	17
enniatin B_4 (**56**)	0.20	3.12	11	12	18
enniatin H (**57**)	1.9	6.25	>20	5.5	38
enniatin I (**58**)	0.24	6.25	>20	18	38
enniatin G (**59**)	0.46	6.25	>20	>20	45
enniatin C (**60**)	1.1	6.25	>20	>20	>50
compound **61**	0.22	1.56	11	8.1	1.4

[a] IC_{50} values of the standard antimalarial compounds, chloroquine diphosphate and artemisinin, were 0.16 and 0.0011 μg/mL, respectively.

[b] MIC value of the standard drug, isoniazide, was 0.050 μg/mL.

[c] IC_{50} values of the standard compound, ellipticine, were 0.46 μg/mL for KB cells, 0.60 μg/mL for BC-1 cells, and 1.0 μg/mL for Vero cells.

[d] Not tested.

Conclusion

Chemical investigation of the insect pathogenic fungus *Verticillium hemipterigenum* BCC 1449 resulted in the isolation and structural determination of eight compounds of three different chemical classes: two new epipolythiodiketopiperazines **51** and **52**; two pyrone derivatives, pyrenocines A (**53**) and B (**54**); four cyclohexadepsipeptides, enniatins B (**55**), B_4 (**56**), H (**57**) and I (**58**). Enniatins H (**57**) and I (**58**) are new "unusual" analogs, possessing one and two 2-hydroxy-3-methylpentanoic acid residues instead of 2-hydroxyisovaleric acid. These results demonstrate that the fungus *Verticillium hemipterigenum* is a unique source for bioactive metabolites.

Studies on precursor-directed biosynthesis using *V. hemipterigenum* BCC 1449 led to the discovery of a unique substrate specificity by enniatin synthetase of this fungus: L-leucine is selectively incorporated in enniatin molecule as *N*-methyl amino acid residues, in contrast, L-isoleucine is predominantly employed as 2-hydroxy acid residues via (2*R*,3*S*)-2-hydroxy-3-methylpentanoic acid (Hmp). The latter result was used to determine the stereochemistry of the new naturally occurring enniatins H (**57**) and I (**58**). Moreover, these preliminary results will be useful for application to systematic studies on the production of a series of "unnatural" natural analogs.