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Unusual enniatins produced by the insect pathogenic fungus *Verticillium hemipterigenum*: isolation and studies on precursor-directed biosynthesis

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Abstract—Two new enniatins H (**3**) and I (**4**), whose substituents on 2-hydroxycarboxylic acid moieties were different from those of known compounds, were isolated, together with known enniatins B (**1**) and B_4 (**2**), from the insect pathogenic fungus *Verticillium hemipterigenum* BCC 1449. Structures of these compounds were elucidated by spectroscopic means. Studies on precursor-directed biosynthesis with strain BCC 1449 led to the production and identification of three analogs, enniatins G (**5**), C (**6**) and MK1688 (**7**), as well as the stereochemical elucidation of **3** and **4**. Enniatins **1**–**7** were evaluated for their antiplasmodial and antimycobacterial activities.

1. Introduction

Enniatins are well-known cyclohexadepsipeptide antibiotics produced by various *Fusarium* species.[1] This class of compounds have been known to exhibit antibiotic,[1b,c,2] insecticidal,[1d,3] and phytotoxic[1e,4] activities, and also inhibit acyl-CoA: cholesterol acyltransferase (ACAT).[5] Enniatins consist of three each of D-2-hydroxyisovaleric acid (Hiv) and L-*N*-methylamino acid residues linked alternately to furnish an 18-membered cyclodepsipeptide structure. Several isomers of enniatins, e.g. enniatin B (**1**) (Fig. 1), have previously been isolated and their differences are the R^1, R^2 and R^3 substituents on the three L-*N*-methylamino acid residues: L-*N*-methylvaline (*N*MeVal), L-*N*-methylleucine (*N*MeLeu) or L-*N*-methylisoleucine (*N*MeIle).[1] However, all the naturally occurring enniatins reported in the literature have fixed substructure at the D-2-hydroxycarboxylic acid residues; R^4=R^5=R^6=*i*-Pr.

In our search for novel bioactive compounds from insect pathogenic fungi,[6] we came across an enniatin mixture as antimalarial constituents in the extract from *Verticillium hemipterigenum* BCC 1449. Together with known enniatins B (**1**) and B_4 (**2**; also reported as enniatin D),[1c,f] two new analogs, enniatins H (**3**) and I (**4**), which, respectively, bear one and two 2-hydroxy-3-methylpentanoic acid (Hmp) residues instead of Hiv, were subsequently isolated. We report herein the isolation and structural elucidation of these unusual enniatins, production of other analogs by precursor-directed biosynthesis employing the fungus BCC 1449, and the evaluation of their biological activities. During the late stage of our experimental works, Zocher's group reported a related study on the precursor-directed biosynthesis of unnatural enniatins using *Fusarium scirpi* (enniatin B producer) and *F. sambucinum* (enniatin A producer).[7]

2. Results and discussion

Enniatins **1**–**4** were isolated from the methanolic extract of mycelia of *V. hemipterigenum* BCC 1449. Structures of known enniatins B (**1**) and B_4 (**2**)[1f] were elucidated by spectroscopic analyses (NMR, MS, IR) with their physicochemical properties being identical to those reported in the literature in all respects. The IR spectrum of enniatin H (**3**), $C_{34}H_{59}N_3O_9$ as shown by HRMS, ^{1}H and ^{13}C NMR, was very similar to those of **1** and **2**, showing absorptions of esters (ν 1743 cm^{-1}) and amides (ν 1663 cm^{-1}). NMR analyses (^{1}H, ^{13}C, DEPTs, COSY, HMQC and HMBC; in $CDCl_3$) revealed that this compound consists of three *N*MeVal, two Hiv and one Hmp residues. Thus, in the ^{1}H NMR spectrum of enniatin H (**3**), protons of three *N*MeVal residues and two Hiv residues appeared as superimposed signals with the chemical shifts very close to those of

Keywords: *Verticillium hemipterigenum*; enniatin; insect pathogenic fungus.

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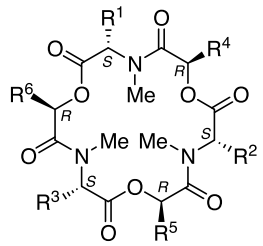

	R^1	R^2	R^3	R^4	R^5	R^6
enniatin B (**1**)	i-Pr	i-Pr	i-Pr	i-Pr	i-Pr	i-Pr
enniatin B_4 (**2**)	i-Bu	i-Pr	i-Pr	i-Pr	i-Pr	i-Pr
enniatin G (**5**)	i-Bu	i-Bu	i-Pr	i-Pr	i-Pr	i-Pr
enniatin C (**6**)	i-Bu	i-Bu	i-Bu	i-Pr	i-Pr	i-Pr
enniatin H (**3**)	i-Pr	i-Pr	i-Pr	*s*-Bu	i-Pr	i-Pr
enniatin I (**4**)	i-Pr	i-Pr	i-Pr	*s*-Bu	*s*-Bu	i-Pr
MK1688 (**7**)	i-Pr	i-Pr	i-Pr	*s*-Bu	*s*-Bu	*s*-Bu

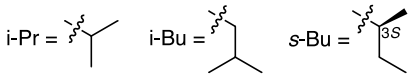

Figure 1. Structures of enniatins.

enniatin B (**1**). 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 Hmp, and this was consistent with HMBC correlations: H-2 to a carbonyl (δ_C 170.3), C-3 and 3-CH_3; and H-4 to C-2, C-5 and 3-CH_3. ^{1}H and ^{13}C NMR assignments of the three *N*MeVal residues for enniatin H 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–CH_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 1; full HMBC data are shown in Section 3). 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. 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 enniatin H structure bearing one *N*MeIle instead of *N*MeVal in **1**, thus enniatin B_1 (R^1=*s*-Bu, R^2=R^3=*i*-Pr; R^4=R^5=R^6=*i*-Pr), was clearly ruled out by these spectroscopic analyses. Furthermore, NMR spectral data (^{1}H and ^{13}C) of enniatin H in $CDCl_3$ were apparently different from those reported for enniatin B_1.[1g,5]

Enniatin I (**4**), molecular formula $C_{35}H_{61}N_3O_9$ (HRMS), showed IR and UV spectra similar to those of **3** and other

Table 1. NMR data for enniatins H and I and compound **7** in $CDCl_3$

Position	Enniatin H (**3**)		Enniatin I (**4**)		**7**	
	^{13}C	^{1}H (mult, J in Hz)	^{13}C	^{1}H (mult, J in Hz)	^{13}C	^{1}H (mult, J in Hz)
*N*MeVal	3 units		3 units		3 units, symmetrical	
1 *C*=O	170.3×3	–	170.3×3	–	170.4	–
2	63.3, 63.2, 63.1	4.57–4.55 (3H, m)	63.1×3	4.56–4.55 (3H, m)	63.1	4.59 (3H, brd, 9.4)
3	28.0, 27.9, 27.8	2.29–2.28 (3H, m)[a]	27.9×2, 27.8	2.30–2.28 (3H, m)[a]	27.8	2.29 (3H, m)
4	20.4, 20.3×2	1.06 (9H, m)	20.3×3	1.06 (9H, m)	20.3	1.06 (9H, d, 6.1)
4′	19.5, 19.4, 19.3	0.90–0.89 (9H, m)	19.4, 19.3, 19.2	0.89 (9H, m)	19.3	0.89 (9H, d, 6.9)
N–CH_3	33.1, 32.9×2	3.14 (3H, s)	32.9×2, 32.7	3.12 (3H, s)	32.7	3.10 (9H, s)
		3.13 (3H, s)		3.11 (3H, s)		
		3.11 (3H, s)		3.09 (3H, s)		
Hiv	2 units		1 unit			
1 *C*=O	169.3×2	–	169.2	–		
2	75.9, 75.6	5.15–5.13 (2H, m)	75.7	5.15 (1H, d, 8.2)		
3	29.9×2	2.28 (2H, m)[a]	29.9	2.29 (1H, m)[a]		
4	18.7[b], 18.6[b]	0.98 (6H, m)[a]	18.6[b]	0.98 (3H, m)[a]		
4′	18.5×2[b]	0.96 (6H, m)[b]	18.5[b]	0.95 (3H, m)[a]		
Hmp	1 unit		2 units		3 units, symmetrical	
1 *C*=O	169.3	–	169.2×2	–	169.2	–
2	74.3	5.27 (1H, d, 6.8)	74.4, 74.2	5.28–5.27 (2H, m)	74.3	5.28 (3H, brd, 5.6)
3	36.1	2.00 (1H, m)	36.1×2	2.02 (2H, m)	36.2	2.02 (3H, m)
4	25.4	1.46 (1H, m)	25.3×2	1.46 (2H, m)	25.4	1.45 (3H, m)
		1.19 (1H, m)		1.19–1.18 (2H, m)		1.19 (3H, m)
5	11.3	0.92 (3H, m)	11.3×2	0.92 (6H, t, 7.5)	11.3	0.92 (9H, t, 7.4)
3-CH_3	14.6	0.96 (3H, m)[a]	14.6×2	0.96 (6H, m)[a]	14.6	0.96 (9H, d, 6.4)

[a] ^{1}H signals are overlapping.
[b] Assignments can be interchanged.

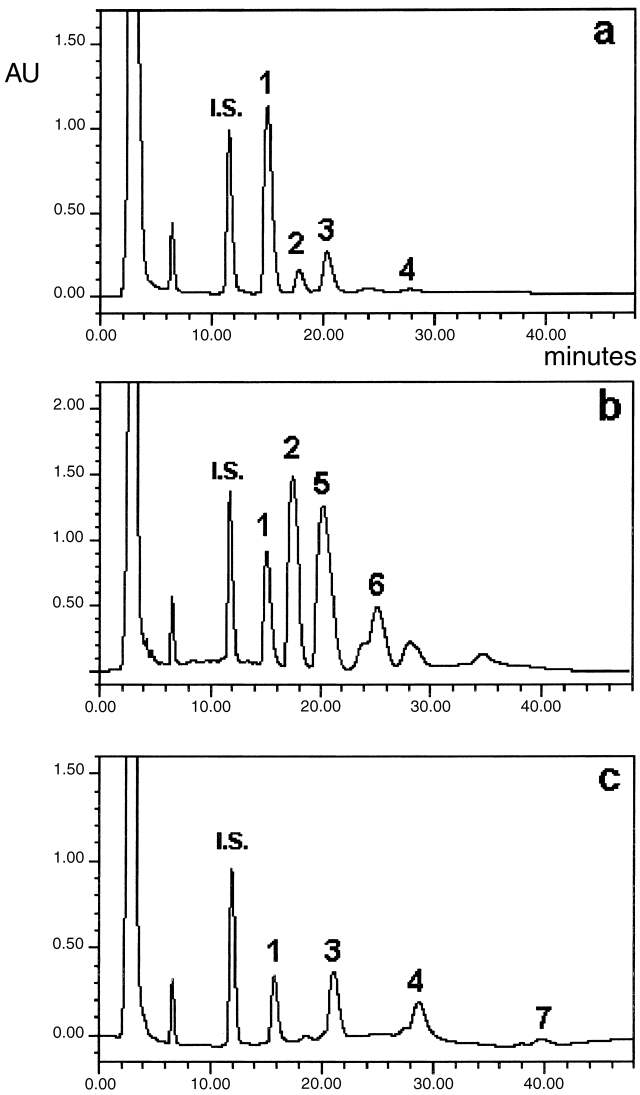

Figure 2. HPLC chromatogram of the EtOAc extracts from supernatant (detection at 210 nm): (**a**) control (non-additive); (**b**) L-leucine fed (20 mM); (**c**) L-isoleucine fed (20 mM). Internal standard (I.S.): ethyl 4-phenylbenzoate (0.50 mg).

enniatins. ^{1}H and ^{13}C NMR spectra of enniatin I were also similar to those of **3** where chemical shifts of the protons and carbons in each residue were superimposed but with different composition: three *N*MeVal, one Hiv, and two Hmp. Analyses of 2D NMR spectra (COSY, NOESY, HMQC and HMBC) further confirmed the connectivity and assignment of each residue (Table 1; HMBC correlations are shown in Section 3).

Enniatin synthetase, a multifunctional enzyme catalyzing enniatin biosynthesis, has previously been isolated from *F. oxysporum*,[8] and the cell-free synthesis of enniatins has also been reported.[8,9] It is known that the biosynthetic precursor for L-*N*MeVal residues of the major metabolite enniatin B (**1**) is L-valine as shown by the uptake of radioactive substrate. The D-Hiv residues are also derived from L-valine via 2-ketoisovalerate as reported in the literature.[10] Production of the unusual analogs **3** and **4** by *V. hemipterigenum* BCC 1449 suggested that the nature of enniatin synthetase of this strain might be different from those of previously investigated enniatins-producing *Fusarium* species, especially at the region associated with Hiv substrate recognition. With the characteristic enniatins-producing strain (BCC 1449) in hand, we decided to examine the precursor-directed biosynthesis,[11] since it was anticipated that feeding the substrate analog, L-leucine and L-isoleucine, might result in the incorporation of these mimics as either L-*N*-methylamino acid or D-2-hydroxy-carboxylic acid residues in the enniatin molecules.

A feeding experiment with 20 mM of L-leucine (fermentation: 4×1 L Erlenmeyer flasks, each containing 250 mL of potato dextrose broth) led to the enhanced production of enniatins as compared to controlled fermentation (no additive) (Fig. 2). HPLC/UV analysis (ODS column: MeCN/H_2O=70:30; detection at 210 nm) of the extract from supernatant showed that enniatin B_4 (**2**) and a new analog (**5**), corresponding to the peak at t_R 20 min, were produced in higher amounts relative to enniatin B (**1**). 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. Thus, extracts from supernatant and mycelia were combined and subjected to chromatographic separation. Compounds **1**, **2**, **5** and a minor product corresponding to the HPLC peak at t_R 25 min (**6**) were isolated (see Section 3). Although the HPLC retention time (in MeCN/H_2O) of compound **5** was very close to **3**, the prep. HPLC fraction corresponding to this peak contained mainly compound **5** and a trace amount of **3** which was removed by subsequent re-chromatography employing MeOH/H_2O as the solvent system. Spectral data for enniatins B (**1**) and B_4 (**2**) obtained from this feeding experiment were identical to those obtained from non-additive fermentation. NMR analyses of another major product, **5**, 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, assigned as depicted (Fig. 1), was identical to enniatin G which was very recently isolated from the mangrove fungus *Halosarpheia* sp. (strain 732).[12] The minor product, **6**, exhibited a C_3-symmetric structure as indicated by its molecular formula ($C_{36}H_{63}N_3O_9$, HRMS) and ^{1}H and ^{13}C NMR spectra. Analyses 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.[13]

A feeding experiment with L-isoleucine (20 mM) gave dramatically different results in which enhanced production of enniatins H (**3**) and I (**4**), and the appearance of another derivative, **7**, at t_R 40 min, were observed by HPLC/UV analysis (Fig. 2). Due to the small amounts of enniatin products, a 10 L fermentation (250 mL×40 flasks) was conducted from which four compounds, **1**, **3**, **4**, and **7**, were isolated. Spectral data of **1**, **3** and **4**, obtained from the L-isoleucine-fed culture were identical in all respects to those from non-additive fermentation. The newly produced analog, **7**, molecular formula $C_{36}H_{63}N_3O_9$ (HRMS), possessed a C_3-symmetric structure as indicated by its

Table 2. Antiplasmodial, antimycobacterial and cytotoxic activities of enniatins **1**–**7**

Compound	*P. falciparum* K1[a] (IC_{50}, μg/mL)	*M. tuberculosis* H37Ra[b] (MIC, μg/mL)	Cytotoxicity[c] (IC_{50}, μg/mL)		
			KB cells	BC-1 cells	Vero cells
Enniatin B (**1**)	0.27	3.12	16	18	17
Enniatin B_4 (**2**)	0.20	3.12	11	12	18
Enniatin G (**5**)	0.46	6.25	>20	>20	45
Enniatin C (**6**)	1.1	6.25	>20	>20	>50
Enniatin H (**3**)	1.9	6.25	>20	5.5	38
Enniatin I (**4**)	0.24	6.25	>20	18	38
Compound **7**	0.22	1.56	11	8.1	1.4

[a] IC_{50} values of the standard antimalarial compouds, 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.

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 (**3**) and I (**4**), and the missing analog, **7**. A related compound MK1688, obtained from *F. oxysporum* D338, was claimed as an antifungal substance in a Japanese patent,[14] 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 **7**, with those of MK1688 in the patent, we have concluded that they are the same compound. Therefore, MK1688 possesses (2*R*,3*S*)-configuration at the Hmp residues.

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 (**3**), I (**4**) and MK1688 (**7**) in the feeding experiment as well as by the production of **3** and **4** 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.[7]

Enniatins **1**–**7** were tested for their activities against *Plasmodium falciparum* (K1 strain) and *Mycobacterium tuberculosis* (H37Ra strain), and also for their cytotoxic activity towards two cancer cell-lines (KB, BC-1) and Vero cells. Enniatins **1**–**7** 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) (Table 2). To the best of our knowledge, 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*.[1h] It should also be commented that these enniatins also exhibited cytotoxic activities, but these were rather weak when compared to their antimalarial activities.

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 Perkin–Elmer system 2000 spectrometer. Mass spectra (ESI-TOF) were measured with a Micromass LCT mass spectrometer. ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), DEPTs and 2D NMR spectra (COSY, NOESY, HMQC and HMBC) were taken on a Bruker DRX400 spectrometer.

3.2. 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. The fungus was deposited at the Thailand BIOTEC Culture Collection as BCC 1449.

3.3. Extraction and isolation

The flask cultures (40×1 L Erlenmeyer flask), each containing 250 mL of potato dextrose broth, were incubated at 22°C for 21 days, and filtered to separate into mycelia and supernatant. The mycelial cakes were extracted with methanol (2 L, rt, 2 days) and the solvent concentrated to 500 mL. H_2O (50 mL) was added, washed with hexane (300 mL), and the aqueous methanol 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 semi-solid (0.85 g). This mycelial extract was subjected to Sephadex LH20 column chromatography (CH_2Cl_2/MeOH) followed by CC on silica gel (MeOH/CH_2Cl_2) to obtain a mixture of enniatins. Continuous separation by preparative HPLC using a reversed-phase column (MeCN/H_2O, then MeOH/H_2O) gave enniatin isomers in the following order of elution: enniatins B (**1**, 52 mg), B_4 (**2**, 14 mg), H (**3**, 8.3 mg) and I (**4**, 2.8 mg).

3.3.1. Enniatin H (3). Colorless solid; mp 105–106°C;

$[\alpha]_D^{29}=-102$ (*c* 0.22, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 206 (4.23) nm; IR (KBr) ν_{max} 2967, 1743, 1663, 1470, 1203, 1012 cm^{-1}; HRMS (ESI-TOF) *m/z* 676.4121 $[M+Na]^+$ (calcd for $C_{34}H_{59}N_3O_9Na$ 676.4149; $\Delta=2.8$ mmu); ^1H and ^{13}C NMR data, Table 1. HMBC correlations ($CDCl_3$, 400 MHz, 50 and 100 ms): *N*MeVal (3 units), H-2 to C-1 (δ_C 170.3), C-3, C-4, C-4′, δ_C 169.3 (amide) and N–CH_3; H-4 to C-2, C-3 and C-4′; H-4′ to C-2, C-3 and C-4; N–CH_3 to C-1 and δ_C 169.3 (amide): Hiv (2 units), H-2 to C-3, C-4 and C-4′; H-3 to C-2, C-4 and C-4′; H-4 to C-2, C-3 and C-4′; H-4′ to C-2, C-3 and C-4: Hmp (1 unit), H-2 to C-3, 3-CH_3 and δ_C 170.3 (ester); H-4a (δ_H 1.46) to C-2, C-5 and 3-CH_3; H-5 to C-3: 3-CH_3 to C-3 and C-4.

3.3.2. Enniatin I (4). Colorless gum; $[\alpha]_D^{29}=-87$ (*c* 0.12, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 207 (4.23) nm; IR (KBr) ν_{max} 2965, 1745, 1665, 1468, 1281, 1192, 1012 cm^{-1}; HRMS (ESI-TOF) *m/z* 690.4277 $[M+Na]^+$ (calcd for $C_{35}H_{61}N_3O_9Na$ 690.4306; $\Delta=2.9$ mmu); ^1H and ^{13}C NMR data, Table 1. HMBC correlations ($CDCl_3$, 400 MHz, 100 ms): *N*MeVal (3 units), H-2 to C-1 (δ_C 170.3), C-3, C-4, C-4′, δ_C 169.2 (amide) and N–CH_3; H-3 to C-2, C-4 and C-4′; H-4 to C-2, C-3 and C-4′; H-4′ to C-2, C-3 and C-4; N–CH_3 to C-1 and δ_C 169.2 (amide): Hiv (1 unit), H-3 to C-4 and C-4′; H-4 to C-2, C-3 and C-4′; H-4′ to C-2, C-3 and C-4: Hmp (2 units), H-4a (δ_H 1.46, 2H) to C-3, C-5 and 3-CH_3; H-5 to C-3 and C-4; 3-CH_3 to C-2, C-3 and C-4.

3.4. Precursor-directed biosynthesis

V. hemipterigenum BCC 1449 was incubated in 4×1 L Erlenmeyer flasks, each containing 250 mL of potato dextrose broth with 20 mM of L-leucine. The flask cultures were filtered to separate into mycelia and supernatant (ca. 1 L). Extraction from mycelia was performed as described above. Supernatant was extracted with EtOAc (1 L), dried over $MgSO_4$, and concentrated in vacuo to obtain a crude extract. To each extract from mycelia and supernatant was added ethyl 4-phenylbenzoate (0.50 mg) as an internal standard. Each sample was subjected to HPLC/UV analysis using a reversed-phase column (NovaPak 8NV4μ; 8×100 mm), elution with MeCN/H_2O=70:30 with a flow rate of 1 mL/min (detection at 210 nm). Calibration was made for pure enniatin B, and for the calculation of the amounts of enniatin analogs. The L-isoleucine-feeding and control (no additive) experiments were carried out in the same manner. After HPLC analyses, the extracts from mycelia and supernatant of the L-leucine-fed culture were combined, and subjected to chromatographic separation/purification to obtain pure enniatins B (**1**, 2.5 mg), B_4 (**2**, 5.7 mg), G (**5**, 6.8 mg) and C (**6**, 2.2 mg). The L-isoleucine-feeding experiment was repeated on a larger scale (40 flasks; total 10 L) from which enniatins B (**1**, 8.1 mg), H (**3**, 22 mg) and I (**4**, 25 mg), and MK1688 (**7**, 10 mg) were isolated.

3.4.1. Enniatin G (5). Colorless solid; mp 143–145°C; $[\alpha]_D^{26}=-75$ (*c* 0.21, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 206 (4.28) nm; HRMS (ESI-TOF) *m/z* 690.4301 $[M+Na]^+$ (calcd for $C_{35}H_{61}N_3O_9Na$ 690.4306; $\Delta=0.5$ mmu); IR and NMR spectral data were identical to those listed in the literature.[12]

3.4.2. Enniatin C (6). Colorless solid; mp 159–160°C; $[\alpha]_D^{27}=-47$ (*c* 0.11, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 205 (4.23) nm; IR (KBr) ν_{max} 2964, 1748, 1659, 1471, 1268, 1204, 1014 cm^{-1}; HRMS (ESI-TOF) *m/z* 704.4443 $[M+Na]^+$ (calcd for $C_{36}H_{63}N_3O_9Na$ 704.4462; $\Delta=1.9$ mmu); ^1H NMR (400 MHz, $CDCl_3$) δ *N*MeLeu (3 units, symmetrical) 5.33 (3H, brd, *J*=7.1 Hz, H-2), 3.10 (9H, s, NCH_3), 1.73 (3H, m, H-3a), 1.66 (3H, m, H-3b), 1.45 (3H, m, H-4), 0.95 (9H, d, *J*=6.5 Hz, H-5), 0.91 (3H, d, *J*=6.5 Hz, H-5′): Hiv (3 units, symmetrical) 4.91 (3H, d, *J*=8.2 Hz, H-2), 2.20 (3H, m, H-3), 1.01 (9H, d, *J*=6.4 Hz, H-4), 0.94 (9H, d, *J*=6.7 Hz, H-4′); ^{13}C NMR (100 MHz, $CDCl_3$) δ, *N*MeLeu (3 units, symmetrical) 171.1 (s, C-1), 54.2 (d, C-2), 37.8 (t, C-3), 31.4 (q, NCH_3), 25.3 (d, C-4), 23.4 (q, C-5), 20.9 (q, C-5′): Hiv (3 units, symmetrical) 170.3 (s, C-1), 75.7 (d, C-2), 30.0 (d, C-3), 18.6 (q, C-4), 18.0 (q, C-4′).

3.4.3. Compound 7 (MK1688). Colorless gum; $[\alpha]_D^{26}=-89$ (*c* 0.25, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 207 (4.17) nm; IR ($CHCl_3$) ν_{max} 2970, 1737, 1662, 1465, 1191, 1007 cm^{-1}; HRMS (ESI-TOF) *m/z* 704.4458 $[M+Na]^+$ (calcd for $C_{36}H_{63}N_3O_9Na$ 704.4462; $\Delta=0.4$ mmu); ^1H NMR (400 MHz, methanol-d_6) (symmetrical) δ 5.43 (3H, d, *J*=6.6 Hz), 4.80 (3H, d, *J*=9.8 Hz), 3.21 (9H, s), 2.33 (3H, m), 2.00 (3H, m), 1.51 (3H, m), 1.29 (3H, m), 1.12 (9H, d, *J*=6.6 Hz), 1.04 (9H, d, *J*=6.7 Hz), 1.00 (9H, t, *J*=7.4 Hz), 0.97 (9H, d, *J*=6.7 Hz); ^1H and ^{13}C NMR data in $CDCl_3$, Table 1.

3.5. Biological assays

The assay for activity against *P. falciparum* K1 was performed using a standard protocol,[15] which follows the microculture radioisotope technique as described by Desjardins.[16] 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*. Growth inhibitory activity against *M. tuberculosis* H37Ra was performed using the Microplate Alamar Blue Assay (MABA).[17] Cytotoxic activities of the purified compounds against human epidermoid carcinoma (KB cells), human breast cancer (BC-1 cells) and African green monkey kidney fibroblast (Vero cells) were evaluated using colorimetric method.[18]

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