2. EXPERIMENTAL

2.1 Chemicals and instruments

Melting points were measured with an Electrothermal IA9100 digital melting point apparatus and were uncorrected. Optical rotation was determined either in analytical chloroform ($CHCl_3$) or methanol (MeOH) solution with sodium D line (589 nm) on a JASCO DIP-370 digital polarimeter. Ultra violet (UV) spectra were recorded on a Cary 1E UV-visible spectrophotometer (UVIDEC-650) in analytical solvents, ethanol, methanol and chloroform. FT-IR spectra were determined by a Perkin-Elmer system 2000 spectrometer. ^{1}H and ^{13}C NMR, and 2D NMR (COSY, NOESY, HMQC and HMBC) spectra were recorded on a Bruker DRX-400 spectrometer. Those spectra were recorded by using deuterated chloroform ($CDCl_3$; tetramethylsilane as internal standard), deuterated methanol (MeOH-d_4) and deuterated acetone (acetone-d_6) solvents. Time of flight-electro-spray ionization (ESI -TOF) mass spectra were taken on a Micromass LCT mass spectrometer. Silica gel $60PF_{254}$ (Merck) was used for thin-layer chromatography (TLC). Column chromatography (CC) was performed by using Sephadex LH-20 (Code No. 17-0090-01) and silica gel 60H (Merck Code No. 7736). HPLC (high performance liquid chromatography) was performed using a Waters system (Waters 600 HPLC pump controller and Waters 996 Photodiode array detector) with a reversed-phase cartridge columns: NovaPak® 8NV4μ (8 × 100 mm) for analysis, and Prep NovaPak® HRC_{18} (6 μm, 40 × 100 mm) for preparation. MeCN/H_2O or MeOH/H_2O was used as mobile phase for HPLC.

2.2 Fungal Material

The fungus *Verticillium hemipterigenum* was collected from Khlong Nakha Wildlife Sanctuary, Ranong province, Thailand, on Homoptera-adult leafhopper, and identified by Dr Nigel L. Hywel-Jones of the Mycology Research Unit, BIOTEC

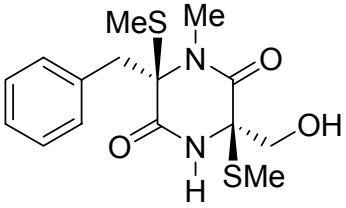
(Hywel-Jones, 1997). This fungus is deposited at the BIOTEC Culture Collection (BCC) as BCC1449.

2.3 Fermentation and extraction

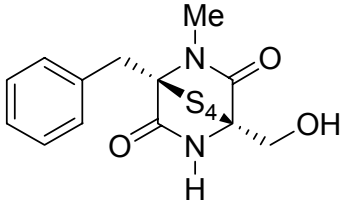
Verticillium hemipterigenum BCC1449 was maintained on potato dextrose agar at 25 ^{o}C for 20 days, which was inoculated into 4 × 1 liter Erlenmeyer flasks, each containing 250 mL of potato dextrose broth (PDB). After incubation at 25 ^{o}C for 7 days on a rotary shaker (200 rpm), these primary cultures were transferred into 40 × 1 liter Erlenmeyer flasks each containing 250 mL of PDB (total 10 liters). The fermentation was carried out at 25 ^{o}C for 21 days. The cultures were filtered to separate filtrate and mycelia. The filtrate was extracted twice with an equal volume of EtOAc. The EtOAc layer was concentrated under reduced pressure to obtain a brown gum (481 mg). The mycelial cakes were extracted with methanol (2 L) at r.t. for 2 days, then filtered. The filtrate was concentrated to ca 500 mL. H_2O (50mL) was added, and the mixture was washed with hexane (300mL). The aqueous MeOH layer was concentrated under reduced pressure. The residual oil was dissolved in EtOAc (500 mL), washed with H_2O (150 mL), and the organic layer was concentrated under reduced pressure to obtain a brown semi-solid (1.13 g). Crude extracts from the culture filtrate and mycelia were separately subjected to chromatographic fractionation.

2.4 Isolation

Compounds **51**-**56** were isolated as constituents of the extract from culture filtrate. Compounds **55**-**58** were isolated as constituents of the extract from mycelia.



51



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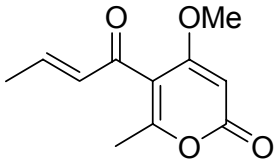

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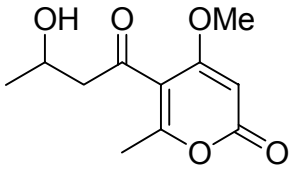

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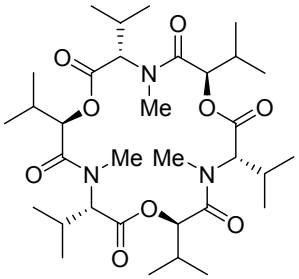
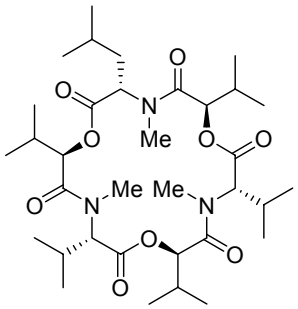
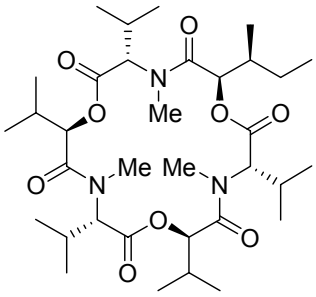


55



56



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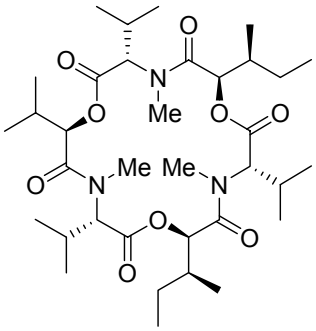


58

2.4.1 Purification of the extract from culture filtrate

The extract from filtrate (481 mg) was triturated in MeOH (2 mL) and filtered. The residual light yellow solid (123 mg) was recrystallized from EtOH-H_2O to obtain pure compound **55** (enniatin B) as colorless crystals (80 mg). The filtrates were combined and concentrated to obtain a brown gum (320 mg) which was passed

through a Sephadex LH-20 column (3.5 × 30 cm) with MeOH as eluent to provide three major fractions A, B and C. Fraction A (36 mg) mainly consisted of triglyceride and fatty acids (^{1}H NMR), fraction C (54 mg) contained mainly sugars (^{1}H NMR), therefore further purification of these two fractions was not conducted. Fraction B (210 mg) was fractionated by silica gel column (3.5 × 30 cm) chromatography using step gradient elution with MeOH/CH_2Cl_2 = 0:100, 1:99, 2:98, 5:95, 10:90, 20:80, 40:60 to collect five fractions in the order of elution: Fr-B-1 (62 mg), Fr-B-2 (17 mg), Fr-B-3 (21 mg), Fr-B-4 (24 mg) and Fr-B-5 (33 mg).

Fr-B-1 (62 mg) was subjected to preparative HPLC using a reversed-phase column (Prep NovaPak® HRC_{18}, 6 μm, 40 × 100 mm) with MeCN/H_2O = 30:70 as eluent at a flow rate of 20 mL/min. Compound **53** (pyrenocine A) eluted at retention time (t_R) 6 minute. Further purification by silica gel chromatography (1.5 × 15 cm), using step gradient elution with EtOAc/hexane = 5:95, 10:90, 20:80, 40:60, gave pure compound (**53**; 43 mg).

Fr-B-2 (17 mg) was subjected to preparative HPLC (Prep NovaPak® HRC_{18}, 6 μm, 40 × 100 mm) with MeCN/H_2O = 30:70 as eluent at a flow rate of 20 mL/min, to obtain compound **54** (pyrenocine B; 6.5 mg, t_R 3.7 min) and compound **52** (3.9 mg, t_R 14 min). Each compound was further purified by silica gel chromatography (1.0 × 10 cm) using step gradient elution with EtOAc/hexane = 5:95, 10:90, 20:80, 40:60: **54** (5.9 mg), **52** (3.6 mg).

Fr-B-3 (21 mg) was subjected to preparative HPLC (Prep NovaPak® HRC_{18}, 6 μm, 40 × 100 mm) with MeCN/H_2O = 60:40 as eluent at a flow rate of 20 mL/min, to obtain compound **51** (8.9 mg, t_R 3 min). Further purification by silica gel chromatography (1.0 × 10 cm) using step gradient elution with EtOAc/hexane = 5:95, 10:90, 20:80, 40:60, gave pure compound (**51**, 8.1 mg).

Fr-B-4 (24 mg) was subjected to preparative HPLC (Prep NovaPak® HRC_{18}, 6 μm, 40 × 100 mm) with MeCN/H_2O = 55:45 as eluent at a flow rate of 20 mL/min, to afford compound **55** (5.9 mg, t_R 35 min) and compound **56** (4.3 mg, t_R 46 min). Each compound was further purified by silica gel chromatography (1.0 × 10 cm) using step gradient elution with EtOAc/hexane = 5:95, 10:90, 20:80, 40:60: **55** (enniatin B; 5.2 mg), **56** (enniatin B_4, 3.3 mg).

Fr-B-5 (33mg) was subjected to preparative HPLC (Prep NovaPak® HRC_{18}, 6 μm, 40 × 100 mm) with MeCN/H_2O = 55:45 as eluent at a flow rate of 20 mL/min, to obtain compound **55** (22.1 mg, t_R 35 min), which was further purified by silica gel chromatography (1.5 × 15 cm) using step gradient elution with EtOAc/hexane = 5:95, 10:90, 20:80, 40:60: **55** (enniatin B; 20 mg).

Compound **51**:

Colorless crystals	
mp	154-157 °C
$[\alpha]^{26}_D$	-70 (*c* 0.21, $CHCl_3$)
UV (EtOH) λ_{max} (log ε)	205 (4.35), 258 (2.94) nm
HRMS (ESI-TOF) *m*/*z*	339.0841 (Δ = 0.4 mmu) $[M–H]^-$
IR(KBr) ν_{max}	3399, 3205, 3108, 1693, 1634, 1435, 1389, 1042, 701 cm^{-1}

^1H and ^{13}C NMR data, in Table 4 (Results and Discussion).

Compound **52**:

Colorless crystals	
mp	152-156 °C
$[\alpha]^{26}_D$	-123 (*c* 0.16, $CHCl_3$)
UV (EtOH) λ_{max} (log ε)	204 (4.35), 299 (3.59) nm
HRMS (ESI-TOF) *m*/*z*	372.9820 (Δ = 1.2 mmu) $[M–H]^-$
IR(KBr) ν_{max}	3290, 3102, 1694, 1634, 1436, 1388, 1054, 752, 711 cm^{-1}

^1H and ^{13}C NMR data, in Table 5 (Results and Discussion).

Compound **53** (pyrenocine A):

Colorless solid	
mp	104-106 °C
UV (MeOH) λ_{max} (log ε)	205 (4.41), 228 (4.20), 273 (3.96) nm
EIMS *m*/*z*	208 $[M]^+$, 193, 165, 69, 43

IR(KBr) ν_{max} 3079, 2945, 1728, 1674, 1629, 1603, 1558, 1449, 1402, 1374, 1088, 629 cm^{-1}

^{1}H and ^{13}C NMR data, in Table 6 (Results and Discussion).

Compound **54** (pyrenocine B):

Pale yellow solid

mp 98-100 ^{o}C

UV (MeOH) λ_{max} (log ε) 204 (4.34), 220 (4.08), 258 (3.98), 284 (3.86) nm

EIMS *m*/*z* 226 $[M]^{+}$, 208, 193, 182, 167, 154, 139, 125, 69, 43

IR (KBr) ν_{max} 3455, 1713, 1684, 1604, 1547, 1449, 1401, 1265, 1084, 634 cm^{-1}

^{1}H and ^{13}C NMR data, in Table 7 (Results and Discussion).

Compound **55** (enniatin B):

Colorless solid

mp 173-175 ^{o}C

$[\alpha]^{29}_{D}$ -96 (*c* 1.04, $CHCl_3$)

UV (EtOH) λ_{max} (log ε) 206 (4.57), 291 (3.16) nm

EIMS *m*/*z* 639 $[M]^{+}$, 624, 596, 538, 496, 409, 296, 282, 196, 169, 141

IR (KBr) ν_{max} 3448, 2967, 1741, 1664, 1384, 1204, 1017 cm^{-1}

^{1}H and ^{13}C NMR data, in Table 8 (Results and Discussion).

Compound **56** (enniatin B_4):

Colorless amorphous solid

mp 124-126 ^{o}C

$[\alpha]^{27}_{D}$ -57 (*c* 0.09, $CHCl_3$)

UV (EtOH) λ_{max} (log ε) 207 (4.30), 279 (2.88) nm

EIMS *m*/*z* 653 $[M]^+$, 571, 552, 524, 470, 457, 410, 310, 296, 282, 210, 196, 169, 155

IR (KBr) ν_{max} 2964, 1733, 1662, 1387, 1130, 1016 cm^{-1}

^1H and ^{13}C NMR data, in Table 9 (Results and Discussion).

2.4.2 Purification of the mycelia extract

The extract from mycelia (1.13 g) was subjected to silica gel column chromatography (3.5 × 15 cm; step gradient elution with MeOH/CH_2Cl_2, 0:100, 2:98, 5:95 then 10:90). The MeOH/CH_2Cl_2 = 5:95 elute (fraction 9 – fraction 12) contained enniatins, as confirmed by TLC and ^1H NMR. These fractions were combined and dried (0.85 g), then passed through a Sephadex LH-20 column (3.5 × 30 cm) using CH_2Cl_2/MeOH (50:50) as eluent. The enniatin-containing fractions (fraction 3 – fraction 6) were combined (dry weight, 243 mg), and this process was repeated once again to furnish an enniatins mixture (224 mg). This sample was subjected to preparative HPLC using a reversed-phase column (40 × 100 mm) with MeCN/H_2O = 65:35 as eluent at a flow rate of 20 mL/min, wherein each enniatin eluted in following order: **55** (enniatin B, 52 mg, t_R 15 min), **56** (enniatin B_4, 19 mg, t_R 17 min), **57** (enniatin H, 11 mg, t_R 20 min) and **58** (enniatin I, 7 mg, t_R 27 min). Compounds **56**, **57** and **58** were further purified by preparative HPLC using the same solvent system: **56** (14.1 mg), **57** (8.3 mg), and **58** (2.8 mg).

Compound **55** (enniatin B): colorless solid. ^1H NMR spectral data was identical to that obtained in section **2.4.1.**

Compound **56** (enniatin B_4): colorless amorphous solid. ^1H NMR spectral data was identical to that obtained in section **2.4.1.**

Compound **57** (enniatin H):

Colorless solid

mp 105-106 ^oC

$[\alpha]^{29}_D$ -102 (*c* 0.22, $CHCl_3$)

UV (EtOH) λ_{max} (log ε) 206 (4.23)

HRMS (ESI-TOF) *m*/*z* 676.4121 (Δ = 2.8 mmu) $[M + Na]^+$

IR (KBr) ν_{max} 2967, 1743, 1663, 1470, 1203, 1012 cm^{-1}

^1H and ^{13}C NMR data, in Table 10 (Results and Discussion).

Compound **58** (enniatin I):

Colorless gum

$[\alpha]^{29}_D$ -87 (*c* 0.12, $CHCl_3$)

UV (EtOH) λ_{max} (log ε) 207 (4.23)

HRMS (ESI-TOF) *m*/*z* 690.4277 (Δ = 2.9 mmu) $[M + Na]^+$

IR(KBr) ν_{max} 2965, 1745, 1665, 1468, 1281, 1192, 1012 cm^{-1}

^1H and ^{13}C NMR data, in Table 11 (Results and Discussion).

Figure 1. Extraction of *V. hemipterigenum* BCC 1449 culture

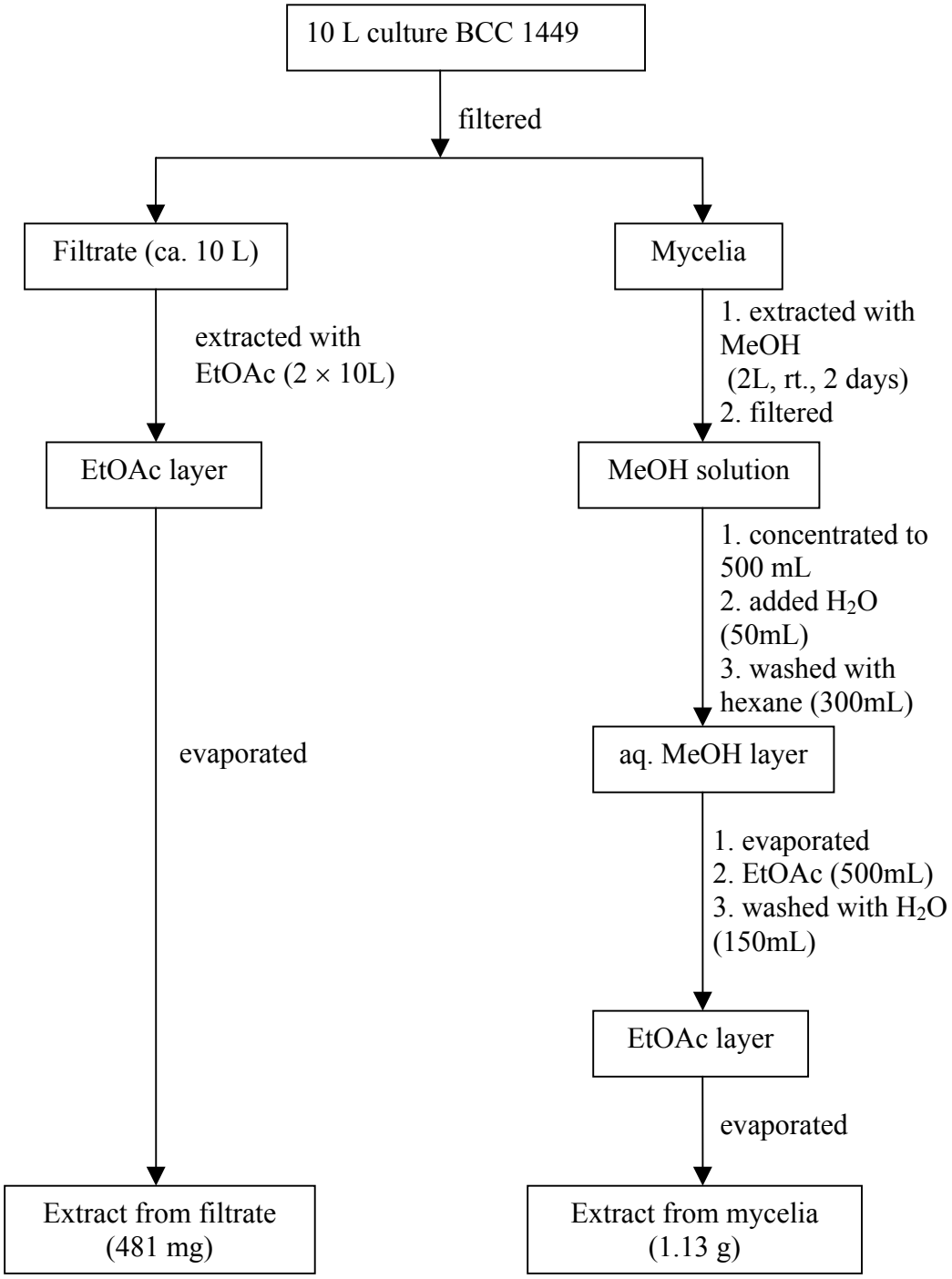


Figure 2. Isolation of compounds **51**-**56** from the filtrate extract

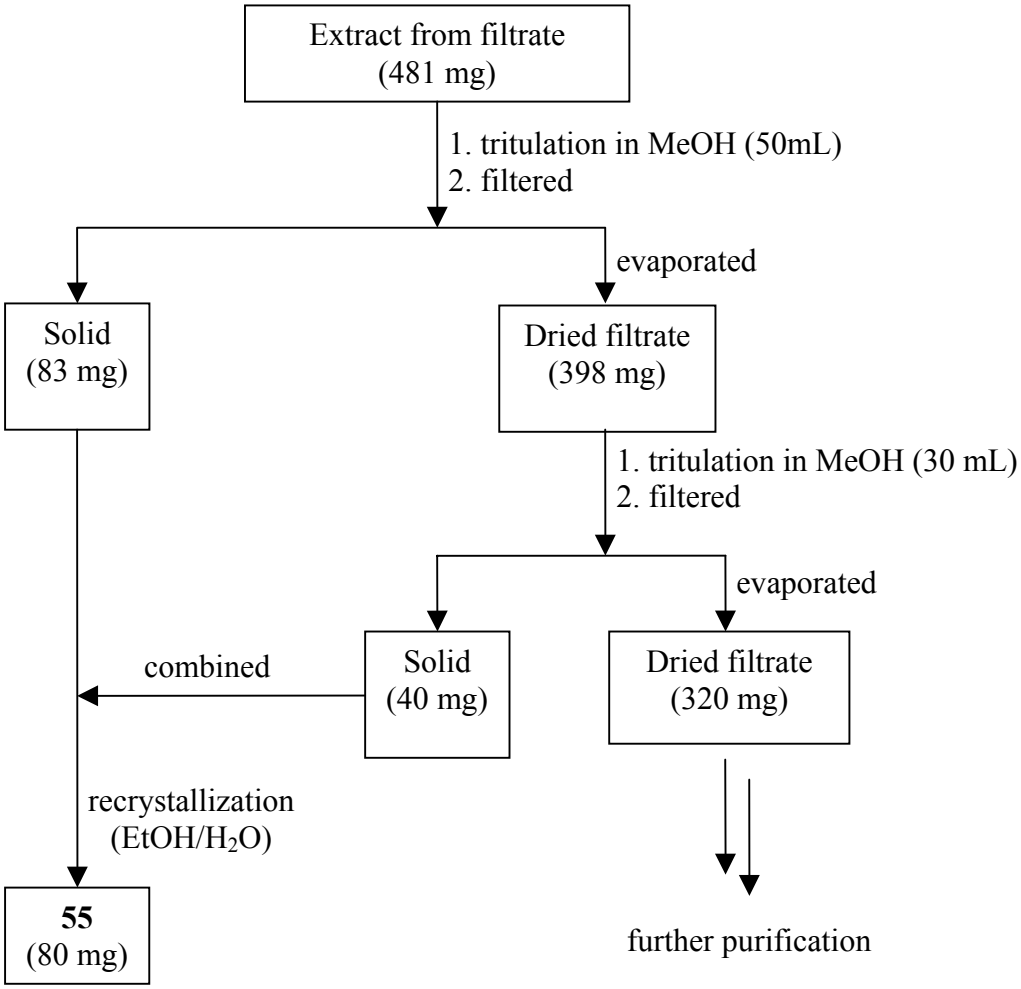
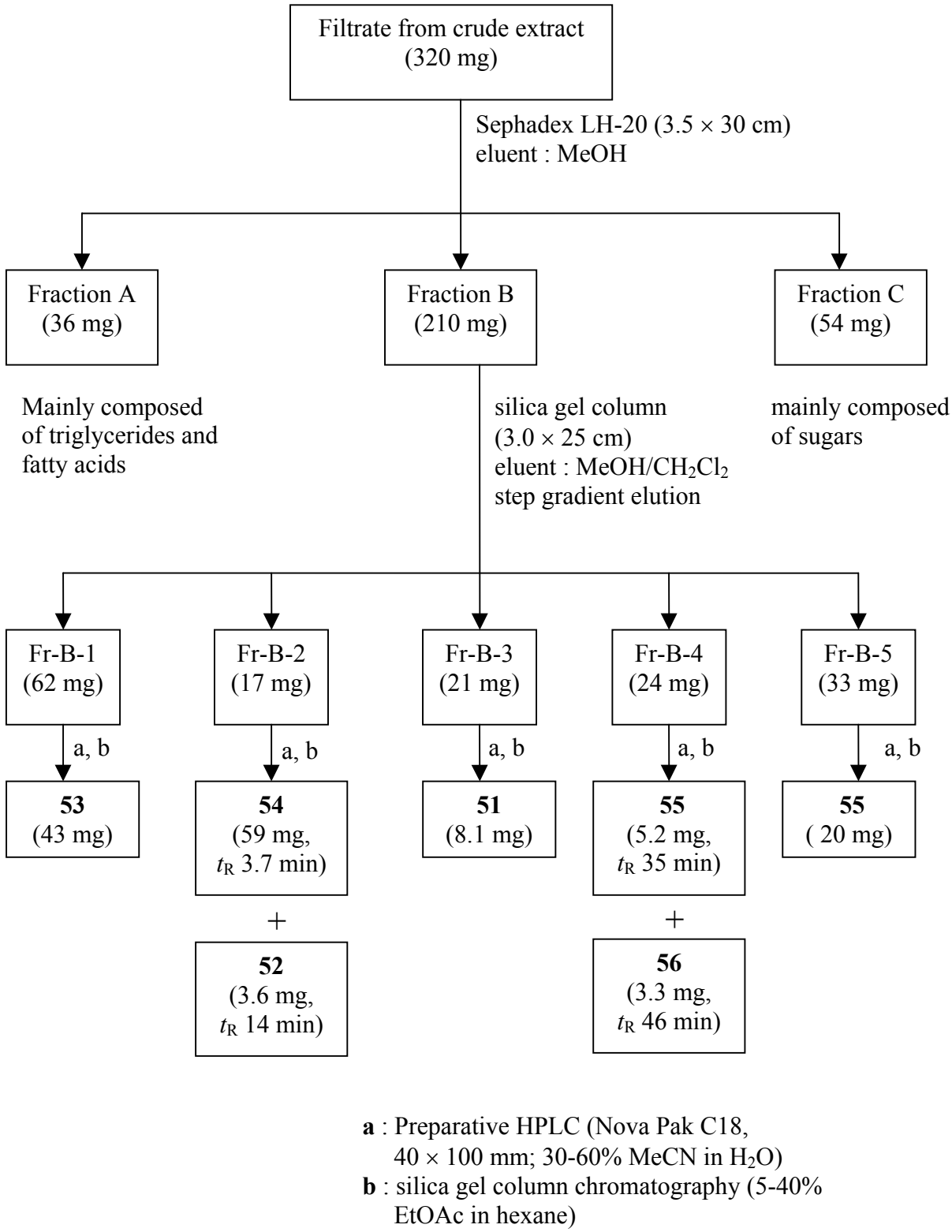
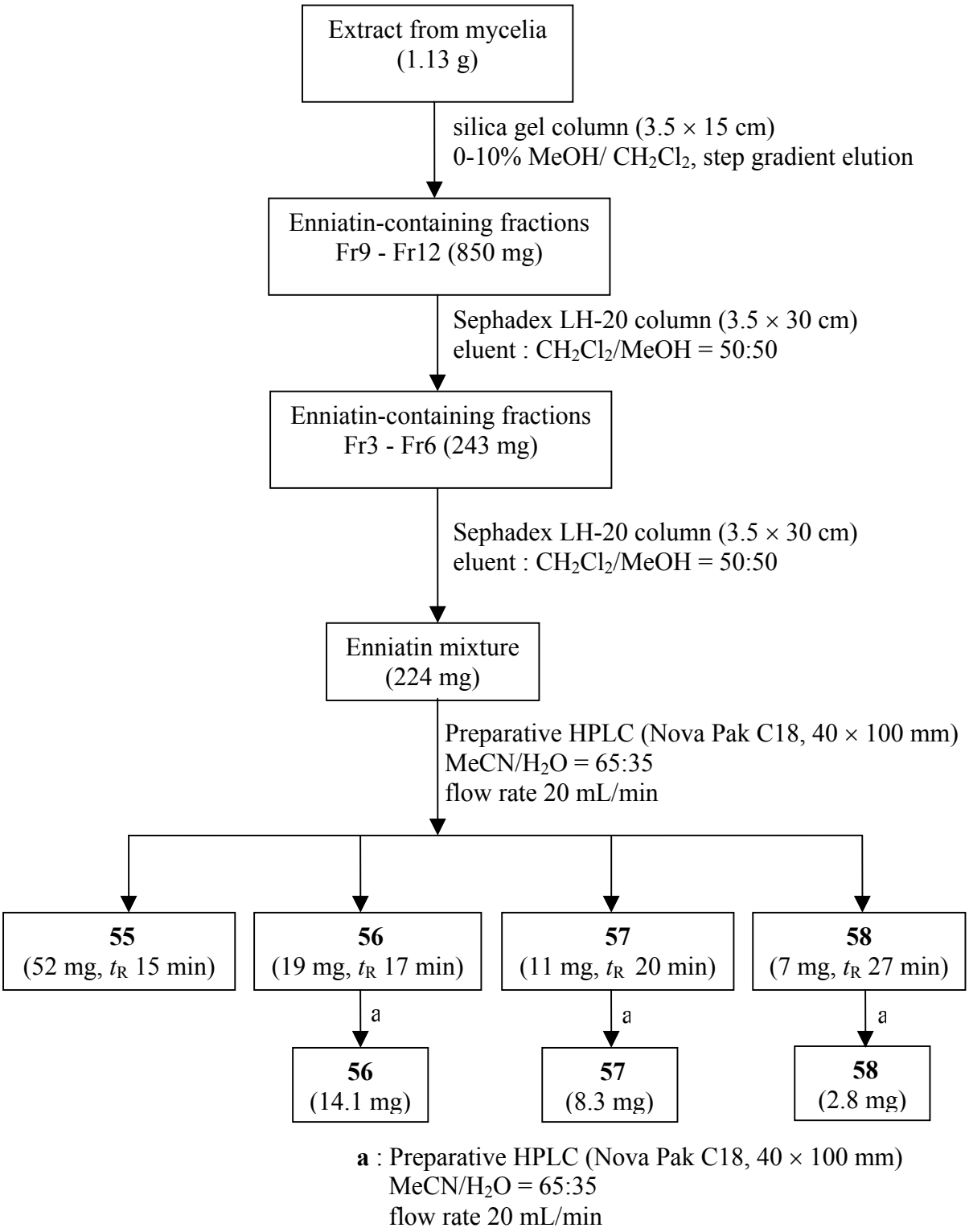


Figure 2. Isolation of compounds 51-56 from the filtrate extract (continued)



a : Preparative HPLC (Nova Pak C18, 40 × 100 mm; 30-60% MeCN in H_2O)
b : silica gel column chromatography (5-40% EtOAc in hexane)

Figure 3. Isolation of compounds **55-58** from the mycelial extract



2.5 Precursor-directed biosynthesis

Precursor-directed biosynthesis using BCC 1449 was examined to determine the stereochemistry of the new compounds, **57** and **58**, and to obtain unnatural analogs (see, Results and Discussion).

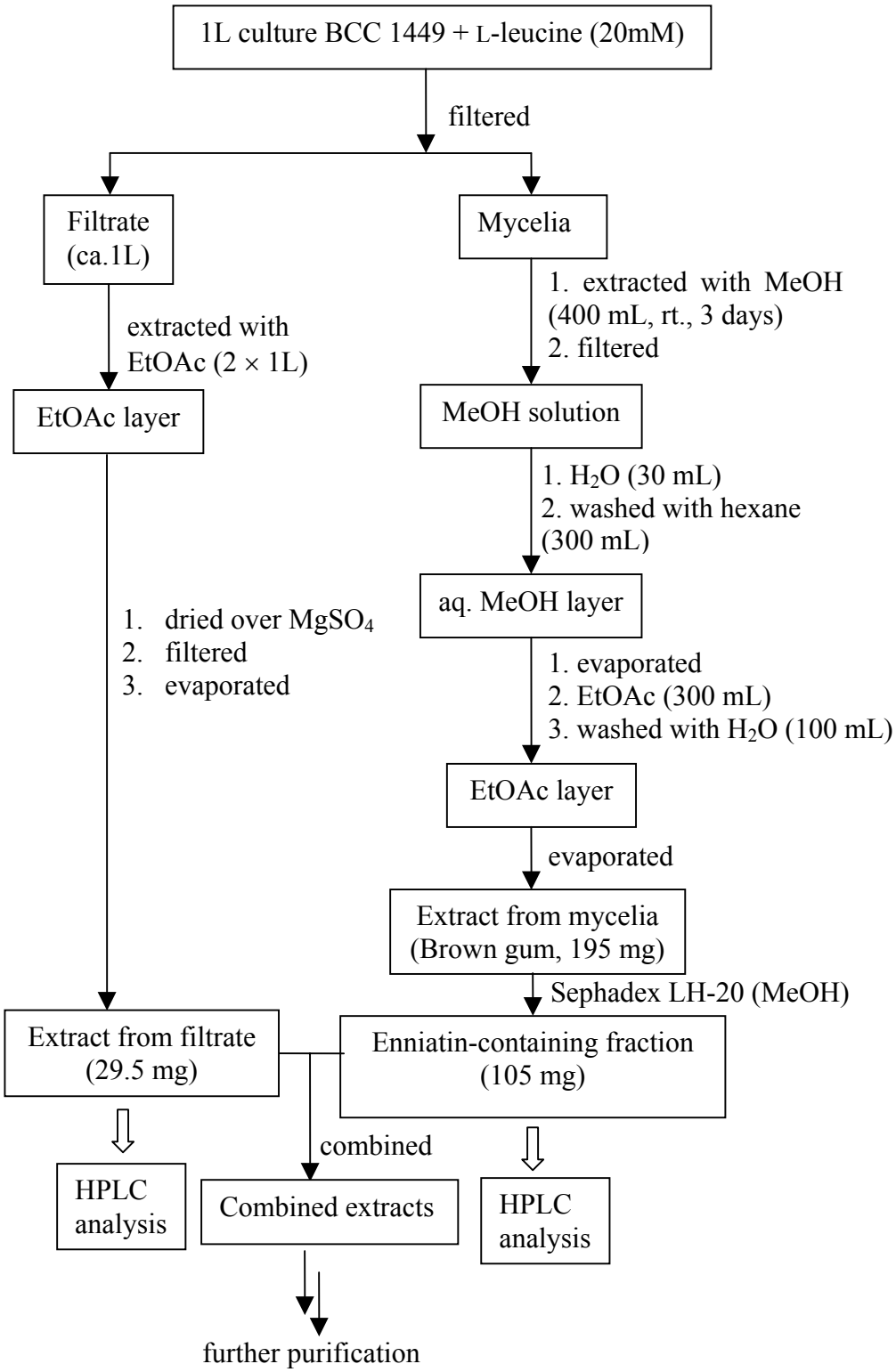
2.5.1 Feeding experiments and HPLC analysis

V. hemipterigenum BCC 1449 was incubated in 4 × 1L Erlenmeyer flasks, each containing 250 mL of PDB with 20 mM of L-leucine. The flask cultures were filtered to separate filtrate (ca. 1 L) and mycelia. The filtrate was extracted with EtOAc (1 L), dried over $MgSO_4$, and concentrated in vacuo to obtain a crude extract (29.5 mg). Mycelia were digested in MeOH (400 mL, r.t., 3 days) and filtered. To the filtrate was added H_2O (30 mL), washed with hexane (300 mL), and the aqueous MeOH layer was concentrated. The residue was dissolved in EtOAc (300 mL) and washed with H_2O (100 mL). The organic layer was concentrated in vacuo to obtain a brown gum (195 mg). This extract was passed through a Sephadex LH-20 column (MeOH), and enniatin-containing fractions were combined (105 mg). To each extract from culture filtrate and mycelia was added with ethyl 4-phenylbenzoate (0.50 mg) as an internal standard and dissolved in MeCN. 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. The result for HPLC analysis is described in Chapter 3 (Results and Discussion).

culture media	extract from filtrate	extract from mycelia
PDB [control]	16.6 mg	14.0 mg
PDB + L-leucine (20mM)	29.5 mg	105 mg
PDB + L-isoleucine (20mM)	14.4 mg	31.2 mg

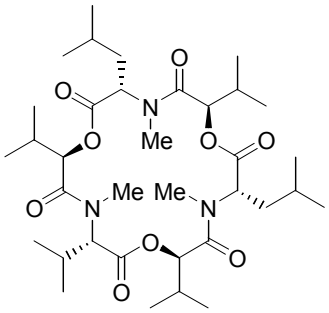
Figure 4. Extraction procedure in precursor-directed biosynthesis

Extraction of *V. hemipterigenum* BCC 1449 culture + L-leucine (20 mM)

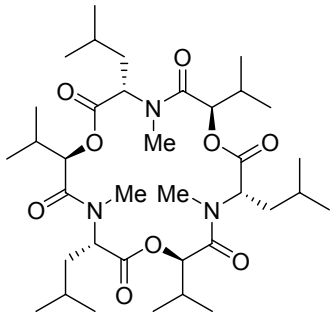


2.5.2 Isolation from L-leucine-fed culture

After HPLC analysis, the extracts from filtrate and mycelia of the L-leucine-fed culture were combined, and subjected to silica gel column chromatography ($MeOH/CH_2Cl_2$ = 2:98, then 4:96) to obtain an enniatin mixture (30 mg). Each enniatin was separated by preparative HPLC (40 × 100 mm; $MeCN/H_2O$ = 60:40; flow rate 20 mL/min): **55** (enniatin B, 2.5 mg), **56** (enniatin B_4, 6.6 mg), **59** (enniatin G, 8.5 mg), and **60** (enniatin C, 3.4 mg). Compounds **56**, **59** and **60** were further purified by preparative HPLC using $MeOH/H_2O$ = 75:25 as eluent: **56** (5.7 mg), **59** (6.8 mg), and **60** (2.2 mg).



59



60

Compound **55** (enniatin B): colorless solid. ^1H NMR spectral data was identical to that obtained in section **2.4.1.**

Compound **56** (enniatin B_4): colorless solid. ^1H NMR spectral data was identical to that obtained in section **2.4.1.**

Compound **59** (enniatin G):

Colorless solid	
mp	143-145 ^{o}C
$[\alpha]^{26}_{D}$	-75 (*c* 0.21, $CHCl_3$)
UV (MeOH) λ_{max} (log ε)	206 (4.28)
HRMS (ESI-TOF) *m/z*	690.4301 (Δ = 0.5 mmu) $[M + Na]^+$
IR (KBr) ν_{max}	2964, 1749, 1655, 1471, 1388, 1204, 1016 cm^{-1}

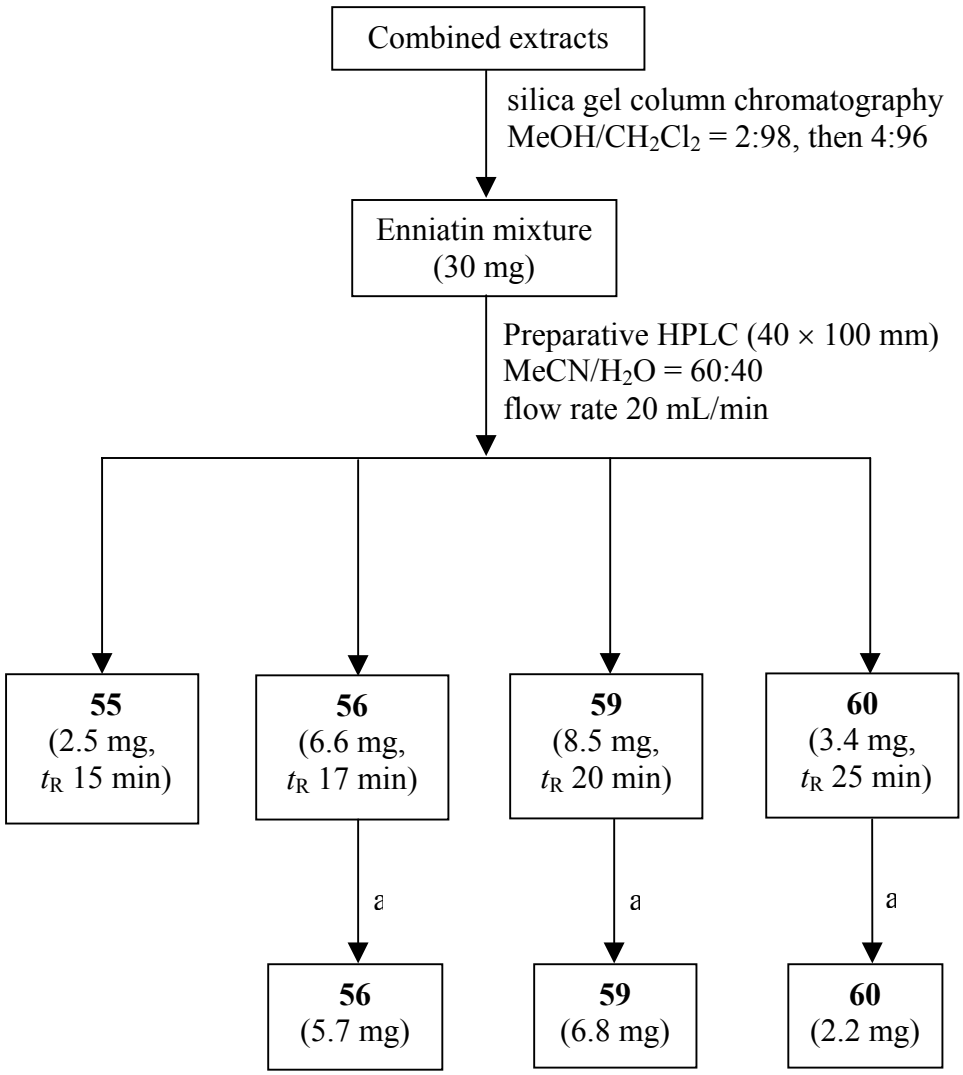
^{1}H and ^{13}C NMR data, in Table 13 (Results and Discussion).

Compound **60** (enniatin C):

Colorless solid	mp 159-160 ^{o}C
$[\alpha]^{27}_{D}$	-47 (*c* 0.11, $CHCl_3$)
UV (MeOH) λ_{max} (log ε)	205 (4.23)
HRMS (ESI-TOF) *m/z*	704.4443 (Δ = 1.9 mmu) $[M + Na]^+$
IR(KBr) ν_{max}	2964, 1748, 1659, 1471, 1268, 1204, 1014 cm^{-1}

^{1}H and ^{13}C NMR data, in Table 14 (Results and Discussion).

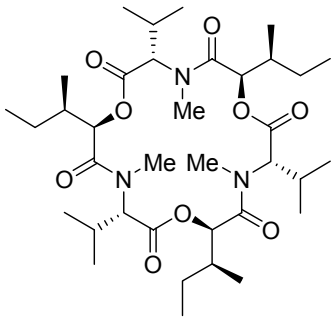
Figure 5. Isolation of compounds **55, 56, 59** and **60,** from the combined extracts of *V. hemipterigenum* BCC 1449 + L-leucine



a : Preparative HPLC (Nova Pak C18, 40 × 100 mm)
MeOH/H_2O = 75:25
flow rate 20 mL/min

2.5.3 Isolation from L-isoleucine-feeding culture

The L-isoleucine-feeding experiment was repeated on a larger scale (40 flasks; total 10 L). Extraction procedure followed that described in section **2.4.2**., giving extracts from culture filtrate (127 mg) and mycelia (505 mg). These extracts were combined and subjected to Sephadex LH-20 column chromatography (MeOH), then chromatographed on a silica gel column (MeOH/CH_2Cl_2 = 2:98, 4:96) to obtain an enniatin-containing fraction (93 mg, brown gum). Fractionation by preparative HPLC (40 × 100 mm; MeCN/H_2O = 70:30) afforded compounds **55** (enniatin B, 8.1 mg), **57** (enniatin H, 22.2 mg), **58** (enniatin I, 25.9 mg), and **61** (MK1688, 17.5 mg). Compound **58** was further purified by preparative HPLC (40 × 100 mm; MeOH/H_2O = 80:20) to obtain a pure sample (25 mg). Compound **61** was further purified by preparative HPLC (40 × 100 mm; MeOH/H_2O = 80:20), followed by re-injections using MeCN/H_2O = 65:35, to furnish a pure sample (10.0 mg).



61

Compound **55** (enniatin B): colorless solid. ESI-TOF MS and ^{1}H NMR spectral data were identical to that obtained in section **2.4.1.**

Compound **57** (enniatin H): colorless solid. ^{1}H NMR spectral data was identical to that obtained in section **2.4.2.**

Compound **59** (enniatin I): colorless solid. ^{1}H NMR spectral data was identical to that obtained in section **2.4.2.**

Compound **61** (MK1688):

Colorless gum	
$[\alpha]^{26}_{D}$	-89 (*c* 0.25, $CHCl_3$)
UV (MeOH) λ_{max} (log ε)	207 (4.17)
HRMS (ESI-TOF) *m*/*z*	704.4458 (Δ = 0.4 mmu) $[M + Na]^+$
IR (KBr) ν_{max}	2970, 1737, 1662, 1465, 1191, 1007 cm^{-1}

^{1}H and ^{13}C NMR data, in Table 15 (Results and Discussion).

Figure 6. Extraction of *V. hemipterigenum* BCC 1449 culture + L-isoleucine (20mM)

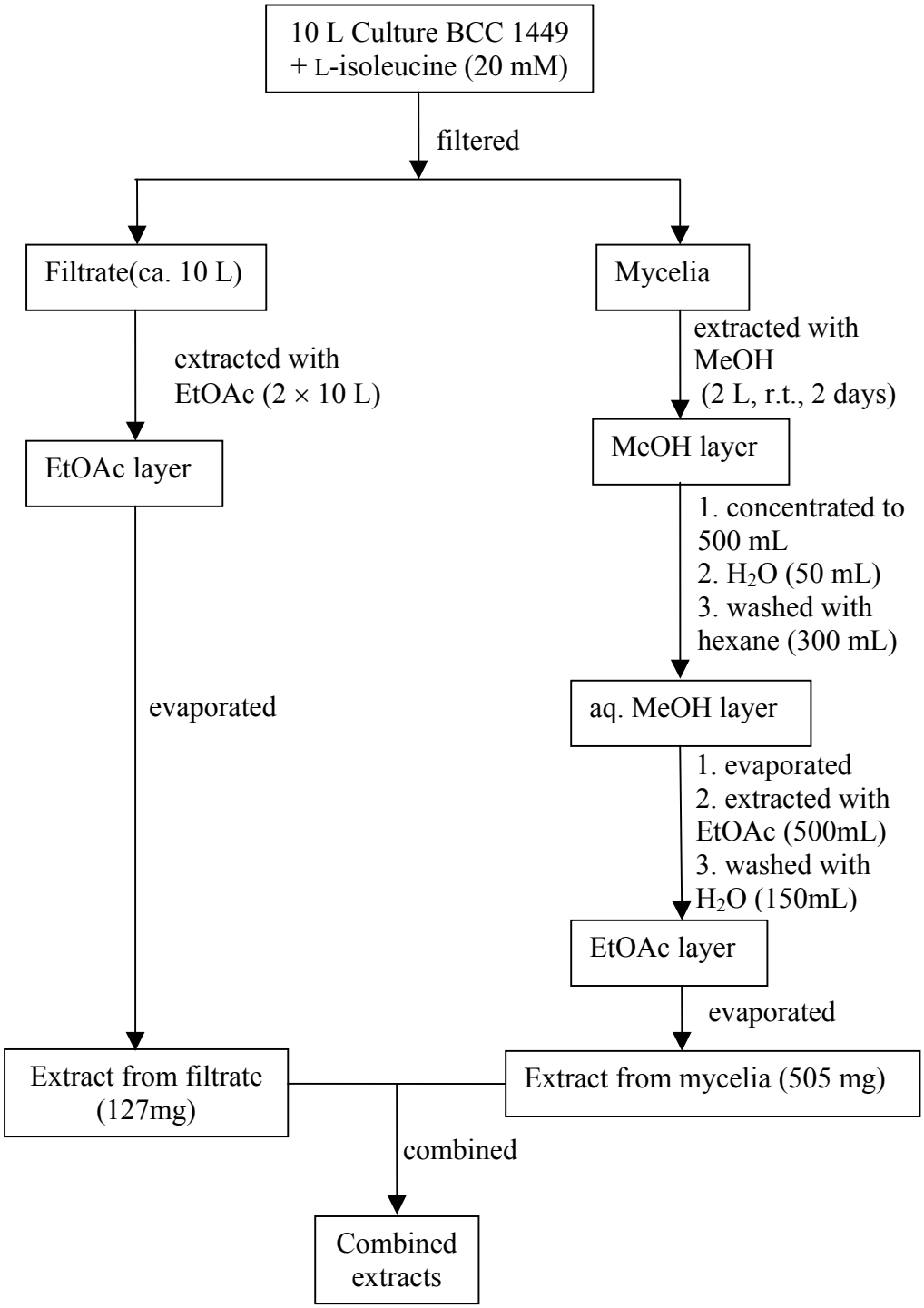
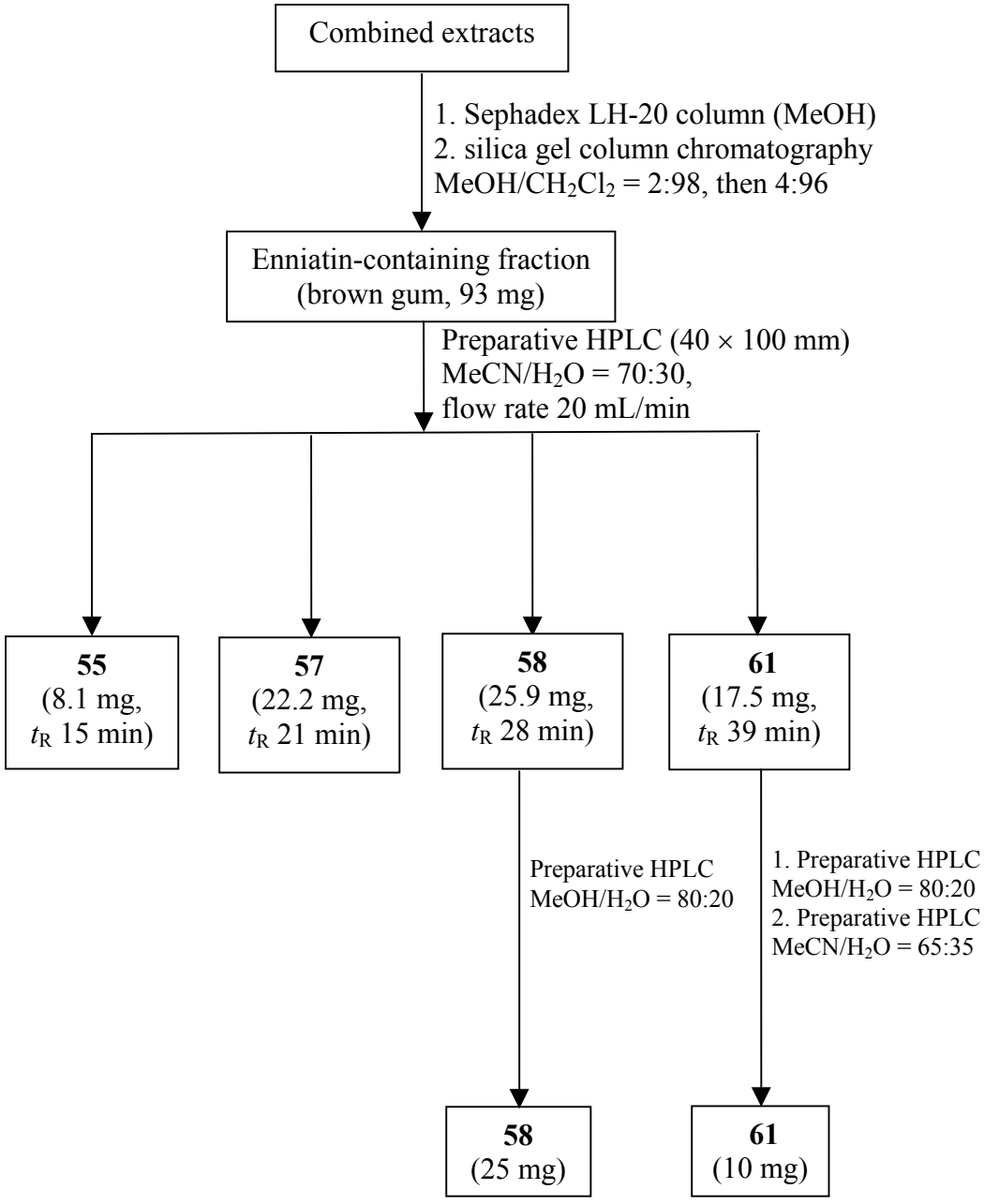


Figure 7. Isolation of compounds **55, 57, 58 and 61** from the combined extracts of *V. hemipterigenum* BCC 1449 + L-isoleucine



2.6 Biological assays

All biological assays for this study were performed by the staffs of the BIOTEC Bioassay Research Laboratory. The assay for activity against *P. falciparum* K1 was performed using a standard protocol (Jaturapat, *et al*., 2001) which follows the microculture radioisotope technique as described by Desjardins (Desjardins, *et al*., 1979). 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* H_{37}Ra was performed using the Microplate Alamar Blue Assay (MABA) (Collins and Franzblau, 1997). Cytotoxic activities of the purified compounds against oral human epidermoid carcinoma (KB cells), human breast cancer (BC cells) and African green monkey kidney fibroblast (Vero cells) were evaluated using colorimetric method (Skehan, *et al*., 1990).