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Precursor-directed biosynthesis of beauvericin analogs by the insect pathogenic fungus *Paecilomyces tenuipes* BCC 1614

Chongdee Nilanonta,[a] Masahiko Isaka,[b,*] Prasat Kittakoop,[b] Srisuda Trakulnaleamsai,[b] Morakot Tanticharoen[b] and Yodhathai Thebtaranonth[a,b]

[a]*Department of Chemistry, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand*
[b]*National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Rama 6 Road, Bangkok 10400, Thailand*

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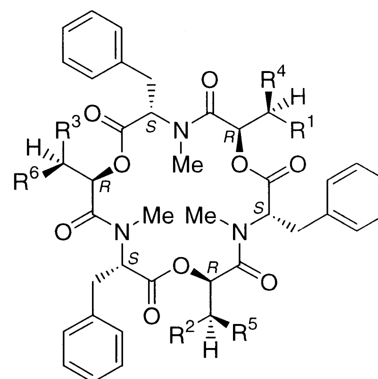
Abstract—Precursor-directed biosynthesis of beauvericin analogs, cyclohexadepsipeptide antibiotics, was investigated using the insect pathogenic fungus *Paecilomyces tenuipes* BCC 1614. Feeding L-isoleucine or D-alloisoleucine in liquid medium led to the enhanced production of beauvericin A and beauvericin B together with beauvericin and a missing analog, beauvericin C. Feeding experiments with D-isoleucine or L-alloisoleucine resulted in the production of diastereomers of beauvericins A, B and C, named allobeauvericins A, B and C, respectively. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

Beauvericin (**1**), a cyclohexadepsipeptide antibiotic consisting of three L-*N*-methylphenylalanine (*N*MePhe) units connected alternatively with three D-2-hydroxyisovaleric acid (Hiv) residues has commonly been isolated from *Beauveria bassiana*[1] and several other fungi.[2–6] Beauvericin is an insecticide toxic to mosquito larvae,[1,2] brine shrimp,[1] blowfly[7] and Colorado potato beetle,[2] and is also active against Gram-positive bacteria and fungi.[1] Gupta et al. isolated new minor analogs, beauvericin A (**2**) and beauvericin B (**3**), possessing one and two 2-hydroxy-3-methylpentanoic acid (Hmp) residues, respectively, instead of Hiv, as minor metabolites from *B. bassiana*.[8] Recently, we reported the isolation of **1** and **2** from an insect pathogenic fungus *Paecilomyces tenuipes* BCC 1614 and evaluation of their antimycobacterial and antiplasmodial activities.[9]

Biosynthetic studies using radio-active substrates have been reported[10,11] where [^{14}C]-2-hydroxyisovaleric acid and [^{14}C]-L-phenylalanine were incorporated into the beauvericin molecule. [^{14}C]-L-valine was also effectively taken up in beauvericin biosynthesis, therefore, it is almost certain that it is the precursor of the D-Hiv residues. Our hypothesis was that the recognition of Hiv (derived from L-valine) by the multifunctional enzyme, beauvericin synthetase, might not be very specific and allow the inclusion of one or two Hmp (possibly derived from L-isoleucine) into the biosynthetic pool leading to the production of minor analogs **2** or **3**. Therefore, directed-biosynthetic study of feeding four isomers of isoleucines, independently as precursors, was conducted employing the fungus *P. tenuipes* BCC 1614.



	R^1	R^2	R^3	R^4	R^5	R^6
beauvericin (**1**)	Me	Me	Me	Me	Me	Me
beauvericin A (**2**)	Et	Me	Me	Me	Me	Me
beauvericin B (**3**)	Et	Et	Me	Me	Me	Me
beauvericin C (**4**)	Et	Et	Et	Me	Me	Me
allobeauvericin A (**5**)	Me	Me	Me	Et	Me	Me
allobeauvericin B (**6**)	Me	Me	Me	Et	Et	Me
allobeauvericin C (**7**)	Me	Me	Me	Et	Et	Et

Figure 1. Structures of beauvericins.

Keywords: beauvericin; cyclodepsipeptides; precursor-directed biosynthesis; antiplasmodial activity; antimycobacterial activity.

* Corresponding author. Tel.: +66-2-6448103; fax: +66-2-6448107; e-mail: isaka@biotec.or.th

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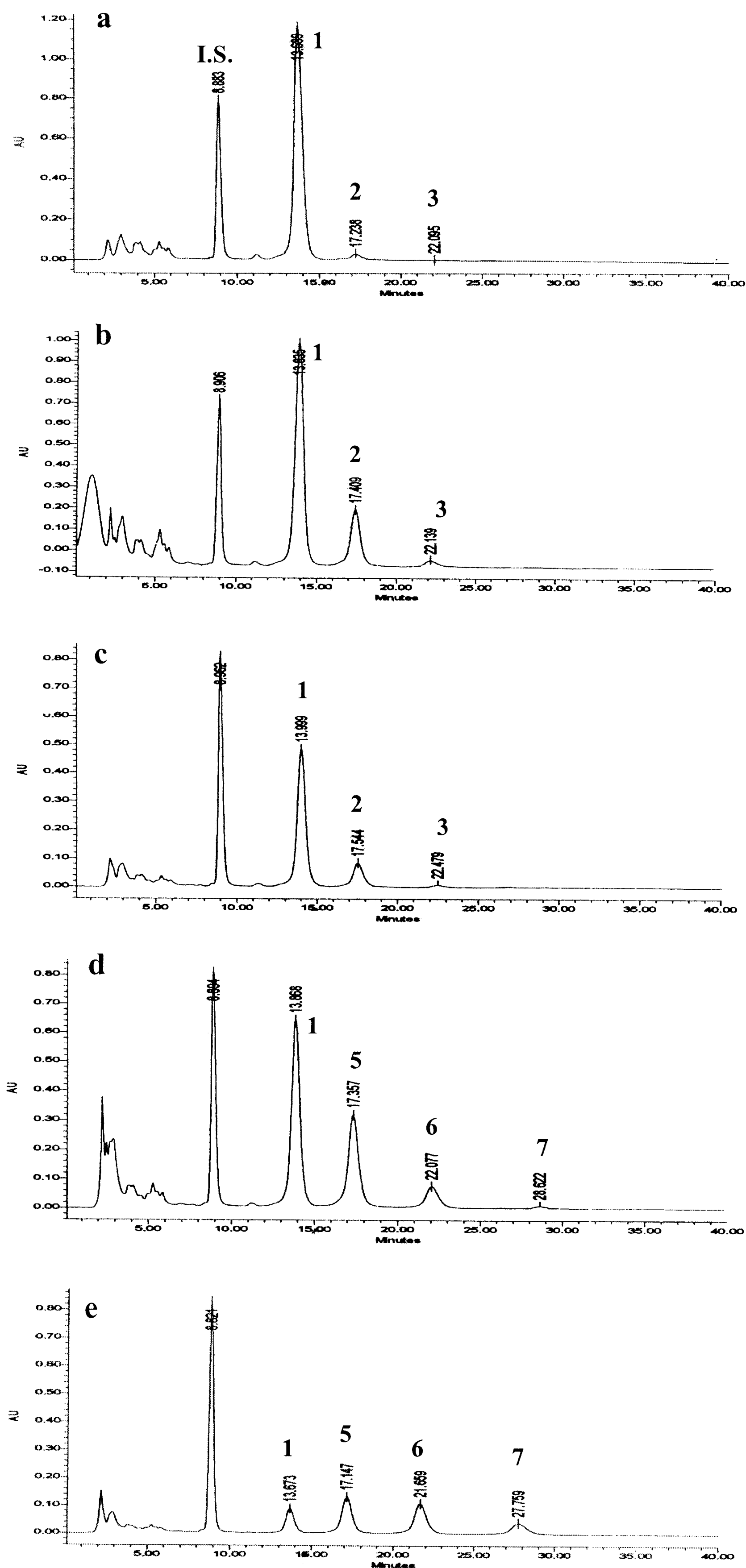

Figure 2. HPLC chromatogram of crude extracts from mycelia (Nova-Pak 8NV4μ, 8×100 mm; eluent, MeCN/H_2O=70:30; flow rate, 1 mL/min; detection, 210 nm): (a) control; (b) fed L-isoleucine (50 mM); (c) fed D-alloisoleucine (50 mM); (d) fed D-isoleucine (50 mM); (e) fed L-alloisoleucine (50 mM). Internal standard (IS): mesitylene.

2. Results and discussion

Isoleucine-fed (50 mM) cultivation of *P. tenuipes* BCC 1614 was assessed in 25 mL of liquid medium (MM), and the mycelial extract was directly analyzed for the production of beauvericins by HPLC–UV (reversed-phase column) using an internal standard (mesitylene, 1.1 mg).

Feeding L-isoleucine ((2*S*,3*S*)-2-amino-3-methylpentanoic acid), the most plausible biosynthetic precursor of **2** and **3**, led to the increased production of **2** and appearance of **3** (Fig. 2b) when compared to the control experiment (no additive; Fig. 2a). Large scale fermentation (40×1 L flasks, each containing 250 mL of L-isoleucine-fed medium; total 10 L) was conducted and from the extract were obtained compounds **1**, **2** and **3** by chromatographic separation/purification. Spectral data of beauvericin (**1**) and beauvericin A (**2**) were identical to those previously isolated from standard fermentation and also to the literature data.[8] Spectral data of beauvericin B (**3**) were identical to literature data.[8] In addition, a third beauvericin isomer (**4**), named beauvericin C, which eluted after **3** on preparative reversed-phase HPLC, was isolated in very small amounts (0.3 mg). The C_3-symmetry of beauvericin C, indicated by the ^{1}H and ^{13}C NMR spectra and its molecular formula of $C_{48}H_{63}N_3O_9$ (HRMS), was consistent with the assigned structure (**4**, in Fig. 1). A similar result was obtained by feeding D-alloisoleucine ((2*R*,3*S*)-2-amino-3-methylpentanoic acid), but with a higher composition of **2** and **3** (Fig. 2c). Physico-chemical data of **1**, **2** and **3**, isolated from the large-scale culture (fed D-alloisoleucine, 500 mL), were identical to those from L-isoleucine-fed experiment. Since both the precursors, L-isoleucine and D-alloisoleucine, have (3*S*)-configuration at the β-positions, it is apparent that Hmp residues in **2**, **3** and **4** have identical absolute configuration, (*S*), at the corresponding chiral carbon center.

Feeding D-isoleucine ((2*R*,3*R*)-2-amino-3-methylpentanoic acid) or L-alloisoleucine ((2*S*,3*R*)-2-amino-3-methylpentanoic acid) resulted in the production, along with **1**, of three analogs with close retention times in HPLC analysis to those of **2**, **3** and **4** (Fig. 2d and e). The three compounds (**5**, **6** and **7**), isolated from large scale cultures (fed D-isoleucine, 100 mL culture, and fed L-alloisoleucine, 500 mL culture), had identical molecular formulae (HRMS) to those of **2**, **3** and **4**, respectively, and also showed indistinguishable IR and UV spectra. NMR spectra of **5**–**7** were, respectively, similar to those of **2**–**4**, but were apparently different compounds as displayed by the different ^{1}H and ^{13}C chemical shifts of the Hmp residues. Structures of **5**–**7** assigned by 2D-NMR analyses were consistent with those shown in Fig. 1. In particular, compound **7** possessed a C_3-symmetry as indicated by its NMR spectra. Compounds **5**, **6** and **7**, having (3*R*)-configuration at the β-position of the Hmp residues, are diastereomeric to beauvericins A, B and C, hence, named allobeauvericins A, B and C, respectively.

The absolute configuration (2*R*) at the α-positions of the Hmp residues in compounds **2**–**7** was deduced by analogy to beauvericin (**1**). This was strongly supported by the NOESY analyses. The crystal structure of beauvericin (**1**) was previously reported, where α-protons of Hiv residues and *N*-methyl groups are both directed to the same side and vertically oriented to the depsipeptide ring, and are opposite to the three α-protons of *N*MePhe residues.[12] In our NOESY

Table 1. ^{13}C NMR data of compounds **3**–**7** (in $CDCl_3$)

Position	**3**	**4** (symmetrical)	**5**	**6**	**7** (symmetrical)
Hiv	1 unit	–	2 units	1 unit	–
1 *C*=O	169.53		169.78[a], 169.64[a]	169.74[b]	
2	75.53		75.64×2	75.76	
3	29.72		29.74, 29.70	29.75	
4	17.43		17.43×2	17.42	
4′	18.29		18.32×2	18.37	
Hmp	2 units (2*R*,3*S*)	3 units (2*R*,3*S*)	1 unit (2*R*,3*R*)	2 unit (2*R*,3*R*)	3 units (2*R*,3*R*)
1 *C*=O	169.53×2	169.35×3	169.61[a]	169.89×2[b]	170.01×3
2	74.36, 74.31	74.23×3	74.27	74.39×2	74.62×3
3	35.89×2	35.86×3	35.62	35.65, 35.62	35.69×3
4	24.41, 24.37	24.47×3	24.53	24.60, 24.57	24.62×3
5	11.34×2	11.33×3	10.63	10.62×2	10.65×3
3-CH_3	14.38×2	14.33×3	13.27	13.24×2	13.29×3
*N*MePhe (3 units)					
1 *C*=O	169.96×2, 169.93	169.94×3	169.99[a], 169.94[a], 169.92[a]	169.94×3[b]	170.01×3
2	57.28, 57.17×2	57.38×3	57.36×2, 56.64	57.47, 56.72×2	56.89×3
3	34.75, 34.69, 34.69	34.66×3	34.84, 34.73, 34.62	34.86, 34.76, 34.63	34.75×3
4	136.58×2, 136.55	136.60×3	136.64, 136.58, 136.48	136.64, 136.53, 136.45	136.50×3
5, 9	128.53×2 128.46×4	128.46×6	128.53×6	128.53×6	128.56×6
6, 8	128.85×6	128.91×6	128.81×6	128.80×2 128.77×4	128.75×6
7	126.77×3	126.76×3	126.77×3	126.78×3	126.82×3
N-CH_3	32.15×3	32.25×3	32.33×2, 31.86	32.40, 31.91×2	31.96×3

[a,b] assignments can be interchanged.

Table 2. ^{1}H NMR data of compounds **4–7** (in $CDCl_3$)

Position	**4** (mult, *J* in Hz) (symmetrical)	**5** (mult, *J* in Hz)	**6** (mult, *J* in Hz)	**7** (mult, *J* in Hz) (symmetrical)
Hiv	–	2 units	1 unit	–
2		4.86 (2H, d, 8.5)	4.83 (1H, d, 8.4)	
3		1.98 (2H, m)	1.98 (1H, m)	
4		0.41 (6H, d, 6.7)	0.42 (3H, d, 6.8)	
4′		0.81 (6H, d, 6.6)	0.81 (3H, d, 6.6)	
Hmp	3 units (2*R*,3*S*)	1 unit (2*R*,3*R*)	2 units (2*R*,3*R*)	3 units (2*R*,3*R*)
2	5.01 (3H, d, 7.7)	4.96 (1H, d, 9.1)	4.94 (2H, d, 9.0)	4.91 (3H, d, 8.7)
3	1.74 (3H, m)	1.79 (1H, m)	1.78 (2H, m)	1.77 (3H, m)
4	0.76–0.67 (6H, m)	1.37 (1H, m) 0.94 (1H, m)	1.38 (2H, m) 0.95 (2H, m)	1.39 (3H, m) 0.96 (3H, m)
5	0.67 (9H, m)	0.75 (3H, t, 7.4)	0.74 (6H, t, 7.4)	0.75 (9H, t, 7.4)
3-CH_3	0.76 (9H, d, 6.7)	0.32 (3H, d, 6.8)	0.32 (3H, d, 6.8) 0.31 (3H, d, 6.8)	0.33 (9H, d, 6.6)
*N*MePhe (3 units)				
2	5.51 (3H, dd, 11.6, 4.4)	5.67 (1H, dd, 12, 4.8) 5.51 (2H, m)	5.65 (2H, m) 5.47 (1H, dd, 11.6, 4.3)	5.63 (3H, m)
3	3.36 (3H, dd, 14.6, 4.9) 2.97 (3H, dd, 14.6, 11.8)	3.38 (3H, dd, 14.6, 4.8) 2.95 (3H, m)	3.38 (3H, dd, 14.6, 4.9) 2.95 (3H, m)	3.39 (3H, dd, 14.6, 4.8) 2.93 (3H, dd, 14.5, 12.2)
5–9	7.17–7.28 (15H, m)	7.16–7.28 (15H, m)	7.15–7.28 (15H, m)	7.13–7.27 (15H, m)
N-CH_3	2.98 (9H, s) 3.01 (3H, s) 3.05 (3H, s)	3.00 (3H, s) 3.03 (3H, s) 3.05 (3H, s)	2.99 (3H, s)	3.03 (9H, s)

analyses of **1–7** (in $CDCl_3$), intense correlations between α-protons of the 2-hydroxycarboxylic acid (Hiv and Hmp) residues and *N*-methyl protons were observed while there was only a very weak intensity of the cross signals between the α-protons of *N*MePhe and *N*-methyl protons. Therefore, all compounds (**1–7**) should possess very similar cyclodepsipeptide stereo-structure (Tables 1 and 2).

Peeters et al. previously reported their work on substrate specificity in cell-free beauvericin biosynthesis using purified beauvericin synthetase from *B. bassiana*, where they argued that phenylalanine moieties were replaced by a number of amino acids, e.g. β-phenylserine, *ortho*-, *meta*-, *para*-fluorophenylalanine, isoleucine, norleucine and leucine.[13] The difference with our results could be the fact that the transformation system from α-amino acid to 2-hydroxycarboxylic acid is lacking in the biosynthesis using purified beauvericin synthetase.

The beuvericin isomers were tested for activities against *Mycobacterium tuberculosis* H37Ra and *Plasmodium falciparum* K1, and also for their cytotoxic activity towards two cancer cell-lines (KB, BC-1) and Vero cells (Table 3). All the beauvericins showed similar antimycobacterial (MIC 0.8–1.6 μg/mL) and antiplasmodial (IC_{50} 1.3–2.4 μg/mL) activities, however, these activities may be related to their cytotoxic behavior.

Precursor-directed biosynthesis is a common method for production of unnatural derivatives by feeding substrate analogs.[14] The novelty of our system is that all three positions in beauvericin molecule are equally subjected to replacement by the precursor, thus, three analogs of **1** can be produced in the same culture. Scrambled feeding using two or more precursors may well result in the production of multiple analogs. In this context, we are currently investigating the scope and limitation of this strategy applying various α-amino acids and 2-hydroxycarboxylic acids as precursors.

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

Table 3. Antimycobacterial, antiplasmodial and cytotoxic activities of beauvericin isomers

Compound	*M. tuberculosis*[a] (MIC, μg/mL)	*P. falciparum*[b] (IC_{50}, μg/mL)	Cytotoxicity[c] (IC_{50}, μg/mL)		
			KB	BC-1	Vero
1	1.6	1.3	>20	15	10
2	1.6	1.8	13	14	9.1
3	1.6	2.3	10	9.0	9.1
5	0.8	2.0	>20	15	5.9
6	0.8	2.4	>20	4.4	4.4
7	0.8	1.6	14	3.3	5.2

Assays for beauvericin C (**4**) were not conducted due to the small sample amount.
[a] MIC value of the standard drug, isoniazide, was 0.050 μg/mL.
[b] IC_{50} values of the standard antimalarial compounds, chloroquine diphosphate and artemisinin, were 0.16 and 0.0011 μg/mL, respectively.
[c] IC_{50} values of 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.

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

P. tenuipes was collected from Khlong Naka Wildlife Sanctuary, Ranong province, southern Thailand, and identified by Dr Nigel L. Hywel-Jones of Mycology Research Unit, BIOTEC. The culture is deposited at the Thailand BIOTEC Culture Collection as BCC 1614.

3.3. Feeding experiments in analytical scale

L-Isoleucine-fed (50 mM) cultivation of *P. tenuipes* BCC 1614 was assessed in a 100 mL Erlenmeyer flask containing 25 mL of minimum salt medium (MM; composition, glucose 20.0 g, NH_4NO_3 3.0 g, KH_2PO_4 0.5 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $CaCl_2$ 0.5 g and yeast extract 1.0 g, per 1000 mL of distilled water). After static incubation for 21 days, the culture was filtered and the residue (mycelial cake) was extracted with MeOH (200 mL). After partial concentration to 100 mL, H_2O (10 mL) was added, washed with hexane (60 mL), and the aqueous MeOH layer was concentrated under reduced pressure. The residual oil was dissolved in EtOAc (100 mL), washed with H_2O (10 mL), then concentrated to obtain a light-brown amorphous solid. This extract was analyzed for the production of beauvericins by HPLC/UV using a reversed-phase column (Nova-Pak 8NV4μ, 8×100 mm; eluent, MeCN/H_2O=70:30; flow rate, 1 mL/min; detection, 210 nm) with an internal standard, mesitylene (1.1 mg, t_R 9 min). Other feeding experiments and control (no additive) fermentation were conducted at the same time and in the same manner.

3.4. Typical procedure for extraction and isolation from the large scale cultures

Large scale fermentation of *P. tenuipes* BCC 1614 with L-alloisoleucine (20 mM) was conducted in 2×1 L Erlenmeyer flasks each containing 250 mL of liquid medium (MM). The flask cultures were filtered to separate into mycelia and supernatant. The mycelial cakes were extracted with MeOH (400 mL, rt, 2 days). After partial concentration to 100 mL, H_2O (10 mL) was added, washed with hexane (50 mL), and the aqueous methanol layer was concentrated under reduced pressure. The residual oil was dissolved in EtOAc (100 mL), washed with H_2O (30 mL), then concentrated to obtain a light-brown gum (121 mg). This mycelial extract was subjected to Sephadex LH20 column chromatography (MeOH as eluent). The beauvericins-containing fraction was subjected to preparative HPLC using a reversed-phase column (Prep Nova-Pak HR C_{18}, 6 μm, 40×100 mm) with 70% MeCN/H_2O as eluent at a flow rate of 20 mL/min to obtain beauvericin isomers in the following order of elution: beauvericin (**1**, 10.2 mg, t_R 20 min), allobeauvericin A (**5**, 13.6 mg, t_R 25 min), allobeauvericin B (**6**, 10.4 mg, t_R 33 min) and allobeauvericin C (**7**, 3.1 mg, t_R 44 min). Other feeding experiments on large scale were carried out in the same manner.

3.4.1. Beauvericin (1). Colorless crystals; mp 94–97°C (MeOH–H_2O); $[\alpha]_D^{28}$=+69 (*c* 1.08, MeOH); UV, IR, MS and NMR data were identical to those reported in the literature.[8]

3.4.2. Beauvericin A (2). Colorless powder; mp 93–95°C; $[\alpha]_D^{25}$=+57 (*c* 0.18, MeOH); UV, IR, MS and NMR data were identical to those reported in the literature.[8]

3.4.3. Beauvericin B (3). Colorless powder; mp 81–83°C; $[\alpha]_D^{25}$=+69 (*c* 0.09, MeOH); UV, IR, MS and ^{1}H NMR data were identical to those reported in the literature;[8] ^{13}C NMR data, Table 1.

3.4.4. Beauvericin C (4). Colorless amorphous solid; UV (MeOH) λ_{max} 204 nm; IR (KBr) ν_{max} 2964, 2929, 1741, 1651, 1455, 1209, 1010, 745, 699 cm^{-1}; HRMS (ESI-TOF) *m/z* 848.4461 [M+Na]$^+$ (calcd for $C_{48}H_{63}N_3O_9Na$ 848.4462); NMR data, Tables 1 and 2.

3.4.5. Allobeauvericin A (5). Colorless crystals; mp 91–94°C; $[\alpha]_D^{25}$=+65 (*c* 0.20, MeOH); UV (MeOH) λ_{max} 206 nm; IR (KBr) ν_{max} 2965, 2934, 1742, 1655, 1456, 1197, 1017, 750, 699 cm^{-1}; HRMS (ESI-TOF) *m/z* 820.4160 [M+Na]$^+$ (calcd for $C_{46}H_{59}N_3O_9Na$ 820.4149); NMR data, Tables 1 and 2.

3.4.6. Allobeauvericin B (6). Colorless powder; mp 90–92°C; $[\alpha]_D^{25}$=+83 (*c* 0.21, MeOH); UV (MeOH) λ_{max} 205 nm; IR (KBr) ν_{max} 2965, 2934, 1741, 1655, 1456, 1197, 1017, 749, 699 cm^{-1}; HRMS (ESI-TOF) *m/z* 834.4290 [M+Na]$^+$ (calcd for $C_{47}H_{61}N_3O_9Na$ 834.4306); NMR data, Tables 1 and 2.

3.4.7. Allobeauvericin C (7). Colorless powder; mp 79–82°C; $[\alpha]_D^{25}$=+102 (*c* 0.055, MeOH); UV (MeOH) λ_{max} 206 nm; IR (KBr) ν_{max} 2964, 2933, 1741, 1651, 1456, 1211, 1017, 747, 699 cm^{-1}; HRMS (ESI-TOF) *m/z* 848.4482 [M+Na]$^+$ (calcd for $C_{48}H_{63}N_3O_9Na$ 848.4462); NMR data, Tables 1 and 2.

3.5. Biological assays

Growth inhibitory activity against *M. tuberculosis* H37Ra was performed using the Microplate Alamar Blue Assay (MABA).[15] The assay for activity against *P. falciparum* K1 was performed using the standard protocol,[16] which follows the microculture radioisotope technique as described by Desjardins.[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 the colorimetric method.[18]

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