Cytotoxic and Antihaptotactic Beauvericin Analogues from Precursor-Directed Biosynthesis with the Insect Pathogen *Beauveria bassiana* ATCC 7159

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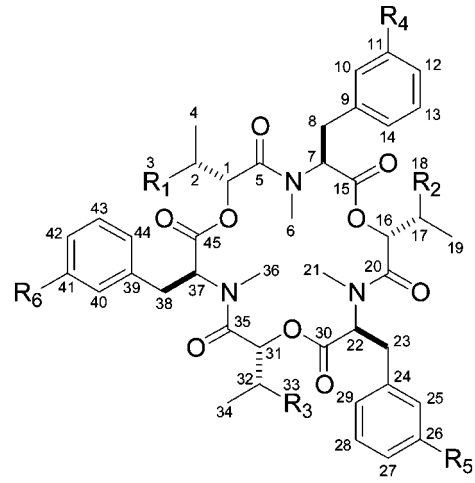
Precursor-directed biosynthesis was used to produce analogues of the cyclic depsipeptide mycotoxin beauvericin (**1**) using the filamentous fungus *Beauveria bassiana* ATCC 7159. Feeding 30 analogues of D-2-hydroxyisovalerate and L-phenylalanine, the natural 2-hydroxycarboxylic acid and amino acid precursors of beauvericin, led to the biosynthesis of novel beauvericins. Six of these were isolated and characterized, and their cytotoxicity and directional cell migration (haptotaxis) inhibitory activity against the metastatic prostate cancer cell line PC-3M were evaluated. Replacement of one, two, or all three of the D-2-hydroxyisovalerate constituents in beauvericin (**1**) with 2-hydroxybutyrate moieties (beauvericins G_{1-3}, compounds **2**–**4**) caused a parallel decline of cell migration inhibitory activity and cytotoxicity, suggesting a requirement for a branched side chain for both of these biological activities at the corresponding positions of beauvericins. Replacement of one, two, or all three *N*-methyl-L-phenylalanine residues of beauvericin with *N*-methyl-L-3-fluorophenylalanine moieties (beauvericins H_{1-3}, compounds **5**–**7**) increased cytotoxicity without affecting antihaptotactic activity.

The filamentous fungus *Beauveria bassiana* (Hyphomycetes) was identified by A. Bassi in 1835 as the causative agent of the white muscardine disease in *Bombyx mori* (domestic silkworm). The discovery of a link between a microorganism and pathogenicity contributed to the development of our modern understanding of communicable diseases in the later work of Pasteur and Koch. The broad spectrum and potent insect pathogenicity of this fungus has since then been exploited to assess and develop biocontrol strategies against many insect pests.[1] *B. bassiana* produces several secondary metabolites, including the 2-pyridone tenellin,[2] the cyclic peptides beauvericin (**1**, Figure 1),[3] bassianolide,[4] and beauverolides,[5] and the dibenzoquinone oosporein.[2] The biosynthetic gene cluster for the production of the mycotoxin tenellin has been cloned, but a tenellin nonproducer knockout strain of *B. bassiana* has been found to display undiminished virulence toward *Galleria mellonella* (wax moth) larvae.[6] Beauvericin (**1**) is the main fermentation product of *B. bassiana* under laboratory conditions. Although beauvericin (**1**) has also been isolated from larvae of silkworm killed by *B. bassiana* infection,[7] its role in insect pathogenesis is not clear. It was shown to be toxic to the larvae of the Colorado potato beetle[8] and mosquitoes,[3] but was apparently not toxic to larvae of the corn earworm.[9] It was also shown to be toxic to the brine shrimp (*Artemia salina*), to display moderate inhibitory activity against filamentous fungi and Gram-positive bacteria,[3] and to potentiate the fungicidal activity of fluconazole against *Candida albicans.*[10–12] Importantly, beauvericin (**1**) was shown to display potent cytotoxic activity against different human cell lines.[13] A cationophore, beauvericin (**1**) increases cytoplasmic Ca^{2+} concentration, disturbs the physiological ion balance and pH, causes ATP depletion, and eventually activates calcium-sensitive cell apoptotic pathways involving pro- and antiapoptotic Bcl-2 family proteins, the release of mitochondrial cytochrome *c*, and the activation of caspase 3.[14,15]

We have previously reported that beauvericin (**1**) also impedes the haptotactic motility of cancer cells at subcytotoxic concentrations, as indicated by the inhibition of the closure of a "wound" made in a confluent cell monolayer.[16] Haptotaxis (directional cell motility) is an essential process in the formation of new blood vessels in tumors (angiogenesis), cancer cell invasion, and metastasis.[17] Inhibition of angiogenesis is a validated cancer chemotherapeutic strategy, as shown by thalidomide and bevacizumab, and is one of the established mechanisms of actions of the marketed drugs sunitinib, sorafenib, and paclitaxel.[18,19] Efficient inhibition of cell motility might arrest cancer development at the stage of the angiogenic switch. Any side effects caused by such an intervention might also prove clinically manageable, as cell migration is rather infrequent in adults under ordinary physiological conditions.[20] Natural products such as withaferin A,[21] and migrastatin[22] as well as its synthetic analogues,[20] constitute potent inhibitors of cell migration. The antihaptotactic and the cell proliferation inhibition



Compound	R_1	R_2	R_3	R_4	R_5	R_6
Beauvericin (**1**)	Me	Me	Me	H	H	H
Beauvericin G_1 (**2**)	H	Me	Me	H	H	H
Beauvericin G_2 (**3**)	H	H	Me	H	H	H
Beauvericin G_3 (**4**)	H	H	H	H	H	H
Beauvericin H_1 (**5**)	Me	Me	Me	F	H	H
Beauvericin H_2 (**6**)	Me	Me	Me	F	F	H
Beauvericin H_3 (**7**)	Me	Me	Me	F	F	F

Figure 1. Structures of beauvericins.

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Table 1. Precursor Analogues Used for Fermentations

analogues of D-Hiv[a]	final concentration[b]	analogues of L-Phe	final concentration
DL-2-hydroxybutyric acid (Hbu)	10.0 mM	DL-3-fluorophenylalanine	0.3 mM
D-2-hydroxybutyric acid (D-Hbu)	10.0 mM	D-3-fluorophenylalanine	0.3 mM
L-2-hydroxybutyric acid (L-Hbu)	10.0 mM	L-3-fluorophenylalanine	0.3 mM
L-isoleucine (L-Ile)	10.0 mM	DL-2-fluorophenylanine	1.0 mM
L-leucine (L-Leu)	10.0 mM	DL-4-fluorophenylalanine	1.0 mM
2-hydroxy-4-methylpentanoate (Hic)	10.0 mM	DL-2-chlorophenylalanine	0.3 mM
lactic acid	10.0 mM	L-3-chlorophenylalanine	0.3 mM
2-oxovaleric acid	10.0 mM	DL-4-chlorophenylalanine	10.0 mM
β-chlorolactic acid	10.0 mM	DL-3-bromophenylalanine	0.3 mM
L-threonine (L-Thr)	10.0 mM	L-tyrosine (L-Tyr)	10.0 mM
L-serine (L-Ser)	10.0 mM	DL-*m*-tyrosine	10.0 mM
DL-mandelic acid	10.0 mM	DL-*o*-tyrosine	10.0 mM
(*S*)-(+)-hexahydromandelate	1.0 mM	L-4-aminophenylalanine	10.0 mM
(*R*)-(−)-hexahydromandelate	3.0 mM	(*S*)-4-nitrophenylalanine	10.0 mM
		3-(2-thienyl)-DL-alanine	10.0 mM
		DL-3-phenylserine	10.0 mM

[a] D-Hiv, D-hydroxyisovalerate [(2*R*)-2-hydroxy-3-methylbutanoate]. [b] Final concentration of the precursor analogue supplemented during the fermentation.

activities of other natural products such as the taxanes[23] and the vinca alkaloids[24] at subcytotoxic concentrations were shown to have distinct mechanisms of actions. The cytotoxic and antiangiogenic activities of the bacterial polyketide borrelidin were successfully separated by preparing borrelidin analogues via precursor-directed biosynthesis.[25]

Beauvericin (**1**) is a cyclic ester of a trimer of (2*R*)-2-hydroxy-3-methylbutanoyl-*N*-methyl-L-phenylalanine, formed by a nonribosomal peptide synthetase enzyme.[26] This multienzyme activates the α-hydroxycarboxylic acid (2*R*)-2-hydroxy-3-methylbutanoate (D-hydroxyisovalerate, D-Hiv) and the amino acid phenylalanine (L-Phe) as adenylates, captures these activated substrates as the corresponding thioesters on thiolation domains of the synthetase, *N*-methylates the enzyme-bound L-Phe residue, catalyzes the condensation of the two substrates to form enzyme-bound intermediates, and finally releases the free cyclic trimeric lactone. The biosynthesis of related depsipeptide mycotoxins enniatins (cyclic trimeric esters of D-Hiv–L-Ile/Val/Leu) follows a similar enzymatic logic.[27] Beauvericin (**1**) is produced by several different fungal strains in addition to *B. bassiana,* including *Paecilomyces fumosoroseus*,[28] *Polyporus sulphureus*,[29] and *Fusarium* spp.[8,16] Beauvericin analogues have been isolated as minor products in fermentations. Thus, beauvericins A and B, with (2*R*)-2-hydroxy-3-methylpentanoate (D-2-hydroxy-3-methylvalerate, D-Hmv) residues replacing one or two D-Hiv residues, respectively, are produced by *B. bassiana* ARSEF 4122 and were evaluated in an insecticidal assay.[30] Beauvericins D (L-Phe replacing one *N*-methyl-L-Phe residue), E (L-Leu instead of one *N*-methyl-L-Phe residue), and F [(2*R*)-2-hydroxy-4-methylpentanoate or D-2-hydroxyisocaproate, D-Hic, instead of one D-Hiv] were isolated from *Beauveria* sp. FKI-1366 and evaluated in an antifungal assay.[10,11] Precursor-directed biosynthesis with *Paecilomyces tenuipes* BCC 1614 yielded beauvericins A and B and the missing congener beauvericin C upon feeding with L-Ile, while feeding with D-alloisoleucine yielded the corresponding diastereomers, allobeauvericins A, B, and C, all of which have shown slightly increased cytotoxicity against selected cancer cell lines.[28]

Beauvericin analogues with less toxicity and improved antihaptotactic activity might constitute interesting lead compounds for anticancer drug discovery. In an attempt to produce such compounds, we have used precursor-directed biosynthesis by feeding 30 hydroxycarboxylic acid and amino acid precursor analogues to *Beauveria bassiana* ATCC 7159 (Table 1). Herein, we report these feeding experiments and the isolation, structure elucidation, and biological characterization of six new beauvericin analogues, beauvericins G_{1-3} (**2**–**4**) and H_{1-3} (**5**–**7**) in cytotoxicity and haptotaxis inhibition assays.

Results and Discussion

Precursor-directed biosynthesis is a widely used method to produce structural analogues of natural products.[31] We have fed hydroxycarboxylate and amino acid analogues of D-Hiv and L-Phe to *B. bassiana* ATCC 7159 (Table 1), a beauvericin producer that is also widely used for biotransformations.[32–34] Amino acids might replace L-Phe, while 2-hydroxycarboxylic acids might substitute for D-Hiv in beauvericin (**1**). Branched-chain amino acids can also be converted to 2-ketocarboxylic acids by *B. bassiana* branched-chain amino acid aminotransferase (BCAAT) and further to 2-hydroxycarboxylic acids by branched-chain 2-ketocarboxylic acid reductase (BCKR),[35] which could displace D-Hiv in beauvericin (**1**). Incorporation of each precursor analogue is expected to yield up to three products by replacement of one, two, or all three positions of the corresponding natural amino- or hydroxycarboxylic acid constituent of beauvericin (**1**) or might even yield up to six products if a precursor analogue was able to substitute both constituents.

A control experiment with feeding L-Ile afforded the known analogue beauvericin A in a good yield (approximately 2 mg/L, corresponding to ~10% of the yield of beauvericin) and its known congeners beauvericins B and C in minor amounts (less than 0.05 mg/L), all of which were identified by LC-MS (data not shown). This indicated that our *B. bassiana* strain, similar to *Paecilomyces tenuipes,*[28] is able to take up L-Ile, convert it to D-Hmv, and incorporate this precursor analogue into beauvericin-like products. L-Val failed to yield new beauvericin analogues by replacing L-Phe, as noted previously during *in vitro* labeling experiments with a purified beauvericin synthetase enzyme.[26] Since L-Val is the biosynthetic precursor of D-Hiv via the concerted actions of BCAAT and BCKR of the fungus,[35] feeding L-Val to *B. bassiana* ATCC 7159 nevertheless led to a substantial increase in the production of beauvericin (**1**). L-Leu feeding failed to yield the known analogue beauvericin E[11] by replacing an L-Phe moiety, nor did it replace D-Hiv as a D-Hic moiety: 2-ketoisocaproic acid, presumably derived from L-Leu by BCAAT, has been shown to be a very weak substrate for the *Beauveria* BCKR enzyme.[35] Another gatekeeper against the substitution of D-Hiv by D-Hic in beauvericins must be the beauvericin synthetase itself: direct feeding of 2-hydroxy-4-methylpentanoate (D-Hic) to *B. bassiana* ATCC 7159 also failed to yield beauvericin analogues (data not shown).

Supplementation of *B. bassiana* fermentations with DL-2-hydroxybutyric acid (Hbu) afforded compounds **2**–**4** (Figures 1 and 2A) in good yields (approximately 1.0, 2.0, and 1.0 mg/L, respectively). The $[M + 1]^+$ ion peak at *m/z* 742.3672 in the HRFABMS of **4** showed that this compound has a molecular formula of $C_{42}H_{51}N_3O_9$, with C_3H_6 less than beauvericin, suggesting

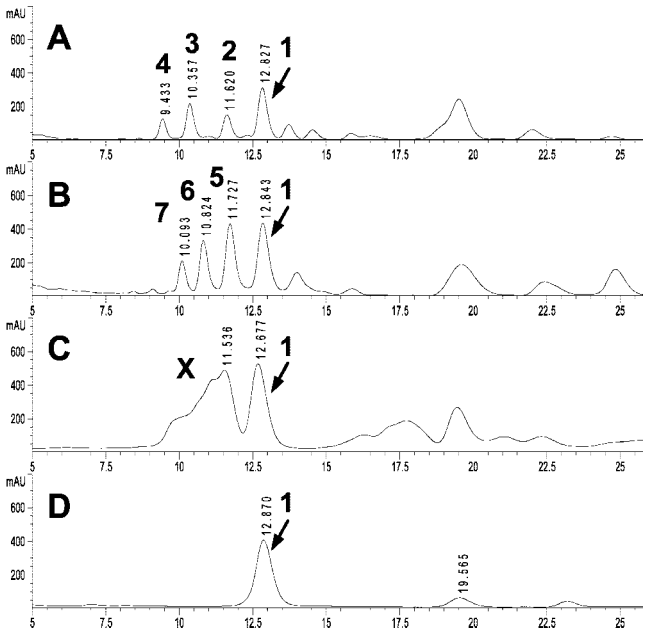

Figure 2. HPLC chromatograms of crude extracts from mycelia of *B. bassiana* ATCC 7159 after feeding during the fermentations with (A) DL-2-hydroxybutyric acid (Hbu), (B) DL-3-fluorophenylalanine, (C) DL-2-fluorophenylalanine, and (D) no precursor analogues. **1**, Beauvericin; **2**, beauvericin G_1; **3**, beauvericin G_2; **4**, beauvericin G_3; **5**, beauvericin H_1; **6**, beauvericin H_2; **7**, beauvericin H_3; X, unseparated mixture of putative beauvericins with one, two, and three *N*-methyl-L-2-fluorophenylalanine(s) instead of *N*-methyl-L-Phe.

the replacement of all three D-Hiv residues of beauvericin (**1**) with Hbu. This deduction was supported by the 11 carbon signals observed in its ^{13}C NMR spectrum, which indicated that this compound has a symmetrical structure.[28] In the ^1H NMR spectrum, the only triplet (including three CH_3 groups) at δ 0.64 instead of two CH_3 doublets at δ 0.83 and 0.47, like that of beauvericin (**1**), further confirmed that compound **4** is a new cyclic hexadepsipeptide consisting of alternating *N*-methyl-L-Phe and Hbu residues. This compound was named beauvericin G_3. Compound **3** has a molecular formula of $C_{43}H_{53}N_3O_9$, as indicated by HRFABMS, suggesting that it contains one CH_2 unit more than compound **4**. A triplet representing two methyl groups and two separated CH_3 doublets were observed in its ^1H NMR spectrum at δ 0.75, 0.66, and 0.39, respectively. This observation suggested that two Hbu and one D-Hiv were incorporated into the structure of compound **3**. The structure was confirmed by its ^{13}C NMR spectrum. Similarly, compound **2** was found to have one unit of Hbu and two units of D-Hiv, with a molecular formula of $C_{44}H_{55}N_3O_9$. Compounds **2** and **3** were named beauvericins G_1 and G_2, respectively. The optical rotations of compounds **2**–**4** were determined and found to show only limited differences compared to that of beauvericin (**1**), implying that the configuration of the C-2 centers of the incorporated hydroxycarboxylic acids are (2*R*), the same as in beauvericin (**1**). A further biosynthetic proof was derived by feeding with enantiomerically pure (>99%) D-Hbu and L-Hbu, respectively: as expected, only (2*R*)-2-hydroxybutyrate (D-Hbu) supported the biosynthesis of beauvericins G_{1-3}. Fittingly, purified BCKR from *Fusarium sambucinum*[35] that generates D-Hiv for beauvericin (**1**) biosynthesis and ketopantoate reductase of *E. coli* that catalyzes an analogous reaction[36] have been shown to generate α-hydroxycarboxylic acids with exclusively the (2*R*) configuration. Feeding experiments with the amino acid Ser or Thr, or those using shorter aliphatic, halogenated, or the bulkier aromatic or saturated C_6 ring-containing hydroxycarboxylic acids, failed to substitute for D-Hiv in compound **1**.

Table 2. Cytotoxic and Antihaptotactic Activities of Compounds **1**–**7**

compound	MTT[a]	WHA[b]	MTT/WHA[c]
beauvericin (**1**)	8.0 ± 0.4	3.5 ± 0.4	2.3
beauvericin G_1 (**2**)	5.0 ± 0.4	3.8 ± 0.2	1.3
beauvericin G_2 (**3**)	10.4 ± 0.1	7.6 ± 0.2	1.4
beauvericin G_3 (**4**)	16.3 ± 0.1	17.2 ± 0.1	0.9
beauvericin H_1 (**5**)	7.4 ± 0.3	3.2 ± 0.1	2.3
beauvericin H_2 (**6**)	6.2 ± 0.1	3.2 ± 0.1	1.9
beauvericin H_3 (**7**)	4.4 ± 0.2	3.2 ± 0.4	1.4

[a] MTT, Cytoxicity, expressed as IC_{50} values in μM as measured in the tetrazolium-based assay.[39] [b] WHA, antihaptotactic activity, expressed as IC_{50} values in μM as measured in the wound-healing inhibition assay.[40] [c] MTT/WHA, cytotoxicity vs antihaptotactic activity index.

Feeding experiments of *B. bassiana* with DL-3-fluorophenylalanine afforded three new unnatural beauvericin analogues (beauvericin H_{1-3}, compounds **5**–**7**, Figures 1 and 2B) in good yields (approximately 2.0, 1.0, and 0.5 mg/L), with molecular formulas of $C_{45}H_{56}FN_3O_9$, $C_{45}H_{55}F_2N_3O_9$, and $C_{45}H_{54}F_3N_3O_9$. Compounds **5**–**7** contain one, two, and three *N*-methyl-3-fluorophenylalanine residues, respectively, replacing the *N*-methyl-L-Phe constituents of beauvericin (**1**). While Hbu was well-tolerated by the cells, 3-fluorophenylalanine caused significant growth inhibition above concentrations of 0.3 mM. *N*-Methylation of the fluorinated Phe analogues by the beauvericin synthetase was nevertheless very efficient, as no *N*-desmethyl beauvericin analogues were encountered. The optical rotation values of compounds **5**–**7** were close to that of beauvericin (**1**), suggesting that **5**–**7** contain L-amino acid residues. Feeding with enantiomerically pure (>99%) L-3-fluorophenylalanine and D-3-fluorophenylalanine were equally efficient in producing **5**–**7**, in apparent contradiction to the above result and to those of Peeters et al.,[26] who have shown that purified beauvericin synthetase (albeit from a different *B. bassiana* strain) is unable to utilize D-Phe for the synthesis of beauvericin (**1**). However, *in vivo* interconversion of D and L amino acids, including Phe, using isomerase or racemase,[37] or an oxidase–transaminase pair of enzymes,[38] is well-documented in microorganisms. Another precursor analogue, DL-2-fluorophenylalanine, was similarly found to be inhibitory to fungal growth in concentrations exceeding 1.0 mM. Although feeding with DL-2-fluorophenylalanine afforded beauvericin-like compounds with molecular weights of 802, 820, and 838 (based on their LC-MS profiles) in good yields (approximately 6 mg/L for the mixture of new compounds, Figure 2C), indicating the efficient replacement of one, two, and three *N*-methyl-L-Phe residues of beauvericin (**1**) with *N*-methyl-2-fluorophenylalanine(s), respectively, we were unable to separate the individual compounds in this mixture under a variety of HPLC conditions tested. No incorporation of *ortho*- or *meta*-chloro- or bromophenylalanine was possible, nor did *para*-halogenated Phe analogues substitute for L-Phe. Similarly, polar substituents (hydroxyl, nitro, amino) on the benzyl group and a heterocyclic ring (thienylalanine) were not tolerated.

Peeters and co-workers had reported the *in vitro* incorporation of 11 labeled L-Phe analogues into beauvericin-like structures by a purified beauvericin synthetase from *B. bassiana* (Bals.) Vuill., as judged by radio-TLC traces. Although these new beauvericins were not isolated and characterized, their results suggest a relaxed substrate specificity of the enzyme *in vitro*.[24] The substrate specificity of the beauvericin synthetase from strain ATCC 7159 appears significantly more constrained in our experiments, indicating that *in vivo* competition from the native substrates D-Hiv and L-Phe restricts utilization of substrate analogues. It is also possible that some of the precursor analogues used in our experiments were unable to penetrate the fungal cell wall and membrane or, conversely, that others might be efficiently metabolized by the cells.

Compounds **1**–**7** were evaluated in a limited SAR study (Table 2) for *in vitro* cytotoxic activity using the MTT assay[39] and for *in*

vitro antihaptotactic activity using the wound-healing assay (WHA)[40] with the inhibition of the cell migration quantified by the NIH ImageJ software,[41] as described previously.[16] The results are summarized in Table 2. Stepwise replacement of the D-Hiv moieties of beauvericin (**1**) with D-Hbu in compounds **2**–**4** led to a progressive decrease of both activities against the metastatic prostate cancer cell line PC-3M, indicating a requirement for a branched side chain at these positions. The antihaptotactic activity was slightly more sensitive to this replacement, as the MTT/WHA IC_{50} ratio decreased from 2.3 (for compound **1**) to 0.9 (beauvericin G_3, compound **4**). Stepwise replacement of the *N*-methyl-L-Phe moieties of beauvericin (**1**) with *N*-methyl-L-3-fluorophenylalanine progressively increased cytotoxicity. As the WHA activity of compounds **5**–**7** remained apparently constant, the MTT/WHA IC_{50} ratio decreased to 1.4 (beauvericin H_3, compound **7**) as a result. This suggests that it might be possible to modulate the cytotoxicity of beauvericins by changing the substituents at the *meta* positions of the L-Phe moieties of beauvericin (**1**) without adversely affecting the antihaptotactic activity.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Jasco DIP-370 digital polarimeter using MeOH as the solvent. 1D and 2D NMR spectra were recorded in $CDCl_3$ or acetone-d_6 on a Bruker DRX-500 instrument at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR. The chemical shift values (δ) are given in parts per million (ppm), and the coupling constants are given in hertz. High-resolution FABMS were obtained with a JEOL HX110A mass spectrometer.

Strain and Culture Conditions. *B. bassiana* ATCC 7159 was maintained on potato dextrose agar (PDA, Difco, Plymouth, MN) plates. Substrate analogues were purchased from Sigma-Aldrich (Allentown, PA), except for D- and L-3-fluorophenylalanine and L-3-chlorophenylalanine (CSPS Pharmaceuticals, San Diego, CA), and were of the highest purity available. D- and L-3-Fluorophenylalanine and D- and L-2-hydroxybutyric acids had an enantiomeric ratio of >99:1 as determined by the manufacturer. The toxicity of substrate analogues was first established by evaluating the growth of *B. bassiana* on PDA plates containing serial dilutions of the precursor analogues. Fermentations were carried out in 250 mL flasks containing 100 mL of potato dextrose broth (PDB) on a rotary shaker at 160 rpm and 28 °C. Main cultures were inoculated with 5% starter cultures that had been cultivated for 3 days. Stock solutions (1.0 M) of amino acid and hydroxycarboxylic acid analogues of D-Hiv and L-Phe were prepared in water, adjusted to pH 9.0 with sodium hydroxide, and filter-sterilized, except for 2-oxovaleric acid, β-chlorolactic acid, and (*S*)- and (*R*)-(+)-hexahydromandelate, which were prepared in 100% methanol and used without sterilization. The analogues were supplemented to the main cultures one day after inoculation, and the cultivation was continued for an additional five days. No precipitation of the analogues was observed. Table 1 shows the analogues used and their final concentrations during fermentation.

Extraction, HPLC Analysis, and Isolation of Metabolites. The cultures were filtered through Whatman No. 1 filter paper, and the mycelial cakes were extracted with MeOH. The clarified supernatants were discarded, as these contained less than 1% of the beauvericins produced by the cultures (results not shown). The extracts were concentrated under reduced pressure; the residual oil was dissolved in EtOAc and then extracted with hexane and $CHCl_3$ separately. The $CHCl_3$ fractions containing beauvericin and its analogues were evaporated to dryness, redissolved in MeOH, and analyzed by a HP-1050 HPLC and a Shimadzu LCMS-QP8000α (Kromasil C18 5 μm, 250 mm × 4.6 mm column; MeOH/H_2O = 80:20 at a flow rate of 0.4 mL/min; detection at 210 nm). Compounds **2**–**7** were isolated from fermentations with multiple flasks with a total culture volume of 2 L, using reversed-phase preparative HPLC (Kromasil C18 5 μm, 250 mm × 10 mm column; MeOH/H_2O = 80:20 at a flow rate of 1.0 mL/min; detection at 210 nm). Separation of the mixture of beauvericins obtained from feeding with 2-fluorophenylalanine was attempted by Sepharose LH-20 column (850 mm × 20 mm) chromatography using 80% MeOH as a solvent and by reversed-phase preparative HPLC with the above column as well as a Kromasil C5 5 μm, 250 mm × 10 mm column, using several different MeOH/H_2O or CH_3CN/H_2O gradients.

Beauvericin G_1 (2): white powder; $[\alpha]^{25}_D$ +16.6 (*c* 0.4, MeOH); ^{1}H NMR ($CDCl_3$, 500 Hz) δ 7.29–7.14 (15H, m, H-10-H-14, H-25–H-29 and H-40–H-44), 5.72 (1H, m, H-7/H-22), 5.65 (1H, m, H-7/H-22), 5.07 (1H, d, J = 9.0 Hz, H-16/H-31), 5.05 (1H, d, J = 9.0 Hz, H-16/H-31), 5.00 (1H, dd, J = 9.2, 4.3 Hz, H-1), 4.46 (1H, brs, H-37), 3.35 (1H, dd, J =14.5, 4.8 Hz, H-8a/H-23a), 3.30 (1H, dd, J = 14.5, 4.6 Hz, H-8a/H-23a), 3.28 (1H, J = 14.6, 9.9 Hz, H-38a), 3.10 (1H, m, H-38b), 3.08 (3H, s, CH_3-6/CH_3-21), 3.04 (3H, s, CH_3-6/CH_3-21), 2.98 (1H, m, H-8b/H-23b), 2.93 (1H, m, H-8b/H-23b), 2.71 (3H, s, CH_3-36), 2.10 (1H, m, H-17/H-32), 1.88 (1H, m, H-17/H-32), 1.73 (1H, m, H-2a), 1.58 (1H, m, H-2b), 0.80–0.74 (9H, m, CH_3-4, CH_3-18, and CH_3-33), 0.37 (3H, d, J = 6.5 Hz, CH_3-19/CH_3-34), and 0.26 (3H, d, J = 6.5 Hz, CH_3-19/CH_3-34); ^{13}C NMR ($CDCl_3$, 125 Hz) δ 170.3, 170.0, and 169.8 (C, C-5, C-15, C-20, C-30, C-35, C-45), 136.5 and 136.2 (C, C-9, C-24, and C-39), 129.1, 128.9, and 128.8 (CH, C-10, C-14, C-25, C-29, C-40, and C-44), 128.6 and 128.5 (CH, C-11, C-13, C-26, C-28, C-41, and C-43), 126.8 and 126.7 (CH, C-12, C-27, and C-42), 74.8 (CH, C-16/C-31), 74.6 (CH, C-16/C-31), 73.0 (CH, C-1), 56.2 (CH, C-7, C-22, and C-37), 35.3 (CH_2, C-8/C-23/C-38), 34.6 (CH_2, C-8/C-23/C-38), 34.3 (CH_2, C-8/C-23/C-38), 34.0 (CH_3, C-36), 31.8 (CH_3, C-6/C-21), 31.6 (CH_3, C-6/C-21), 29.7 (CH, C-17/C-32), 23.6 (CH_2, C-2), 18.2 (CH_3, C-18 and C-33), 17.4 (CH_3, C-19/C-34), 17.1 (CH_3, C-19/C-34), 10.1 (CH_3, C-4); HRFABMS *m*/*z* 770.4033 $[M + 1]^+$ (calcd for $C_{44}H_{56}N_3O_9$ 770.4016).

Beauvericin G_2 (3): white powder; $[\alpha]^{25}_D$ +1.7 (*c* 0.3, MeOH); ^{1}H NMR ($CDCl_3$, 500 Hz) δ 7.29–7.13 (15H, m, H-10–H-14, H-25–H-29, and H-40–H-44), 5.63 (1H, m, H-37), 5.18 (3H, m, H-1, H-16, and H-31), 5.05 (1H, m, H-7/H-22), 4.78 (1H, m, H-7/H-22), 3.30 (3H, m, H-8a, H-23a, and H-38a), 3.07 (3H, s, CH_3-36), 3.04 (2H, m, H-8b and H-23b), 2.95 (1H, m, H-38b), 2.84 (3H, s, CH_3-6/CH_3-21), 2.82 (3H, s, CH_3-6/CH_3-21), 2.05 (2H, m, H-2), 1.61 (3H, m, H-17 and H-32), 0.75 (3H, d, J = 6.5 Hz, CH_3-33), 0.66 (6H, t, J = 7.3 Hz, CH_3-4 and CH_3-19), 0.39 (3H, d, J = 6.5 Hz, CH_3-34); ^{13}C NMR ($CDCl_3$, 125 Hz) δ 170.0 (C, C-5, C-15, C-20, C-30, C-35, and C-45), 136.9 and 136.2 (C, C-9, C-24, and 39), 129.1 and 128.9 (CH, C-10, C-14, C-25, C-29, C-40 and C-44), 128.6 and 128.5 (CH, C-11, C-13, C-26, C-28, C-41, and C-43), 126.8 and 126.7 (CH, C-12, C-27, and C-42), 74.3 (CH, C-1), 72.0 (CH, C-16 and C-31), 56.4 (CH, C-7, C-22, and C-37), 34.9 (CH_2, C-8/C-23/C-38), 34.8 (CH_2, C-8/C-23/C-38), 34.1 (CH_2, C-8/C-23/C-38), 33.9 (CH_3, C-6/C-21), 33.2 (CH_3, C-6/C-21), 31.6 (CH_3, C-36), 29.8 (CH, C-32), 23.8 (CH_2, C-2/C-17), 23.7 (CH_2, C-2/C-17), 18.1 (CH_3, C-33), 17.4 (CH_3, C-34), 9.6 (CH_3, C-4 and C-19); HRFABMS *m*/*z* 756.3830 $[M + 1]^+$ (calcd for $C_{43}H_{54}N_3O_9$ 756.3860).

Beauvericin G_3 (4): white powder; $[\alpha]^{25}_D$ +5.9 (*c* 0.1, MeOH); ^{1}H NMR ($CDCl_3$, 500 Hz) δ 7.30–7.17 (15H, m, H-10–H-14, H-25–H-29, and H-40–H-44), 5.25 (3H, m, H-1, H-16, and H-31), 5.10 (3H, brs, H-7, H-22, and H-37), 3.26 (3H, m, H-8a, H-23a, and H-38a), 3.02 (3H, m, H-8b, H-23b, and H-38b), 2.88 (9H, s, CH_3-6, CH_3-21, and CH_3-36), 1.57 (6H, m, H-2, H-17, and H-32), 0.64 (9H, t, J = 7.1 Hz, CH_3-4, CH_3-19, and CH_3-34); ^{13}C NMR ($CDCl_3$, 125 Hz) δ 169.9 (C, C-5, C-15, C-20, C-30, C-35, and C-45), 136.7 (C, C-9, C-24, and C-39), 129.1 (CH, C-10, C-14, C-25, C-29, C-40, and C-44), 128.5 (CH, C-11, C-13, C-26, C-28, C-41, and C-43), 126.7 (CH, C-12, C-27, and C-42), 71.6 (CH, C-1, C-16, and C-31), 58.6 (CH, C-7, C-22, and C-37), 34.7 (CH_2, C-8, C-23, and C-38), 32.9 (CH_3, C-6, C-21, and C-36), 24.0 (CH_2, C-2, C-17, and C-32), 9.5 (CH_3, C-4, C-19, and C-34); HRFABMS *m*/*z* 742.3672 $[M + 1]^+$ (calcd for $C_{42}H_{52}N_3O_9$ 742.3703).

Beauvericin H_1 (5): white powder; $[\alpha]^{25}_D$ +19.4 (*c* 0.1, MeOH); ^{1}H NMR (600 MHz, acetone-d_6) δ 7. 31 (1H, m, H-12), 7.31–7.16 (10H, m, H-25–H29 and H-40–H-44), 7.15 (1H, d, J = 7.8 Hz, H-14), 7.11 (1H, d, J = 9.1 Hz, H-10), 6.96 (1H, td, J = 7.8, 1.8 Hz, H-13), 5.47–5.38 (3H, m, H-7, H-22, and H-37), 5.06 (2H, d, J = 8.8 Hz, H-1 and H-16), 5.05 (1H, d, J = 8.7 Hz, H-31), 3.32 (1H, dd, J = 14.2, 4.8 Hz, H-8a), 3.28 (2H, dd, J = 14.2, 4.8 Hz, H-23a and H-38a), 3.12 (1H, dd, J = 14.2, 11.4 Hz, H-8b), 3.09 (2H, dd, J = 14.2, 11.4 Hz, H-23b and H-38b), 3.08 (3H, s, CH_3-21/CH_3-36), 3.07 (3H, s, CH_3-21/CH_3-36), 3.05 (3H, s, CH_3-6), 2.01–1.93 (3H, m, H-2, H-17, and H-32), 0.79 (6H, d, J = 6.6 Hz, CH_3-3 and CH_3-18), 0.78 (3H, d, J = 6.6 Hz, CH_3-33), 0.52 (3H, d, J = 6.6 Hz, CH_3-34), 0.48 (3H, d, J = 6.6 Hz, CH_3-4/CH_3-19), 0.47 (3H, d, J = 6.6 Hz, CH_3-4/CH_3-19); HRFABMS *m*/*z* 802.4109 $[M + 1]^+$ (calcd for $C_{45}H_{57}FN_3O_9$ 802.4079).

Beauvericin H_2 (6): white powder; $[\alpha]^{25}_D$ +21.1 (*c* 0.2, MeOH); ^{1}H NMR (500 MHz, acetone-d_6) δ 7.30 (2H, m, H-12 and H-27), 7.29–7.18 (5H, m, H-40–H-44), 7.15 (2H, d, J = 7.7 Hz, H-14 and

H-29), 7.11 (2H, d, $J = 10.1$ Hz, H-10 and H-25), 6.96 (2H, td, $J = 7.7$, 1.8 Hz, H-13 and H-28), 5.42 (1H, m, H-37), 5.39 (2H, m, H-7 and H-22), 5.06 (1H, d, $J = 8.6$ Hz, H-16), 5.05 (2H, d, $J = 8.6$ Hz, H-1 and H-31), 3.30 (2H, dd, $J = 14.2$, 4.9 Hz, H-8a and H-23a), 3.28 (1H, dd, $J = 14.2$, 4.9 Hz, H-38a), 3.11 (2H, dd, $J = 14.2$, 11.6 Hz, H-8b and H-23b), 3.09 (1H, dd, $J = 14.2$, 11.6 Hz, H-38b), 2.98 (9H, s, CH_3-6, CH_3-21, and CH_3-36), 2.03 (3H, m, H-2, H-17, and H-32), 0.81 (3H, d, $J = 6.8$ Hz, CH_3-18), 0.79 (3H, d, $J = 6.8$ Hz, CH_3-3/CH_3-33), 0.78 (3H, d, $J = 6.8$ Hz, CH_3-3/CH_3-33), 0.51 (3H, d, $J = 6.8$ Hz, CH_3-4/CH_3-34), 0.50 (3H, d, $J = 6.8$ Hz, CH_3-4/CH_3-34), 0.46 (3H, d, $J = 6.8$ Hz, CH_3-19); HRFABMS *m/z* 820.4002 $[M + 1]^+$ (calcd for $C_{45}H_{56}F_2N_3O_9$ 820.3984).

Beauvericin H_3 (7): white powder; $[\alpha]^{25}_D$ +35.3 (*c* 0.4, MeOH); ^{1}H NMR (500 MHz, acetone-d_6) δ 7.31 (3H, ddd, $J = 14.2$, 7.8, 2.1 Hz, H-12, H-27, and H-42), 7.15 (3H, d, $J = 7.8$ Hz, H-14, H-29 and H-44), 7.12 (3H, dd, $J = 10.3$, 2.1 Hz, H-10, H-25, and H-40), 6.96 (3H, td, $J = 7.8$, 2.5 Hz, H-13, H-28, and H-43), 5.40 (3H, dd, $J = 11.6$, 4.8 Hz, H-7, H-22, and H-37), 5.05 (3H, d, $J = 8.6$ Hz, H-1, H-16, and H-31), 3.30 (3H, dd, $J = 14.3$, 4.8 Hz, H-8a, H-23a, and H-38a), 3.11 (3H, dd, $J = 14.3$, 11.6 Hz, H-8b, H-23b, and H-38b), 3.07 (9H, s, CH_3-6, CH_3-21 and CH_3-36), 2.03 (3H, m, H-2, H-17, and H-32), 0.81 (9H, d, $J = 6.7$ Hz, CH_3-3, CH_3-18, and CH_3-33), 0.51 (9H, d, $J = 6.7$ Hz, CH_3-4, CH_3-19, and CH_3-34); ^{13}C NMR (125 MHz, acetone-d_6) δ 170.45 (C, C-5, C-20, and C-35), 169.70 (C, C-15, C-30, and C-45), 164.59 (C, C-11, C-26, and C-41), 141.13 and 141.07 (C, C-9, C-24, and C-39), 130.95 and 130.88 (CH, C-13, C-28, and C-43), 126.01 and 125.99 (CH, C-14, C-29, and C-44), 116.81 and 116.64 (CH, C-10, C-25, and C-40), 114.17 and 114.00 (CH, C-12, C-27, and C-42), 75.62 (CH, C-1, C-16, and C-31), 58.07 (CH, C-7, C-22, and C-37), 34.99 (CH_2, C-8, C-23, and C-38), 32.90 (CH_3, C-6, C-21, and C-36), 30.58 (CH, C-2, C-17, and C-32), 18.47 (CH_3,C-3/C-4, C-18/C-19, and C-33/C-34), 17.94 (CH_3,C-3/C-4, C-18/C-19, and C-33/C-34); HRFABMS *m/z* 838.3912 $[M + 1]^+$ (calcd for $C_{45}H_{55}F_3N_3O_9$ 838.3890).

Biological Assays. The *in vitro* tetrazolium-based cytotoxicity assay[39] and the wound-healing assay[40] against a monolayer of the metastatic prostate cancer cell line PC-3M and its quantification[41] were conducted as described previously.[16] The reported values are the averages derived from 2–4 independent experiments with 4 replicates each.

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