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Combinatorial Mutasynthesis of Scrambled Beauvericins, Cyclooligomer Depsipeptide Cell Migration Inhibitors from *Beauveria bassiana*

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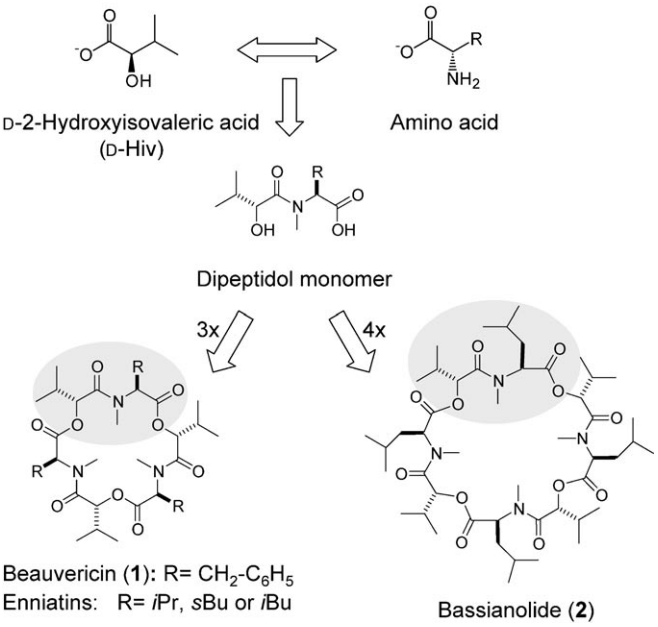
This paper is dedicated to the memory of Dr. Rainer Zocher (1948–2008).

Fungal cyclooligomer depsipeptides such as beauvericin, bassianolide, and enniatins display antibiotic, antifungal, insecticidal, broad-spectrum cancer cell antiproliferative, and cell migration inhibitory activities. We have identified a gene encoding a novel enzyme, ketoisovalerate reductase (KIVR), which is the sole provider of D*-hydroxyisovalerate (*D*-Hiv), a common precursor for cyclooligomer depsipeptide biosynthesis in* Beauveria bassiana*. KIVR and related hypothetical oxidoreductases encoded in fungal genomes are similar to ketopantoate reductases but not to* D*-hydroxycarboxylate dehydrogenases. We demonstrate that a KIVR knockout* B. bassiana *strain can be used for the efficient mutasynthesis of unnatural beauvericin congeners. Simultaneous feeding of precursor analogues enabled the combinatorial mutasynthesis of scrambled beauvericins, some assembled entirely from unnatural precursors. The effects of the introduced structural changes on the antiproliferative and cell migration inhibitory activities of these analogues were evaluated.*

Introduction

The insect pathogen *Beauveria* (teleomorph: *Cordyceps*) *bassiana* (Hypocreales) produces several secondary metabolites, including the cyclooligomer nonribosomal depsipeptides beauvericin (**1**; Scheme 1) and bassianolide (**2**), the diketomorpholine bassiatin, the cyclic peptides beauverolides, the 2-pyridone tenellin, and the dibenzoquinone oosporein.[1–5] Amongst these, the cyclooligomer depsipeptides beauvericin (**1**) and bassianolide (**2**) represent rich pharmacophores with diverse biological activities. Bassianolide (**2**), a tetramer of the dipeptidol monomer D-Hiv-*N*-methyl-L-leucine (Scheme 1), is toxic to insect larvae,[2] inhibits acetylcholine-induced smooth muscle contraction,[6] and displays moderate antimycobacterial, antiplasmodial, and cytotoxic activity.[7] Beauvericin (**1**), the main cyclooligomer depsipeptide product of *B. bassiana*, transports monovalent ions across membranes; this uncouples oxidative phosphorylation.[8] It is a cyclic trimer assembled from three D-Hiv-*N*-methyl-L-phenylalanine dipeptidol monomers (Scheme 1). Beauvericin is insecticidal,[1,9] displays moderate antifungal and antibiotic activity,[1] and reverses multidrug resistance in *Candida albicans*.[10,11] Beauvericin also displays broad-spectrum antiproliferative activity against different human cancer cell lines by activating calcium-sensitive cell apoptotic pathways.[12] We have previously reported that beauvericin is a potent inhibitor of the haptotactic motility of cancer cells at subcytotoxic concentrations.[13] Haptotaxis (directional cell mo-



Scheme 1. Biosynthesis of fungal cyclooligomer depsipeptides.

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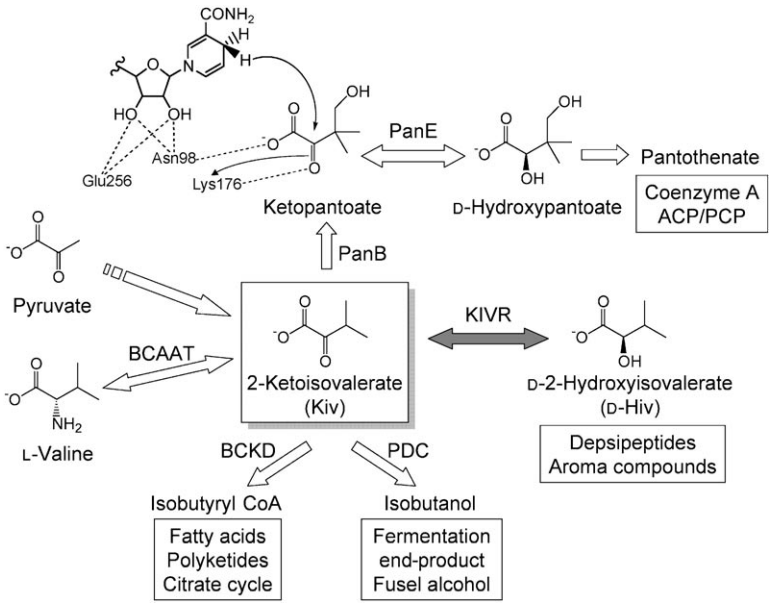
tility) is essential for key rate-limiting processes during cancer progression, including the formation of new blood vessels in tumors (angiogenesis), invasive growth of tumors, and metastasis.[14] Importantly, cytotoxicity is not essential for the inhibition of cell migration; the mechanisms of action for these two processes were shown to be distinct in taxanes and the vinca alkaloids, and demonstrated to be separable in the case of borrelidin analogues.[15] Haptotaxis inhibitors with reduced cytotoxicity would be highly desirable, both to contain the growth of existing tumors and to restrain new tumor formation.

Nonribosomal peptides, including cyclooligomer depsipeptides, encompass an extensive family of biologically active natural products. Their tremendous structural diversity derives in large part from the potential of the nonribosomal peptide synthetase (NRPS) enzymatic machinery to utilize nonproteinogenic amino acids and amino acid derivatives such as 2-hydroxycarboxylic acids as precursors. Most nonribosomal peptides are assembled from these precursors through a process of stepwise and processive condensations. In contrast, the NRPSs for cyclooligomer nonribosomal peptides such as beauvericin catalyze the iterative synthesis of oligopeptide monomer units, followed by the recursive condensation (oligomerization) of the monomers and intramolecular cyclization of the product.[16]

Hydroxyisovalerate (Hiv) is a common 2-hydroxycarboxylic acid constituent of depsipeptides including beauvericin, bassianolide, and other fungal cyclooligomer depsipeptides such as the enniatins (Scheme 1). D-Hiv is formed in fungi by the highly specific chiral reduction of 2-ketoisovalerate (Kiv) by dissociated enzymes.[17,18] Kiv itself is a central intermediate in several metabolic pathways (Scheme 2). It is formed from pyruvate in several steps as a precursor in valine biosynthesis, and is also used during pantothenate biosynthesis in bacteria, fungi, and plants in the production of coenzyme A and the phosphopantetheinyl prosthetic groups of acyl or peptidyl carrier proteins.[19]

A limited number of beauvericin analogues have been isolated as minor congeners from fermentations.[10,20,21] As demonstrated by ourselves and by others, further analogues can be produced by precursor-directed biosynthesis in vivo,[22,23] or by using purified synthetase enzymes in vitro.[24] Although total chemical synthesis of cyclic depsipeptides is also feasible, ester bond formation during solid-phase synthesis is inefficient, and macrolactone ring-closure is problematic.[25,26] Mutasynthesis, the introduction of chemically synthesized "mutasynthons" into complex natural products with the aid of biosynthetic mutants,[27] had not previously been established for cyclooligomer depsipeptides.

In this work we show that the *kivr* gene of *B. bassiana* encodes a putative 2-ketoisovalerate reductase that is the sole supplier of D-Hiv for both beauvericin and bassianolide biosynthesis under routine fermentation conditions. Using the *kivr* knockout *B. bassiana* strain, we have established mutasynthesis and demonstrated a novel combinatorial mutasynthetic strategy for the in vivo production of new beauvericin-like cyclooligomer depsipeptides upon simultaneous feeding with D-Hiv and L-Phe analogues. Finally, we evaluate the influences of the introduced structural changes on the antihaptotactic and antiproliferative activities of these unnatural natural products.



Scheme 2. Metabolism of 2-ketoisovalerate in fungi. A schematic representation of the catalytic mechanism of the *E. coli* PanE ketopantoate reductase[19] is shown with an abridged structure of NADPH. ACP/PCP: acyl carrier protein/peptidyl carrier protein. BCAAT: branched-chain amino acid aminotransferase. BCKD: branched-chain ketoacid dehydrogenase. KIVR: ketoisovalerate reductase. PanB: ketopantoate hydroxymethylase. PDC: pyruvate decarboxylase.

Results

Cloning and sequence analysis of a putative ketoisovalerate reductase

We have recently cloned and functionally characterized two discrete loci of the *B. bassiana* ATCC 7159 genome, each centered on a cyclooligomer depsipeptide synthetase for the biosyntheses of beauvericin[28] and bassianolide (unpublished data). The *bbBeas* gene for the beauvericin synthetase, but not the *bbBsls* gene for the bassianolide synthetase, was found to be clustered with—and to share a putative 2241 bp bidirectional promoter area with—a gene (*kivr*) encoding a deduced 2-ketoisovalerate reductase (KIVR; Figure 1 A).[28] The deduced KIVR protein is 462 amino acids in length and is highly similar to hypothetical fungal ketopantoate reductases in GenBank, such as XP_382715 from *Gibberella zeae* (anamorph: *Fusarium graminearum*; 43% identity/61% similarity). Similarity was also detected with the functionally uncharacterized terminal reductase domain of the *Xylaria* sp. bassianolide synthetase (ABR28366, 25% identity/43% similarity), and also with characterized or putative bacterial ketopantoate reductases such as ABC28760 from *Hahella chejuensis* (26% identity/40% similarity). Ketopantoate reductases catalyze the reversible, NADPH-dependent chiral reduc-

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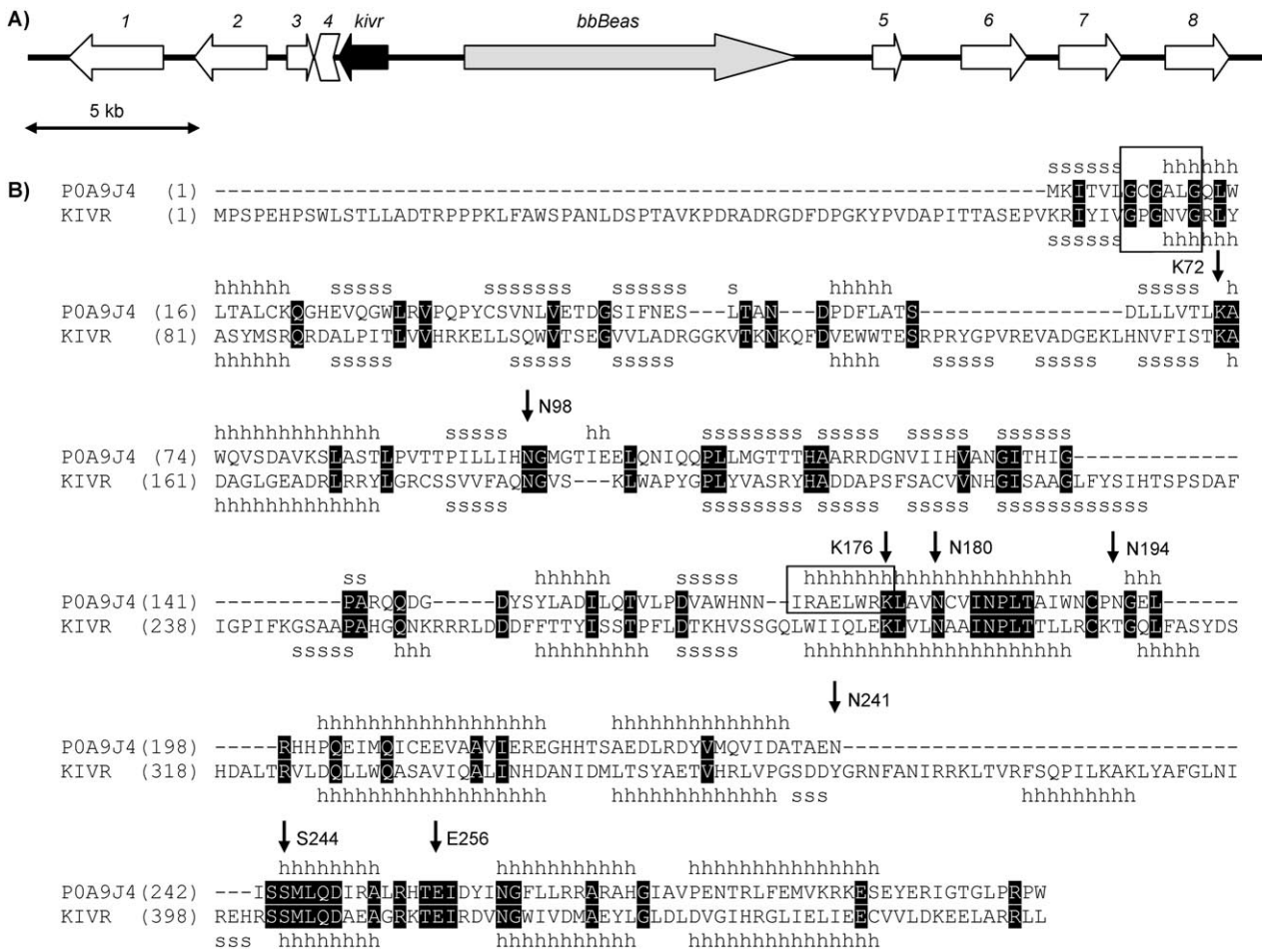

Figure 1. Sequence analysis of *kivr*. A) The beauvericin biosynthetic locus of *B. bassiana* (EU 886 196).[28] B) Structure-based sequence alignment of KIVR and the *E. coli* PanE ketopantoate reductase (P0A9J4). The alignment was optimized and annotated with secondary structure predictions in SWISS-MODEL[48] by use of the determined crystal structure of PanE (2OFP, chain A). Identical amino acids are shown on black background. Key amino acid residues mentioned in the text are labeled with arrows and numbered with reference to the PanE sequence. The nucleotide binding signatures and the hinge region separating the N-terminal Rossmann fold and the C-terminal catalytic domain in PanE[19] are boxed. h: α-helix. s: bend.

tion of ketopantoate to D-hydroxypantoate in vitamin B_5 biosynthesis (Scheme 2).[19] The deduced *B. bassiana* KIVR protein is also similar to COG1893 (ApbA ketopantoate reductases, *E* value $= 5e^{-20}$) and contains a G*X*G*XX*G nucleotide-binding signature (amino acid positions 72–77).

A survey of 30 available fungal genome sequences (Fungal Genome Initiative, (FGI), http://www.broad.mit.edu/annotation/fungi/fgi/) revealed one to nine genes per genome for a total of 118 sequences annotated as encoding COG1893 ketopantoate reductases. A phylogenetic tree constructed from a multiple alignment of these deduced fungal protein sequences and that of the *E. coli* PanE ketopantoate reductase (Figure S1 in the Supporting Information) revealed wide sequence divergence with many clades supported by significant bootstrap values, indicating a possible functional heterogeneity within this protein family. In particular, the *E. coli* ketopantoate reductase and the *B. bassiana* KIVR were found to be members of separate clades (Figure S1 in the Supporting Information).

The structure of the *E. coli* ketopantoate reductase in a ternary complex with NADP and pantoate has been solved to reveal a Glu-Asn-Lys triad active site architecture (Scheme 2).[19] The protonated Lys176 (KIVR: Lys290, Figure 1 B) acts as a general acid to polarize the substrate carbonyl, facilitates the transfer of the *pro-S* hydride of NADPH to the *si* face of ketopantoate, and protonates the developing alkoxide.[29] Asn98 (KIVR: Asn185) forms hydrogen bonds both with the carboxylate of the substrate and with the ribose of the nicotinamide: this orients the cofactor, together with further hydrogen bonds to the NADPH from Glu256 (KIVR: Glu415) and Lys72 (KIVR: Lys159). Further stabilization is provided by a hydrogen bond between the carboxyl group of the substrate and Ser244 (KIVR: Ser403), and between the α-hydroxy group of the product and Asn180 (KIVR: Asn294). All these residues are conserved in the *B. bassiana* KIVR (Figure 1 B) and in most hypothetical fungal COG1893 enzymes. Asn194 and Asn241 of the *E. coli* ketopantoate reductase form hydrogen bonds with the C4 hydroxy group of ketopantoate. These residues, however, are divergent in KIVR (Thr308 and Tyr366, respectively) and in many other hypothetical fungal COG1893 enzymes, again suggesting that the fungal COG1893 enzymes might act on substrates with divergent structures.

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Both beauvericin and bassianolide (as well as enniatins) incorporate D-Hiv as their 2-hydroxycarboxylate constituent.[24] A highly substrate- and stereospecific NADPH-dependent 53 kDa enzyme (termed "D-hydroxyisovalerate dehydrogenase"), which catalyzes the reversible reduction of 2-ketoisovalerate (Kiv) to D-Hiv, has been purified and biochemically characterized from *F. sambucinum*, a producer of enniatins,[18,30] but the corresponding protein and gene sequences were not determined. Concurrently with this work, it was hypothesized that a terminal reductase domain appended to the *Xylaria* sp. bassianolide synthetase might serve a similar function.[7] We propose that the *kivr* gene encodes the "D-hydroxyisovalerate dehydrogenase" of *B. bassiana* that supplies D-Hiv for the biosynthesis of beauvericin and bassianolide. In light of the sequence and the structural (and presumably mechanistic) similarity with ketopantoate reductases, but not with D-lactose or D-hydroxyisocaproate dehydrogenases (see Discussion), we propose to rename these enzymes as ketoisovalerate reductases (KIVRs).

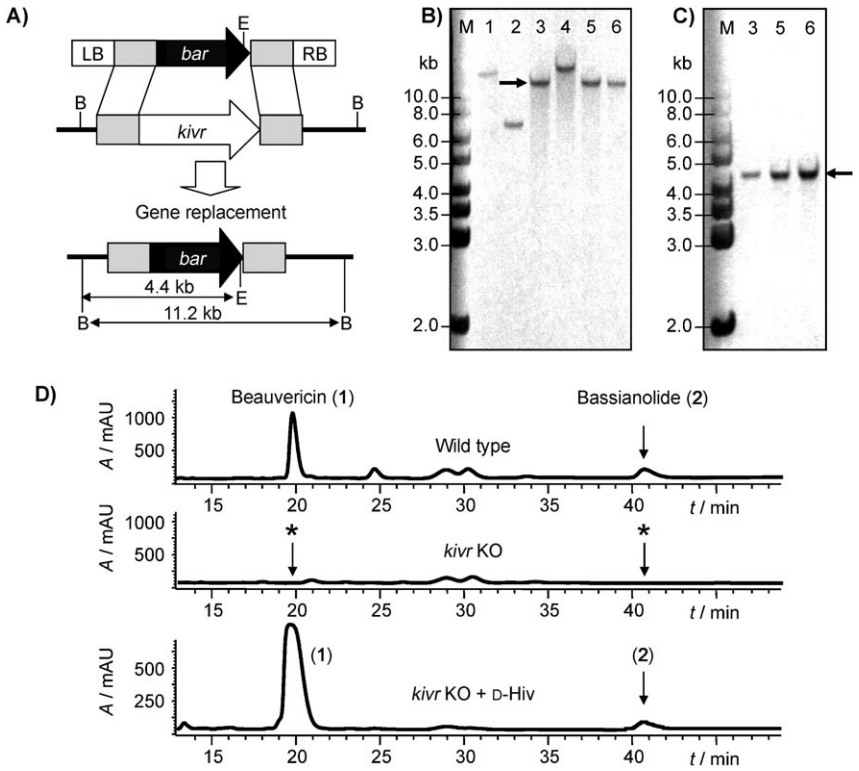

Figure 2. Replacement of the *kivr* gene in *B. bassiana.* A) Scheme for the double homologous recombination between the T-DNA and the *B. bassiana* genome. LB and RB: Left and right borders of the T-DNA. E: EcoRI restriction site. B: BamHI restriction site. B) Southern hybridization against BamHI-digested total DNA of several transformants with the *bar* gene as the probe. The arrow shows the expected hybridizing fragment found in isolates **3**, **5**, and **6**. M: marker. C) Southern hybridization against BamHI–EcoRI double digests of total DNA of selected transformants from panel B. The arrow shows the expected hybridizing fragment. D) Beauvericin (**1**) and bassianolide (**2**) production in *B. bassiana* ATCC 7159 (wild-type) and the *kivr* knockout mutant (*kivr* KO), and chemical complementation of the *kivr* mutant upon feeding with D-Hiv (*kivr* KO + D-Hiv).

Disruption of *kivr* in *B. bassiana*

To ascertain the biological role of KIVR, we disrupted the *kivr* gene on the *B. bassiana* chromosome by employing *Agrobacterium tumefaciens*-mediated transformation.[31,32] Glufosinate-resistant putative transformants were verified by a series of Southern hybridizations (Figure 2A–C). Approximately 80% of the transformants carried the selectable marker gene at an ectopic location, while the rest of the transformants were validated to have undergone gene replacement through double homologous recombination. The production of beauvericin and bassianolide in the ectopic integrant isolates was indistinguishable from that in the wild-type strain. In marked contrast, the biosyntheses of both beauvericin and bassianolide were fully abrogated in the *kivr* knockout isolates, as shown by HPLC and LC-MS analysis of the corresponding extracts (Figure 2D). Feeding of D-Hiv during fermentations restored the ability of the *kivr* mutant isolates to produce both cyclodepsipeptides, with an approximately 30% increase in beauvericin production and a 40% decrease in bassianolide biosynthesis relative to the wild type (Figure 2D). The inability of the *kivr* mutant to produce either of the two D-Hiv-containing cyclodepsipeptides and the efficient rescue of the production of both of these compounds by chemical complementation with D-Hiv unambiguously establishes that KIVR is the only enzyme that supplies D-Hiv for cyclooligomer depsipeptide biosynthesis in *B. bassiana* under the fermentation conditions tested.

Mutasynthesis of beauvericin analogues with a *kivr* knockout *B. bassiana* strain

As a consequence of the oligomeric nature of beauvericin (Scheme 1), precursor-directed biosynthesis with the wild-type strain and an appropriate D-Hiv analogue yields beauvericin (**1**) and a series of three beauvericin analogues in which one, two, or all three D-Hiv moieties are replaced by the externally supplied precursor.[22,23] In contrast, the *kivr* mutant strain is unable to produce any beauvericin-like compounds unless the fermentations are supplemented with an appropriate D-Hiv analogue. Moreover, upon D-Hiv analogue supplementation, this strain biosynthesizes only a single beauvericin compound, in which all the D-Hiv positions are fully substituted by the externally supplied precursor. We have demonstrated this principle by feeding D-2-hydroxybutyrate (D-Hbu) to the *kivr* mutant *B. bassiana* strain and observing the biosynthesis of beauveric-

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in G_3 (**3**) as the sole beauvericin product (Figure 3). Beauvericin G_3 had also been produced by precursor-directed biosynthesis with the wild-type *B. bassiana* strain, and that fermentation

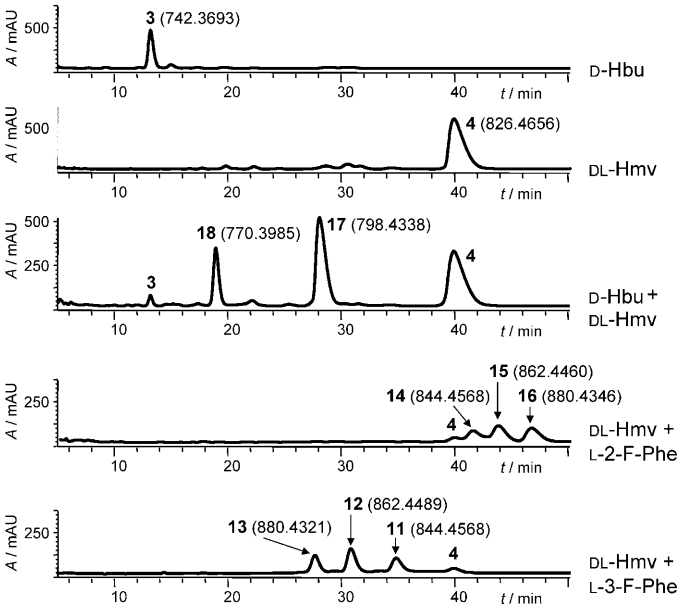

Figure 3. Production of beauvericin analogues by combinatorial mutasynthesis. The *kivr* knockout *B. bassiana* strain was fermented in media supplemented with the precursor analogues indicated to the *right*. Beauvericin analogues are identified with their compound numbers and the *m/z* values for their $[M+H]^+$ ions in HR-FAB-MS.

had also yielded beauvericins G_1, G_2, and G_3 (D-Hbu replacing one, two, or three D-Hiv residues, respectively, in beauvericin), in addition to natural beauvericin (**1**).[22] Production of beauvericin G_3 by mutasynthesis is also significantly more efficient than its production by precursor-directed biosynthesis (approximately 8 mg L^{-1} versus ~1 mg L^{-1},[22] respectively).

Attempts to use the commercially available D-Hiv precursor analogues DL-lactate, DL-2-hydroxyisocaproate, DL-mandelate, and (*R,S*)-hexahydromandelate for mutasynthesis were unproductive, similarly to our previous precursor-directed biosynthesis experiments.[22] Since very few feasible D-Hiv analogues are commercially available, we considered producing a much larger variety of these precursor analogues by synthesis from the corresponding readily available amino acids. We found that the most direct and efficient procedure for conversion of amino acids into the corresponding 2-hydroxycarboxylic acids involves diazotization followed by hydrolysis with $NaNO_2$/H_2SO_4/H_2O under mild conditions (see the Experimental Section). As a proof of principle, we have successfully applied this procedure on a gram scale for the synthesis of DL-2-hydroxy-3-methylvalerate (DL-Hmv) from DL-isoleucine. Concurrently with this work, a similar synthetic scheme that uses more vigorous conditions to support in vitro enzymatic synthesis of enniatin variants was described by the Zocher group.[33] After structure confirmation, synthetic DL-Hmv was fed to the *kivr* mutant strain and was found to support the exclusive production of the known analogue beauvericin C (**4**)[22,23] in a good yield (ca. 12 mg L^{-1}; Figure 3). In contrast, only minor amounts of this congener had previously been obtained by ourselves and by others during precursor-directed biosynthesis with the wild-type beauvericin producer strains.[22,23]

Combinatorial mutasynthesis of scrambled beauvericins

To produce a larger variety of beauvericin analogues, we considered combinatorial simultaneous feeding of pairs of precursor analogues during mutasynthesis. To demonstrate the feasibility of this new approach, we used the 2-hydroxycarboxylic acid analogues D-Hbu and DL-Hmv, as well as the L-Phe analogues 3-fluoro-L-Phe (L-3-F-Phe) and 2-fluoro-L-Phe (L-2-F-Phe). Efficient incorporation of these Phe analogues into unnatural beauvericins had been demonstrated in our previous study.[22] While the fed 2-hydroxycarboxylates completely replaced D-Hiv in beauvericins obtained with the *kivr* mutant as described above, the Phe analogues were expected to substitute zero, one, two, or three Phe moieties to provide a beauvericin analogue series (Figure 3). Satisfyingly, combinatorial feeding of the *kivr* mutant with the above precursors enabled the efficient production of 14 new beauvericin analogues belonging to five novel series (Table 1, compounds **5**–**18**). To achieve acceptable yields of the novel beauvericins BG_1 (**17**) and AG_2 (**18**), D-Hbu had to be supplied at a minimum of a tenfold excess over DL-Hmv during simultaneous feeding with these 2-hydroxycarboxylates. All of the new beauvericin analogues produced were characterized by HR-FAB-MS, and eight were isolated by preparative HPLC and their structures were confirmed by ^{1}H NMR spectroscopy (see the Supporting Information). All attempts to separate the remaining six analogues failed, and thus these were isolated as product mixtures. The isolated beauvericin analogues and the beauvericin analogue mixtures were evaluated in a limited SAR study for their in vitro antiproliferative and antihaptotactic activities against the metastatic prostate cancer cell line PC-3M (Table 2 and Figure 4).

Discussion

2-Ketoisovalerate (Kiv) is an intermediate both in valine catabolic pathways and in valine anabolic pathways (Scheme 2). Kiv is also converted into vitamin B_5 (pantothenate) and further into coenzyme A via ketopantoate in prokaryotes, fungi, and plants; this pathway is an attractive target for the development of highly specific antibiotics, antifungals, and herbicides because animals do not biosynthesize pantothenic acid. Kiv is also metabolized to isobutyryl-CoA prior to entry into the citrate cycle and for the biosynthesis of branched-chain fatty acids and polyketides. Yeasts can convert Kiv into isobutanol (a fusel alcohol) during fermentative regeneration of NAD^+.[34] Chiral reduction of Kiv in fungi provides D-Hiv, a precursor for the biosyntheses of nonribosomal depsipeptides and aroma compounds.

2-Hydroxyisovalerate is a common 2-hydroxycarboxylate constituent of depsipeptides. Known bacterial depsipeptide synthetase NRPSs activate and load 2-ketocarboxylates and uti-

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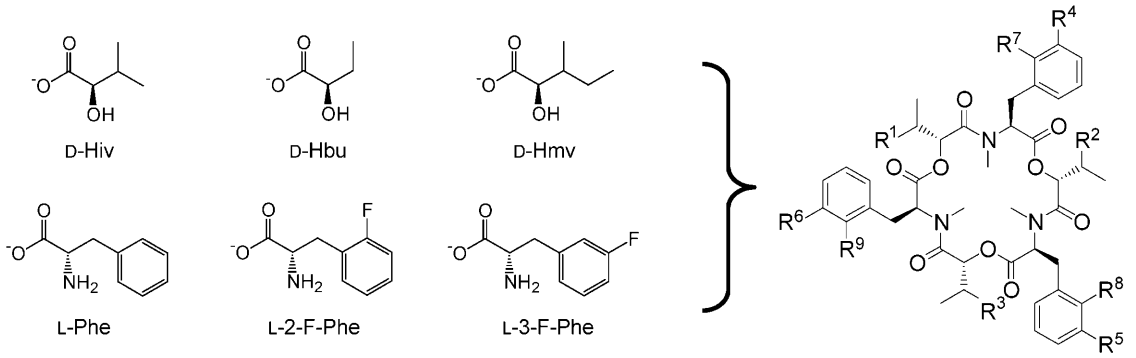
Table 1. Structures of beauvericin analogues. The structures of the precursor analogues fed to the *kivr* knockout strain are also shown.



Compound	Feeding	Yield[a]	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	R^9
beauvericin[b] (**1**)	NA	20	Me	Me	Me	H	H	H	H	H	H
beauvericin G_3 (**3**)	D-Hbu	8	H	H	H	H	H	H	H	H	H
beauvericin C (**4**)	D-Hmv	12	Et	Et	Et	H	H	H	H	H	H
beauvericin G_3H_1 (**5**)	D-Hbu + L-3-F-Phe	3[c]	H	H	H	F	H	H	H	H	H
beauvericin G_3H_2 (**6**)	D-Hbu + L-3-F-Phe		H	H	H	F	F	H	H	H	H
beauvericin G_3H_3 (**7**)	D-Hbu + L-3-F-Phe		H	H	H	F	F	F	H	H	H
beauvericin G_3I_1 (**8**)	D-Hbu + L-2-F-Phe	1	H	H	H	H	H	H	F	H	H
beauvericin G_3I_2 (**9**)	D-Hbu + L-2-F-Phe	1	H	H	H	H	H	H	F	F	H
beauvericin G_3I_3 (**10**)	D-Hbu + L-2-F-Phe	1	H	H	H	H	H	H	F	F	F
beauvericin CH_1 (**11**)	D-Hmv + L-3-F-Phe	1	Et	Et	Et	F	H	H	H	H	H
beauvericin CH_2 (**12**)	D-Hbu + L-3-F-Phe	2	Et	Et	Et	F	F	H	H	H	H
beauvericin CH_3 (**13**)	D-Hbu + L-3-F-Phe	1	Et	Et	Et	F	F	F	H	H	H
beauvericin Cl_1 (**14**)	D-Hbu + L-2-F-Phe	3[c]	Et	Et	Et	H	H	H	F	H	H
beauvericin Cl_2 (**15**)	D-Hbu + L-2-F-Phe		Et	Et	Et	H	H	H	F	F	H
beauvericin Cl_3 (**16**)	D-Hbu + L-2-F-Phe		Et	Et	Et	H	H	H	F	F	F
beauvericin BG_1 (**17**)	D-Hmv + D-Hbu	8	Et	Et	H	H	H	H	H	H	H
beauvericin AG_2 (**18**)	D-Hmv + D-Hbu	5	Et	H	H	H	H	H	H	H	H

[a] Approximate isolated yield of the product in mg L^{-1}. [b] Beauvericin produced by the wild-type strain. [c] Isolated yields of the product mixtures G_3H_{0-3} and Cl_{0-3}, respectively.

Table 2. Cancer cell antiproliferative and antihaptotactic activities of beauvericin analogues.

Compound	MTT[a]	WHA[b]	MTT/WHA[c]	Compound	MTT[a]	WHA[b]	MTT/WHA[c]
beauvericin (**1**)[d]	8.0 ± 0.4	3.5 ± 0.4	2.3	beauvericin G_3I_3 (**10**)	5.1 ± 0.8	5.9 ± 0.7	0.9
beauvericin G_1[d]	5.0 ± 0.4	3.8 ± 0.2	1.3	beauvericin CH_1 (**11**)	2.1 ± 0.1	3.6 ± 0.8	0.6
beauvericin G_2[d]	10.4 ± 0.1	7.6 ± 0.2	1.4	beauvericin CH_2 (**12**)	1.4 ± 0.1	3.2 ± 0.1	0.4
beauvericin G_3 (**3**)[d]	16.3 ± 0.1	17.2 ± 0.1	0.9	beauvericin CH_3 (**13**)	3.4 ± 0.1	4.8 ± 0.3	0.7
beauvericin H_1[d]	7.4 ± 0.3	3.2 ± 0.1	2.3	beauvericin BG_1 (**17**)	4.6 ± 0.1	4.6 ± 0.1	1.0
beauvericin H_2[d]	6.2 ± 0.1	3.2 ± 0.1	1.9	beauvericin AG_2 (**18**)	4.9 ± 0.1	4.0 ± 0.3	0.8
beauvericin H_3[d]	4.4 ± 0.2	3.2 ± 0.4	1.4	beauvericin G_3H_{0-3}[e]	7.8 ± 0.3	5.5 ± 0.2	1.4
beauvericin C (**4**)	2.4 ± 0.2	3.2 ± 0.3	0.8	beauvericin Cl_{0-3}[f]	2.1 ± 0.1	3.4 ± 0.3	0.6
beauvericin G_3I_1 (**8**)	8.0 ± 0.3	5.4 ± 0.5	1.5	beauvericin I_{1-3}[g]	2.8 ± 0.2	2.3 ± 0.2	1.2
beauvericin G_3I_2 (**9**)	7.6 ± 0.9	8.2 ± 0.6	0.9	bassianolide (**2**)	> 30.0	> 30.0	–

[a] MTT: antiproliferative activity expressed as IC_{50} in μM. [b] WHA: antihaptotactic activity expressed as IC_{50} in μM. [c] MTT/WHA: antiproliferative versus antihaptotactic activity index. [d] Data from.[22] [e] Mixture of beauvericins $G_3/G_3H_1/G_3H_2/G_3H_3$ (2:3:2:1). [f] Mixture of beauvericins $C/Cl_1/Cl_2/Cl_3$ (1:3:5:4). [g] Mixture of beauvericins $I_1/I_2/I_3$ (2:3:1).

lize an integrated NRPS domain with similarities to polyketide synthase ketoacyl reductases to reduce the C2 carbonyl of the resulting 2-ketocarboxylate carrier protein thioester. In contrast, fungal cyclooligomer depsipeptide synthetases directly incorporate preformed 2-hydroxycarboxylic acids, conceivably representing an example of convergent evolution. D-Hiv is thus biosynthesized in fungi by distinct, monofunctional, NADPH-dependent dehydrogenase enzymes with high chiral specificity, exemplified by the "D-Hiv dehydrogenase" purified from the enniatin producer *F. sambucinum* (teleomorph: *G. pulicaris*).[17,18,35] While the corresponding gene has not been isolated, the measured size of the purified *F. sambucinum* enzyme (53 kDa for the monomer) is in excellent agreement with the deduced size of KIVR (51 491 Da), shown here to be the

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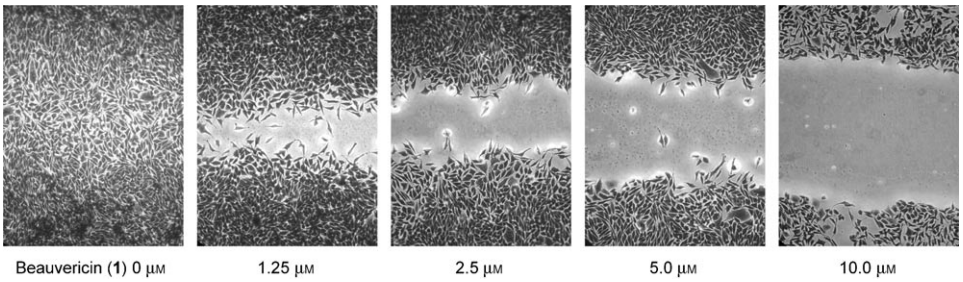

Figure 4. The pictures demonstrate the inhibition of cell migration by increasing concentrations of beauvericin as evident from the reduced closure of a "scrape wound" in a monolayer of PC-3M cells. Cell migration is completely inhibited by 10 μM beauvericin.

enzyme responsible for the supply of D-Hiv for beauvericin and bassianolide biosynthesis in *B. bassiana.*

Different bacterial strains produce D-2-hydroxycarboxylic acids using chiral enzymes belonging to the D-hydroxyacid dehydrogenase superfamily. However, Kiv and other β-branched 2-ketocarboxylic acids are very poor substrates (if at all) for all known D-hydroxyacid dehydrogenases. These bacterial enzymes also utilize NADH, and also feature a conserved Arg–(Asp/Glu)–His active site triad. The *B. bassiana* KIVR reveals a different deduced active site architecture and shows very low sequence similarity (<12% identity) to these enzymes. A malonate decarboxylase enzyme preparation from *Streptococcus bovis* was previously reported to display weak NADH-dependent Kiv dehydrogenase activity;[36] the *B. bassiana* KIVR is again highly divergent (<13% identity) from this enzyme. Similar very low sequence identities and different deduced active site architectures are evident with the *Alternaria alternata* AFTS1 and AMT2 aldo-ketoreductases suggested (but not shown) to convert Kiv into D-Hiv during mycotoxin biosynthesis.[37] In contrast, the *B. bassiana* KIVR shows high sequence similarity to deduced COG1893 ketopantoate reductases of fungi, together with moderate but significant sequence similarity and similar predicted secondary structure (Figure 1B) to prokaryotic ketopantoate reductases such as PanE of *E. coli*.[19] Ketopantoate reductases (E.C. 1.1.1.169), which are members of the 6-phosphogluconate dehydrogenase superfamily, catalyze the enantioselective conversion of 2-ketopantoate into D-hydroxypantoate during coenzyme A biosynthesis. While they show only marginal activity for Kiv,[38] ketopantoate reductases utilize NADPH and show an ordered sequential bi-bi reaction mechanism.[29] Fittingly, the *F. sambucinum* "D-Hiv dehydrogenase"[17] also utilizes NADPH in an ordered bi-bi kinetic mechanism with a sequential order identical to that of ketopantoate reductases during the reduction of Kiv to D-Hiv.[29,39] KIVR and the related fungal hypothetical COG1893 enzymes (Figure S1 in the Supporting Information) are predicted to share the N-terminal αβ-Rossmann fold and the C-terminal α-helical catalytic domain architecture of the *E. coli* ketopantoate reductase, whereas the residues implicated in catalysis and in the orientation of NADPH and the substrate[19] are also strictly conserved (Figure 1B). In comparison with the *E. coli* ketopantoate reductase, the *B. bassiana* KIVR features a ~65 amino acid *N*-terminal extension and four apparent insertions, with the insertion closest to the C terminus contributing an extra α-helix to the predicted catalytic domain (Figure 1B).

Fungal genome sequences available at the FGI website reveal multiple (up to nine) hypothetical COG1893 ketopantoate reductases of significant sequence diversity in each species; only *Uncinocarpus reesii* and *Cryptococcus neoformans* have single encoded enzymes of this type. The hypothetical fungal COG1893 proteins form several apparent sequence clades (Figure S1 in the supporting Information), which hints at a possible divergence in their substrate specificity with retention of general enzyme architecture. Similarly, D-lactate and D-hydroxyisocaproate dehydrogenases show high overall sequence similarity but non-overlapping substrate specificities.[40] We thus propose that the *B. bassiana* KIVR—and presumably the *F. sambucinum* "D-Hiv dehydrogenase" and similar fungal COG1893 proteins—form a novel family within the 6-phosphogluconate dehydrogenase superfamily, with structural and mechanistic[29,39] similarities to ketopantoate reductases, but nearly orthogonal substrate specificities.[19,38]

Inactivation of the *kivr* gene by gene replacement completely abrogated the production of both beauvericin and bassianolide in *B. bassiana*. No significant amounts of beauvericin or bassianolide analogues with alternative 2-hydroxycarboxylic acids were detected in the absence of hydroxycarboxylate supplementation during fermentations with the *kivr* knockout strain. This probably reflects the limited availability of any such metabolites in the strain with no KIVR activity, and the relatively stringent 2-hydroxycarboxylate precursor specificity of the two cyclooligomer depsipeptide synthetases. While genetic complementation is not technically feasible in *B. bassiana*, the ability of the strain to produce both beauvericin and bassianolide could be restored upon chemical complementation by feeding D-Hiv (Figure 1). With the *kivr* mutant, beauvericin production was somewhat increased relative to the wild-type strain upon D-Hiv feeding, while bassianolide production was decreased. Similar variations in the levels of restoration of small-molecule natural product biosynthesis are often encountered upon replacement of an essential innate precursor supply biosynthetic route with the external supply of the precursor,[27] which reflects the absence of information on the intracellular concentration of the innate precursor and the varied efficiencies of the uptake and intracellular compartmentalization of the externally supplied precursor.

Precursor-directed biosynthesis—the feeding of precursor analogues to wild-type strains to effect the biosynthesis of product analogues—is a well established method for generating chemical diversity and has been applied to fungal cyclodepsipeptides.[22,23,41,42] Mutasynthesis, applied here for the first time to fungal cyclooligomer depsipeptide biosynthesis, improves on this technique by eliminating the competing natural

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precursor and allows the uncontested incorporation of "mutasynthons".[27] Mutasynthesis is especially effective in simplifying product profiles for oligomer products such as beauvericin, in which alternative precursors compete to fill more than one position in the product. Mutasynthesis with the *kivr* knockout strain and D-Hbu or DL-Hmv (Figure 2) thus led to the efficient and exclusive biosyntheses of beauvericins G_3 (**3**) and C (**4**), respectively, with all three D-Hiv moieties replaced by the unnatural precursor. We have not monitored the biosynthesis of altered bassianolides in these experiments. Mutasynthesis with the *kivr* knockout strain is comparable in principle to the utilization of D-Hiv analogues during in vitro reconstituted enzymatic synthesis of cyclooligomer depsipeptides.[33,35,41] Whereas in vitro enzymatic synthesis is not constrained by precursor uptake or cell toxicity issues, in vivo mutasynthesis is inherently scalable during fermentation and could provide high yields.[27] Importantly, however, both of these approaches are limited by the inherent substrate specificity and tolerance of the NRPS enzyme involved. Consequently, D-2-hydroxycarboxylic acids with larger substituents at C2, such as hexahydromandelate, or those with γ-branched side chains, such as 2-hydroxyisocaproate, did not support in vivo mutasynthesis of beauvericins in our experiments, and had been only marginally accepted during in vitro synthesis of enniatins.[33] This hurdle might be overcome by reprogramming the NRPSs themselves.[43]

We have demonstrated here for the first time an in vivo combinatorial biosynthesis strategy to produce a library of unnatural beauvericin-type cyclooligomer depsipeptides by simultaneous feeding of pairs of D-Hiv and L-Phe analogues during mutasynthesis (Figures 3 and Table 1). In this format, successful incorporation of a new precursor analogue can be quickly leveraged to produce a novel series of beauvericins by pairing the new precursor with other precursor analogues previously validated as acceptable for the strain. Such an approach, however, would be expected to be practical only in a mutasynthetic format in the case of cyclooligomer depsipeptides. With the wild-type strain, the repeated use of the native and the unnatural precursors during biosynthesis yields an excessive number of cyclooligomer products, due to all the possible circular permutations. Simultaneous feeding of both an L-Phe and a D-Hiv analogue to a wild-type *B. bassiana* strain, for example, will theoretically yield 24 different beauvericins (in comparison with four with the *kivr* knockout strain; Figure 2). Similarly, simultaneous feeding of two different D-Hiv analogues would return eleven product analogues (relative to four with the *kivr* knockout strain; Figure 2). Such complex product mixtures would likely prove difficult to separate and would spread the yield, so that each individual beauvericin congener would be produced only in a minor amount. The simplified product profile obtained with the *kivr* knockout strain during simultaneous feeding of more than one precursor analogue allowed the efficient in vivo combinatorial biosynthesis of 14 new unnatural beauvericin analogues that belong to five novel series; this effectively doubled the number of known beauvericin analogues. In several of these compounds (**7**, **10**, **13**, **16**), all amino acid and hydroxycarboxylic acid positions are completely replaced by the fed precursors, so these are truly "unnatural" beauvericins. Combinatorial mutasynthesis also made it possible to extend the structural variety of fluorinated beauvericin analogues (H and I series). The H series of beauvericins is especially intriguing, because natural products are not known to contain halogen substituents at *meta* positions. Indeed, while organofluorides are very rare in nature, fluorinated analogues are abundant amongst marketed drugs because they often possess increased biological activities and more favorable pharmacokinetic properties.

Interestingly, DL-Hmv is apparently a better substrate than D-Hbu for beauvericin synthesis in vivo, as shown by the need to supply D-Hbu in a ten-fold excess relative to DL-Hmv during simultaneous feeding. Indeed, beauvericins A, B, and C (with D-Hmv instead of D-Hiv) are minor natural products in some beauvericin fermentations,[23] whereas the beauvericin G series (D-Hbu instead of D-Hiv) is detected only in precursor feeding experiments.[22] In contrast, purified enniatin synthetase shows a three-fold preference for D-Hbu over D-Hmv as a substrate in vitro;[33] further experiments should determine the contributions of the hydroxycarboxylate substrate specificities of the beauvericin versus the enniatin synthetases (as opposed to the in vivo substrate uptake efficiencies) to this discrepancy.

Bassianolide, as well as all the nine isolated beauvericin analogues and the three beauvericin analogue mixtures that were inseparable by common chromatographic techniques, were evaluated for their antiproliferative and antihaptotactic activities against the metastatic prostate cancer cell line PC-3M (Table 2). Bassianolide (up to 30 μM) had no activity in either assay. The beauvericin analogues produced by combinatorial mutasynthesis or precursor-directed biosynthesis showed a one log variance in cytotoxicity (1.4–16.3 μM), with a slightly smaller variation in their antihaptotactic activities (2.3–17.2 μM). While none of these analogues proved superior to beauvericin, the introduced structural changes often affected the antiproliferative and the cell migration inhibitory activities of these compounds independently of each other; this allowed us to define the structure–activity relationships of this important scaffold further. As is evident in the G series of beauvericins (e.g., **3**), replacement of the D-Hiv moieties in beauvericin with D-Hbu was detrimental both to the antiproliferative and to the antihaptotactic activities.[22] In contrast, replacement of D-Hiv with D-Hmv (as in **4**) did not affect cell motility but increased cytotoxicity. Replacement of the *N*-Me-L-Phe residues with their *meta*-fluoro or *ortho*-fluoro analogues (H and I series) similarly increased the antiproliferative activities of these compounds, while the antihaptotactic activities remained unchanged. In scrambled beauvericin analogues with both the amino acid and the hydroxycarboxylic acid varied, the presence of a fluorinated amino acid (as in the G_3H and G_3I series) compensated for the detrimental effects of the missing methyl groups in the G_3 analogues for cytotoxicity but only partially balanced the decrease in antihaptotactic activity. The increased cytotoxicities gained from the presence either of the fluorophenylalanines or of the D-Hmv residues (as in the CH and CI series) did not prove to be additive and did not seem to affect the inhibition of cell migration. Finally, asymmetric

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beauvericins with both D-Hbu and D-Hmv moieties (**17**, **18**) were slightly more cytotoxic than beauvericin without significant changes in their antihaptotactic activities. Further research directed towards production of unnatural beauvericins by combinatorial (bio)synthetic strategies to disconnect and to tune the antiproliferative and the antihaptotactic activities of this interesting bioactive scaffold through more drastic structural changes is underway in our laboratories.

Experimental Section

Strains, plasmids, chemicals, and culture conditions: *B. bassiana* ATCC 7159 was maintained on potato dextrose agar (PDA, Difco). *E. coli* Epi300 and fosmid pCCFOS1 (both from Epicentre) were used for *B. bassiana* genomic library construction, and *E. coli* DH10B and plasmid pJET1 (Fermentas) were used for routine cloning and sequencing. *Agrobacterium tumefaciens* LBA4404 (Invitrogen) with the plasmid pAg1-H3[32] and the *bar* (glufosinate resistance) selectable marker gene from plasmid pCB1524[44] were used for *Beauveria* transformation. Chemicals were purchased from Sigma–Aldrich, except for L-(3-fluorophenyl)alanine and L-(2-fluorophenyl)alanine (CSPS Pharmaceuticals, San Diego, CA, USA), and were of the highest purities available. *B. bassiana* fermentations were carried out as described,[22] with supplementation of D-Hbu (5 mM, final concentration), DL-Hmv (5 mM), D-2-F-Phe (0.3 mM), and D-3-F-Phe (0.3 mM) if necessary. For simultaneous double feeding with D-Hbu and DL-Hmv, D-Hbu was used at 10 mM and DL-Hmv was supplied at 1 mM. The fluorophenylalanine precursor analogues are moderately toxic to *B. bassiana* and their feeding leads to decreased beauvericin titers in fermentations.

Preparation of DL-HMV: Sulfuric acid (4 M, 5 mL, 0.02 mmol) was added dropwise to a stirred suspension of DL-isoleucine (1.0 g, 7.59 mmol) in H_2O (10 mL) until a clear solution was obtained. This solution was cooled to 0 °C, followed by the simultaneous addition of aq. $NaNO_2$ (15 mL, 0.03 mmol) and the remainder of the above sulfuric acid solution over a period of 1 h. The reaction mixture was stirred at 0 °C for 2 h, brought to room temperature, and stirred for an additional 16 h. It was then extracted with EtOAc (4× 100 mL), and the combined EtOAc extracts was washed with H_2O (3×100 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude product thus obtained was purified by chromatography over a column of silica gel (20 g) made up in CH_2Cl_2 and eluted with MeOH/CH_2Cl_2 (2:98) followed by MeOH/CH_2Cl_2 (3:97). Fractions eluted with MeOH/CH_2Cl_2 (3:97) were combined and concentrated to afford DL-Hmv (427.1 mg, 42%).

Cloning and sequence analysis: The *kivr* gene is clustered with the *bbBeas* beauvericin synthetase gene of *B. bassiana*.[28] Complete sequencing of the pCCFOS1-based fosmids fBass28 A03 and fBass11F05, covering the beauvericin biosynthetic locus of *B. bassiana*, was achieved through the generation of overlapping *Hae*III fragments by partial digestion and the cloning of these fragments into pJET1. Automated DNA sequencing was performed on double-stranded DNA templates with use of the JET forward and reverse primers (Fermentas) at the University of Arizona Genomic Analysis and Core Technology facility. Custom-designed oligonucleotide primers were used for sequence finishing. Sequencher 4.7 (Gene Codes Corp., Ann Arbor, MI, USA) was used for sequence assembly, and the VectorNTI suite 9.0 (Invitrogen) for sequence analysis. The annotated sequence of the beauvericin biosynthetic locus appears in GenBank as EU886196.

Disruption of the *kivr* gene: The *kivr* disruption cassette (See Supporting Information) contained the *bar* glufosinate resistance marker between two PCR-amplified DNA fragments covering the 5′ and the 3′ regions of the *kivr* gene. This disruption cassette was inserted between the left and right borders of the T-DNA of the pAg-H1 vector and used for *Agrobacterium*-mediated transformation and selection of glufosinate-resistant *B. bassiana* transformants as described.[31] The digoxigenin-labeled (Roche Applied Science) *bar* gene was used as the probe in Southern hybridizations. Ten independent, validated *kivr* knockout isolates were collected, and four of these were evaluated for the production of beauvericin and bassianolide with or without D-Hiv feeding. Three independent ectopic integrant isolates were also used as controls. Since the four homologous recombinant isolates were indistinguishable in these fermentations, only a single isolate was used for the simultaneous combinatorial feeding experiments.

Extraction, isolation, and analysis of metabolites: Metabolites from fermentations were extracted and partially purified by solvent/solvent partition from *B. bassiana* mycelia as described.[22] HPLC analyses and reversed-phase preparative HPLC were carried out on a HP-1050 instrument fitted with a Kromasil C_{18} column (5 μm, 250 mm×4.6 mm), mobile phase MeOH/H_2O (80:20, flow rate 1.0 mL min^{-1}), detection at 210 nm. Separation of beauvericin mixtures was also attempted by chromatography on a Sephadex LH-20 column (850 mm × 20 mm) with MeOH/H_2O (80:20) as solvent, and by reversed-phase preparative HPLC with a Kromasil C_5 column (5 μm, 250 mm×10 mm) with several different MeOH/H_2O or CH_3CN/H_2O gradients. Dilute aliquots of beauvericin product mixtures that could not be separated on a preparative scale were resolved at a reduced flow rate (0.4 mL min^{-1}) by analytical HPLC, and the relative amounts of the components were approximated on the basis of peak area values.

Biological assays: The in vitro tetrazolium-based cytotoxicity assay[45] and the cell migration inhibition assay[46] against a monolayer of the metastatic prostate cancer cell line PC-3M and its quantification[47] were conducted as described previously.[13] The reported values are the averages derived from two to four independent experiments each with four replicates. The MTT/WHA index contrasts the antiproliferative and the antihaptotactic activities of a given compound against the PC-3M cells. Values <1 indicate that the inhibition of cell migration is primarily due to the antiproliferative activity of the compound; larger values indicate specific inhibition of cell migration independent of cytotoxicity.

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