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In vitro chemoenzymatic and *in vivo* biocatalytic syntheses of new beauvericin analogues†

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New beauvericins have been synthesized using the nonribosomal peptide synthetase BbBEAS from the entomopathogenic fungus *Beauveria bassiana*. Chemical diversity was generated by *in vitro* chemoenzymatic and *in vivo* whole cell biocatalytic syntheses using either a *B. bassiana* mutant or an *E. coli* strain expressing the *bbBeas* gene.

The entomopathogenic fungus *B. bassiana*[1] produces various secondary metabolites,[2,3] including the cyclooligomer depsipeptide (COD) beauvericin. This COD is a cyclic trimer of D-Hiv-*N*-methyl-L-phenylalanine dipeptidol monomers. Beauvericin displays structural analogies to the cyclohexadepsipeptide enniatin (*Fusarium oxysporum*) and to the cyclooctadepsipeptides bassianolide (*Beauveria bassiana*) and PF1022A (*Rosellinia sp.*).[4] Fungal CODs are interesting pharmacophores that exhibit a broad range of biological activities including antitumor,[5] antibacterial, antibiotic,[1] antifungal, insecticidal, anthelmintic,[6] antimalarial, anti-inflammatory and immunosuppressant activities.[7] Therefore, analogues and derivatives of these small bioactive natural products may acquire important roles in modern medicine to treat a variety of diseases.

CODs are produced by nonribosomal peptide synthetase (NRPS) enzymes in an iterative and recursive process.[4,8] Beauvericin synthetase (BbBEAS) contains two NRPS modules harbouring domains that catalyze adenylation (A), thiolation (T), methylation (M) and condensation (C) reactions. The A domains activate their dedicated substrates (A_1: D-2-hydroxyisovalerate [D-Hiv]; A_2: L-phenylalanine [L-Phe]) by aminoadenylation, followed by covalent loading of these substrates onto BbBEAS for subsequent assembly of beauvericin.[4,9–11] The beauvericin precursor D-Hiv is synthesized by ketoisovalerate reductase (KIVR) from 2-ketoisovalerate from primary metabolism.[4,12,13]

In this contribution we demonstrate, for the first time, the isolation of recombinant beauvericin synthetase BbBEAS (351 kDa, ~0.4 mg mL^{-1}) from *E. coli* and generate new beauvericin analogues by a chemoenzymatic approach (Fig. 1A). In a subsequent *in vivo* whole cell biocatalytic approach (Fig. 1B), we also investigate the production of beauvericins by mutational biosynthesis (MBS)[14–17] using a KIVR knockout strain of *B. bassiana* (*B. bassiana kivr*$^-$)[12] or an *E. coli* strain expressing BbBEAS (*E. coli bbBeas*$^+$).[18] Previous studies of enzymatic synthesis of CODs with related fungal NRPSs indicated a relaxed substrate specificity for the hydroxy acid-activating A_1 domain, whereas amino acid activation was apparently more restricted.[19,20] Application of these observations to *in vivo* biosynthesis with fungal cultures led to the isolation of new CODs of the enniatin,[21] the PF1022 and the beauvericin series.[22,23]

Beauvericin synthetase BbBEAS was isolated from *E. coli* BL21 *bbBeas*$^+$ (for cultivation conditions see ESI†, General techniques)

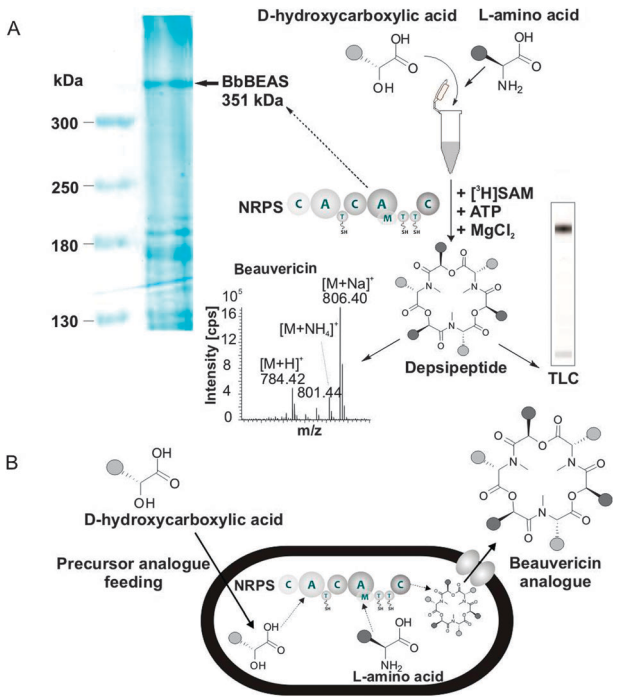

Fig. 1 Synthesis of beauvericin analogues. (A) Concept of the *in vitro* chemoenzymatic synthesis. An SDS-PAGE gel of recombinant BbBEAS isolated from *E. coli bbBeas*$^+$ is also shown. (B) Concept of the *in vivo* whole cell biocatalytic synthesis.

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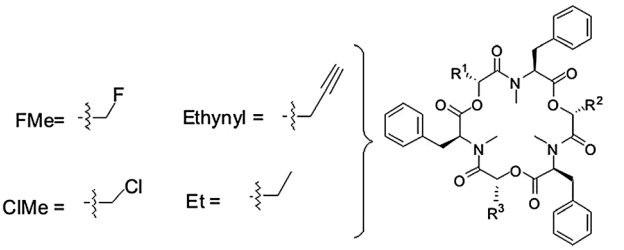
by precipitation with 60% ammonium sulphate as described recently for related COD synthetases.[24] *In vitro* reconstitution of the enzyme and detection of synthesised COD analogues were carried out according to Zocher *et al.* by incorporation of a [^{3}H] label from [^{3}H-methyl]-SAM.[25] Accordingly, 10 μL of a reaction mixture, containing 0.1 M ATP (pH 7), 1 M $MgCl_2$ and 0.1 M L-Phe in 1 M Tris–HCl (pH 8), was added to 3 μL of 0.1 M D-2-hydroxyisovalerate (D-Hiv, 3). The reaction was started by adding 0.55 mCi [^{3}H-methyl]-SAM and 200 μL BbBEAS (0.2 nmol). After 30 min of incubation at 25 °C the reaction was stopped by adding 1 mL H_2O. The radioactively labelled depsipeptide was extracted with 2 mL EtOAc. 100 μL of the organic phase was mixed with 4 mL LumaSafe Plus and measured in a scintillator. The remaining organic extract was analysed by thin layer chromatography (TLC) using silica 60 F_{254} plates at room temperature (eluent EtOAc : MeOH : H_2O = 100 : 5 : 1) after equilibration of the chamber with eluent vapour. Detection and quantification were performed by using a Radio-TLC Scanner (Raytest). Efficient chemoenzymatic synthesis of beauvericin by recombinant BbBEAS was demonstrated using this protocol, and the structure of the product was confirmed by HPLC-ESI-MS and MS/MS (ESI†, Fig. S1 and S2, and General techniques). To our surprise, small amounts of beauvericin were also produced by the BbBEAS enzyme *in vitro* in control reactions where L-Phe was omitted from the reaction mixture (Fig. S1B, ESI†). We propose that L-Phe is present in the beauvericin synthetase preparation, probably as an activated enzyme-bound adenylate or as a thioester, similar to that observed earlier by Zocher *et al.*[26] Subsequently, we applied this chemoenzymatic synthesis strategy to the production of beauvericin analogues by replacing D-Hiv with one of 10 synthetic α-hydroxy acids (Table S1, ESI†). Four α-hydroxy acids, 2-hydroxy-butyric acid (D-Hbu, 2), DL-2-hydroxy-pent-4-ynoic acid (DL-Hpyn, 12), D-fluorolactate (13), and D-chlorolactate (14), were successfully incorporated into CODs (Table 1 and Fig. S1, ESI†).

In an attempt to expand the repertoire of beauvericin analogues, we synthesized and tested 37 hydroxy acids in an *in vivo* whole cell biocatalytic format (Table S1, ESI†). Precursor analogue feeding experiments with *B. bassiana kivr*$^-$ (mutational biosynthesis) have been performed as described by Xu *et al.*[12,22]

As an alternative, we also evaluated the BbBEAS-expressing *E. coli* strain as a whole cell biocatalyst because of its faster growth rate and ease of genetic manipulation. Heterologous production and extraction of beauvericin and its analogues from *E. coli* BL21 *bbBeas*$^+$ was based on the constructs and conditions of Xu *et al.*[18,22] The feeding regimen included simultaneous supplementation with the natural amino acid L-Phe and one of the synthetic hydroxycarboxylic acids. HPLC-ESI-MS was carried out to identify the expected beauvericin analogues by their characteristic molecular masses and retention times. Product structures were confirmed by ESI-MS/MS experiments, providing characteristic fragmentation pattern fingerprints. Further Multiple Reaction Monitoring (MRM) experiments were conducted to estimate the yields of the beauvericin analogues obtained.

Out of the 37 α-hydroxy acids tested, eight were shown to support mutational biosynthesis with the *B. bassiana kivr*$^-$ strain (Table 2). In spite of the radically different cell wall structures of the fungus and the Gram-negative bacterium, six of these eight hydroxy acids were also accepted by *E. coli bbBeas*$^+$ for biocatalytic conversion into beauvericin-like products. Five novel beauvericin analogues (Beau-4, -5, -10, -12, -16) were obtained by using these *in vivo* approaches, while a further three (Beau-2, -8, -9) have previously been described by Xu *et al.*[18] Beau-2 and Beau-12 were also detected during *in vitro* chemoenzymatic synthesis (Table 1). All the beauvericin analogues described here had all three of their α-hydroxy acid positions occupied by the fed precursor analogue, indicating that both the *B. bassiana kivr*$^-$ strain

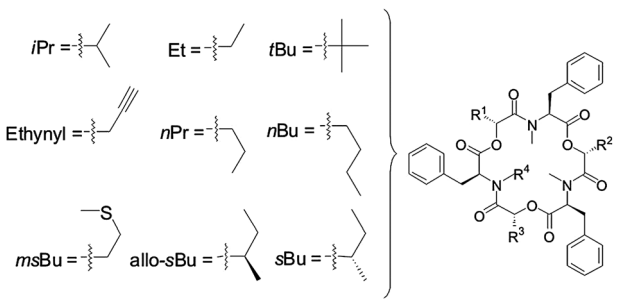
Table 1 Beauvericin analogues produced by *in vitro* chemoenzymatic synthesis with BbBEAS



No.	Precursor	Product	R_F value	$R^1 = R^2 = R^3$
3	D-Hiv	Beauvericin[a]	0.4[b]	*i*Pr
2	D-Hbu	Beau-2	0.7	Et
12	DL-Hpyn	Beau-12	0.6	Ethynyl
13	D-Fluorolactate	Beau-13	0.5	FMe
14	D-Chlorolactate	Beau-14	0.5	ClMe

[a] Beauvericin produced by chemoenzymatic synthesis. [b] Average relative retentions (R_F) calculated from three independent experiments.

Table 2 Beauvericin analogues obtained by *in vivo* whole cell biocatalytic synthesis.



No.	Precursor	Product	*B. bassiana kivr*$^-$ Yield[a]	*E. coli bbBeas*$^+$ Yield[a]	$R^1 = R^2 = R^3$	R^4
3	D-Hiv	Beauvericin	1.11	3.33	*i*Pr	Me
2	D-Hbu	Beau-2	0.23	0.005	Et	Me
4	D-Hval	Beau-4	0.48	2.18	*n*Pr	Me
5	D-Hcap	Beau-5	0.34	0.32	*n*Bu	Me
8	D-allo-Hmv	Beau-8	7.82	1.20	allo-*s*Bu	Me
9	D-Hmv	Beau-9	5.19	2.76	*s*Bu	Me
10	DL-Htbu	Beau-10	0.10	0.04	*t*Bu	Me[b],H[c]
12	DL-Hpyn	Beau-12	0.17	ND[d]	Ethynyl	Me
16	D-Hmsbu	Beau-16	0.04	NT[e]	*ms*Bu	Me

[a] Average yields of the product in mg L^{-1} are calculated from two independent experiments. Final concentrations of the precursors during fermentation: *B. bassiana*, 40 mM; *E. coli*, 30 mM (D-Hiv: 15 mM). [b] *B. bassiana kivr*$^-$. [c] *E. coli bbBeas*$^+$. [d] ND, not detected. [e] NT, not tested.

and the BbBEAS-expressing *E. coli* are devoid of other acceptable 2-hydroxy carboxylic acids. Both producer strains also fully methylated all three amino acid positions of these beauvericin analogues, with the exception of Beau-10 from *E. coli* where one of the amino acid positions remained unmethylated. For example, feeding hydroxy acid 12 (DL-2-hydroxy-pent-4-ynoic acid) to *B. bassiana kivr*$^{-}$ yields Beau-12 with a molecular mass of $[M + H]^+ = 772.6$ and a retention time of 5.3 min, with the corresponding MS/MS spectrum providing a fingerprint where each peak can be assigned to one fragment of the molecule (Fig. S3h, ESI†). Similarly, feeding hydroxy acid 4 (D-2-hydroxy-pentanoic acid) to *E. coli bbBeas*$^{+}$ yields Beau-4 $[M+H]^+ = 784.4$ with a retention time of 5.7 min. Characteristic fragments from MS/MS experiments were assigned accordingly (Fig. S4c, ESI†). The yields of the beauvericin analogues Beau-2, -4, -5, -8, -9, -10, -12 and -16 from *B. bassina kivr*$^{-}$ were estimated by HPLC-ESI-MS (for quantification see ESI†, General techniques) to range between 0.04 and 7.82 mg L^{-1} (Table 2, and Fig. S3a–i, and Table S2, ESI†). Upon comparison, we find that the same strain produces 1.1 mg L^{-1} beauvericin upon feeding the natural hydroxy acid 3 (D-Hiv) under identical fermentation conditions. Product yields in *E. coli* ranged from $\sim$0.005 mg L^{-1} to $\sim$2.8 mg L^{-1} for Beau-2, -4, -5, -8, -9 and -10, as compared to that of beauvericin at $\sim$3 mg L^{-1} with the native substrate 3 in this strain (Table 2, and Fig. S4a–g, and Table S3, ESI†). Surprisingly, precursor analogues 8 and 9 provide for beauvericin analogues yields in *B. bassiana* of up to 7.8 and 5.2 mg L^{-1}, respectively, exceeding that of the native product beauvericin. Although the same precursor analogues also performed well in *E. coli bbBeas*$^{+}$, the yields of Beau-8 and Beau-9 did not exceed that of beauvericin in this host. In contrast to chemoenzymatic synthesis, product analogue yields are determined not only by the innate substrate preferences of BbBEAS during *in vivo* biosynthesis. Rather, the variability of precursor uptake, precursor and product toxicity, and catabolism of the precursor or even the product can all reduce or boost beauvericin analogue yields to different extents in different host strains. Thus, Beau-13 and Beau-14 were only detectable during *in vitro* chemoenzymatic synthesis, but not during *in vivo* biocatalysis. Conversely, while precursor analogues 4, 5, 8, 9, and 10 were apparently acceptable for BbBEAS *in vivo*, the corresponding CODs remained below the detection limits of the chemoenzymatic assay. Such unexpected and unpredictable results emphasize the complementary nature of *in vitro* chemoenzymatic and *in vivo* whole cell biocatalytic approaches[27] towards natural product diversification and "unnatural product" biosynthesis. While the full characterization of the substrate specificity of BbBEAS requires further studies, our results show that the tolerance spectrum of this enzyme includes hydroxy acids with various aliphatic branched side chains, and extends even to halogenated hydroxy acids. Particularly interesting is the biosynthesis, albeit at a low yield, of Beau-12 with the ethynyl side chain amenable to further derivatization by 1,3-dipolar cycloaddition reactions known as "click chemistry" as has also been shown recently for ribosomally synthesized peptide antibiotics.[28,29]

In summary, we successfully generated seven novel beauvericins by *in vitro* chemoenzymatic and *in vivo* biocatalytic syntheses using custom-synthetic hydroxy acid precursor analogues. The production of these new beauvericins could now be optimized and scaled up for characterization in various biological assays.

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