Experimental

Strains and culturing

*Escherichia coli* strain Tam1 (Active Motif) was used as host for plasmids that did not contain a Gateway destination cassette. Gateway destination vectors were propagated in *cdcb* Survival cells (Invitrogen). *Saccharomyces cerevisiae* strain YPH499 (Stratagene) was used as host for plasmid assembly by homologous recombination. *Aspergillus oryzae* strain M-2-3, an arginine auxotroph, was obtained from Professor I. Fuji, University of Tokyo and mycelium was routinely maintained at 28 °C on DPY (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH₂PO₄, 0.05% MgSO₄, 1.5% agar). The following *Beauveria* strains were obtained from Warwick HRI, Wellesbourne, UK: *B. bassiana* 470.96, 544.96, 568.96, 635.96, 719.96, 809.05, 828.05, 911.05, 918.05, 957.05, 965.05, 963.05, 992.05, 1062.05, 1119.05, 1129.05, 1322.05, *B. tenella* 1329.05, 1331.05, *B. brongniartii* 1336.05 and *B. amorpha* 1339.05. *B. bassiana* strain 110.25 and *B. brongniartii* strains 106.22, 109.26, 111.25, 112.42, 120.26, 128.53, 326.67 and 327.67 were obtained from CBS, Utrecht, Netherlands. A *Psoroptes ovis* (sheep scab mite) isolate of *B. bassiana* was obtained from CSL, York, UK. *Beauveria* cultures were maintained at 25 °C on potato dextrose agar (PDA, Difco). Tenellin production medium (TPM): D-mannitol 50 g, KNO₃ 5 g, KH₂PO₄ 1 g, MgSO₄·7H₂O 0.5 g, NaCl 0.1 g, CaCl₂ 0.2 g, FeSO₄·7H₂O 20 mg and mineral ion solution 10 mL (ZnSO₄·7H₂O 880 mg, CuSO₄·5H₂O 40 mg, MnSO₄·4H₂O 7.5 mg, boric acid 6 mg and (NH₄)₆Mo₇O₂₄·4H₂O 4 mg made up to 1 L in deionised water) made up to 1 L in deionised water. Bassianin production medium (BPM).³ CMP: Czapke maltose peptone media (CMP): Czapek-Dox supplemented with 3% maltose and 1% peptone.²

DNA manipulations

Routine cloning was performed according to standard procedures.³ PCR amplifications were routinely performed using Pusbin high fidelity DNA polymerase (NEB) and genomic DNA was extracted from fungal transformants (grown at 28 °C in 10 mL CMP for 7 days with shaking (150 rpm)) using the GenElute Plant Genomic DNA Miniprep Kit (Sigma).

Sequencing, sequence analysis and sequence accession number

DNA sequencing was performed by the Core Genome Facility, University of Sheffield (UK). Sequences were assembled using Sequencher® analysis software, analysed by BLAST (NCBI) and aligned using ClustalW.⁴ Domain analysis was performed using BLAST (NCBI), PKS/NRPS analysis website⁵ and the knowledge based resource for analysis of PKS-NRPS (http://www.nii.res.in/nrps-pks.html). The DMB gene cluster sequence from *B. bassiana* 992.05 has been deposited in the NCBI Database under Accession No HM243222.

Fungal transformation

Plasmid DNA for fungal transformation was prepared using the NucleoBond Midi Prep Kit (Machery-Nagel). *A. oryzae* protoplasts were prepared and transformed with 10 μg DNA as described previously.² Transformants were maintained at 28 °C on DPY or DPY supplemented with basta (75 μg mL⁻¹, Aventis). *B. bassiana* 992.05 was grown on potato dextrose agar plates for 10 days. Sperms were collected into sterile water (1 mL) and inoculated into 50 mL potato dextrose broth and grown at 25 °C for 18 h at 200 rpm. Mycelium was collected by centrifugation at 8000 g for 5 min and protoplasts prepared by incubating with *Trichoderma harzianum* lysing enzymes (20 mg mL⁻¹, Sigma) in 0.8 M NaCl with gentle shaking at room temperature for 2 h. Protoplasts were filtered through miracloth and centrifuged at 2500 g for 5 min. Protoplasts were diluted to 3 × 10⁶ L⁻¹ in solution 1 (0.8 M NaCl, 10 mM CaCl₂, 50 mM Tris-HCl, pH 7.5). Solution 2 (20 μL; 60% PEG 6000, 0.8 M NaCl, 50 mM CaCl₂, 50 mM Tris-HCl, pH 7.5) and 5 μg (10 μL) of appropriate plasmid were added to 100 μL protoplasts and incubated on ice for 30 min. 1 mL solution 2 was then added and the mixtures incubated at RT for 20 min. Transformation mixtures were suspended in CZDS soft top agar (7 mL, 3.5% Czapek-Dox broth, 0.7% agar, 1 M sorbitol), poured onto Czapek-Dox plates supplemented with sorbitol (1 M) and incubated at 25 °C for 24 h. Plates were then overlaid with 7 mL CZDS supplemented with basta (50 μg mL⁻¹) and incubated for a further 7 days. Putative transformant colonies were subcultured on fresh Czapek-Dox plates supplemented with basta (50 μg mL⁻¹) and grown at 25 °C.

Screening of metabolites and LCMS analysis

*Beauveria* strains were grown in 100 mL TPM or BPM at 25 °C for 10 days at 200 rpm. *A. oryzae* transformants were grown in 100 mL CMP medium at 28 °C for 10 days at 200 rpm. *A. oryzae* transformants were grown in 100 mL CMP medium at 28 °C for 10 days at 200 rpm. *A. oryzae* transformants were grown in 100 mL CMP medium at 28 °C for 10 days at 200 rpm. *A. oryzae* transformants were grown in 100 mL CMP medium at 28 °C for 10 days at 200 rpm.
Purification of compounds

Purification of compounds was generally achieved using a Waters mass-directed autopurification system comprising of a Waters 2767 autosampler, Waters 2545 pump system, a Phenomenex LUNA column (5μ, C18, 100 Å, 10 × 250 mm) equipped with a Phenomenex Security Guard precolumn (Luna C3 300 Å) eluted at 4 mL min⁻¹. Solvent A, HPLC grade H2O + 0.05% formic acid; Solvent B, HPLC grade MeOH + 0.045% formic acid; solvent C, HPLC grade CH3CN + 0.045% formic acid. The post-column flow was split (100:1) and the minority flow was made up with solvent A to 1 mL min⁻¹ for simultaneous analysis by diode array detector (Waters 2998), evaporative light scattering (Waters 2424) and ESI mass spectrometry in positive and negative modes (Waters Quatro Micro). Detected peaks were collected into glass test tubes. Combined fractions were evaporated under a flow of dry N2 gas and residues dissolved directly in NMR solvent for NMR analysis.

Desmethylbassianin 5. B. bassiana 992 was grown in TPM medium (10 × 100 mL in 500 mL Erlenmeyer flasks) at 25 °C for 10 days shaken at 200 rpm. The culture was extracted following protocols earlier described to yield a brown solid (1.1 g). The residue was dissolved in acetone (50 mg·mL⁻¹) and subjected to preparative TLC fractionation. The entire extract was loaded onto silica-coated glass TLC plates (20 cm × 20 cm, 11 plates in total), and developed in chloroform/methanol/water (90:18:2). The major bright yellow band at Rf 0.31 corresponding to 5 was scraped off using a spatula onto a filter paper. It was then extracted with acetone, filtered (× 3) to remove silica gel fine particles, and concentrated to yield a dark yellow solid (30.6 mg). The latter was dissolved to 50 mg·mL⁻¹ in HPLC grade methanol, centrifuged for 1 min at 14,000 rpm, and the supernatant subjected to HPLC mass-directed preparative purification. 50-100 μL was injected during successive rounds of a 45 minute program (0 min, 5% B; 13 min, 95% B). Fractions corresponding to the pure compound were evaporated to yield semi-pure compounds. Further refinement following similar HPLC protocols using a gradient of solvents A and B afforded 6 (6.4 mg).

proto-DMB B 8 and proto-DMB C 9. A. oryzae pTAYAarg·dmbS was cultured in CMP medium (15 × 100 mL in 500 mL Erlenmeyer flask) at 25 °C for 8 days with shaking at 200 rpm. The culture was vacuum filtered and the filtrate acidified to pH 3 using 37% HCl, then extracted with ethyl acetate (1.5 L). The organic extract was washed with brine (1 L), and then distilled water (1 L), dried (MgSO4) and concentrated to yield a dark solid (252 mg). The residue was dissolved (to 50 mg·mL⁻¹) in HPLC grade methanol and subjected to HPLC mass-directed purification. The crude solution was injected (100 – 200 μL) during successive rounds of a 20 minute HPLC program (0 min, 25% B; 13 min, 95% B; 15 min, 95% B; 17 min, 25% B; 20 min, 25% B). Eluted fractions were separately collected and evaporated to yield semi-pure compounds. Further refinement following similar HPLC protocols using a gradient of solvents A and B afforded 8 (2.6 mg, 1.7 mg·L⁻¹) and 9 (3.2 mg, 2.1 mg·L⁻¹).

Prototenellin C 12. A. oryzae pTAYAarg·tenS was grown in CMP medium (10 × 100 mL in 500 mL Erlenmeyer flasks) at 25 °C for 7 days with shaking at 200 rpm. Cells and media (1 L) were homogenized and acidified to pH 4.0 using 37% HCl. The resulting homogenate was vacuum filtered and the filtrate extracted with EtOAc (2 × 500 mL). The organic extract was dried (MgSO4) and evaporated to afford a crude extract (70 mg). The extract was dissolved (to 50 mg·mL⁻¹) in HPLC grade methanol and used for mass-directed HPLC preparative purification. 100 – 200 μL of the crude solution was injected during successive rounds of a 20 minute HPLC program (0 min, 25% B; 13 min, 95% B; 15 min, 95% B; 17 min, 25% B; 20 min, 25% B). Fractions corresponding to the pure compound were evaporated to yield 12 (2.4 mg). Similar protocols were used in purifying the same compound from 137 mg of a crude extract of A. oryzae pTAYAarg·tenS·dmbSI obtained from a 1 L fermentation which produced 12 (9.6 mg).

pre-DMB A 13. A. oryzae pTAYAarg·dmbS·pTAYAbar·tenC was cultured in CMP medium (10 × 100 mL in 500 mL Erlenmeyer flasks) at 25 °C for 7 days with shaking at 200 rpm. Cells and media (1 L) were homogenized, acidified to pH 4.0 using 37% HCl, and filtered. The filtrate was extracted with EtOAc (2 × 500 mL). The organic extract was dried (MgSO4) and evaporated to give a dark brown solid (111 mg) which was dissolved (to 50 mg·mL⁻¹) in HPLC methanol and used for HPLC mass-directed purification. 100 – 200 μL of the crude solution was injected during successive rounds of a 20 minute HPLC program (0 min, 25% B; 13 min, 95% B; 15 min, 95% B; 17 min, 25% B; 20 min, 25% B). Collected fractions were evaporated to afford pure 13 (23 mg). The same method was used in purifying 13 (17.6 mg) from a 1 L culture of A. oryzae pTAYAarg·dmbS·pTAYAbar·dmbC. 
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Other data 
Bright yellow powder; mp 140 - 143 °C, IR (neat): \(v_{\text{max}}\) 2960, 2925, 1717, 1640, 1610, 1514, 1426 cm\(^{-1}\); HRESMS calcd. for C\(_{32}\)H\(_{26}\)NO\(_{3}\)Na: 404.1474; found 404.1472 [M]Na\(^{+}\)

HRESMS calcd. for C\(_{32}\)H\(_{26}\)NO\(_{3}\): 580.2547. Found: 580.2540 [M]H\(^{+}\).

Bright yellow amorphous solid; IR (neat): \(v_{\text{max}}\) 3750, 3100, 1750, 1640, 1610, 1570 cm\(^{-1}\); HRESMS calcd. for C\(_{32}\)H\(_{26}\)NO\(_{3}\): 338.1398; found 338.1454 [M - H].
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Cluster sequencing and cloning of dmbS.

The dmbS cluster was cloned by PCR. Initially a series of PCRs using ORF-specific primers for tenA to tenC were used to amplify entire ORFs. Untranslated regions (UTR) were similarly amplified using tenS cluster specific primers. Primers based on tenS were used to amplify short sections of dmbS, and the sequence information obtained from the PCR products used to design gene-specific primers to amplify larger overlapping fragments of the dmbS gene (Figure 1). Fragments P1 and P2 both contain the start (0.8 kb) of the dmbS coding region; P2 starts with a tenS primer (TenSStartFwd) that includes the start of translation of tenS and 16 b of 5' UTR. P1 starts further upstream (with another tenS primer) and was not used in assembling the dmbS gene. The 3' end (1 kb) of the dmbS gene (P6) was amplified with a gene-specific forward primer (992TENDF) and a tenS reverse primer (TenSrev3'end; single-base difference changes dmbS stop codon from TAA to TGA).

The rest of the dmbS coding region (plus introns) was amplified as fragments P3 (3.9 kb), P4 (3.8 kb) and P5 (3.9 kb). P2 and P6 were cloned directly or transferred into Gateway entry vectors. They were then transferred to pTAYAGSarg (pTAex3GS modified to be a yeast-E. coli shuttle vector) by LR recombination to yield pTAYA992Bg and pTAYA992Nd respectively.

Figure 1. Dmb gene cluster sequencing strategy. PCR reactions to produce fragments for sequencing and reconstruction. P1, P2 and P4 were cloned into pENTR/D-TOPO; P3 and P5 were cloned into pZeroBlunt; P6 was cloned into pJet1.2 (Fermentas), excised at flanking restriction sites and ligated into pENTR3F.
Construction and deployment of pTAYAarg-dmbS

The Gateway modified A. oryzae expression vector pTAex3GS was first converted to a yeast-E. coli shuttle vector as follows. The URA3 selectable marker and 2μ origin of replication were amplified from the shuttle vector pENTR-YA using primers (YA-PTLk-F and YA-PTLk-R) that contain 30 base 5’ extensions corresponding to sequences flanking the Stul site in pTAex3GS. pTAYAGSarg was created by transforming yeast with the 2.9 kb PCR product and Stul-linearised pTAex3GS.

The beginning and end fragments of dmbS (P2 and P6, Figure 1) were inserted into pTAYAGSarg by Gateway LR recombination producing plasmids pTAYA992Bg and pTAYA992Nd. To assemble the complete dmbS coding region pTAYA992Nd was linearised with NotI and used to transform yeast together with PCR products covering the rest of the gene (Figure 2); fragment P2 was amplified with flanking amyB promoter sequence from pTAYA992Bg, and fragments P3, P4 and P5 were amplified from their corresponding plasmids. pTAYAarg-dmbS was used to transform A. oryzae M-2-3 (argB) and transformants were selected on minimal medium lacking arginine. Twenty six transformants were grown in 100 mL CMP for eight days, and metabolites were extracted and analysed by LCMS.

Construction of pEYdmbSKO/Sil (dmbS dual silencing/knockout plasmid)

The dual silencing/knockout plasmid pEYdmbSKO/Sil was constructed by homologous recombination in yeast using the yeast assembly vector pEYA, which was first linearised by digestion with NotI + AscI. A 3.8 kb genomic fragment of dmbS (fragment P4 in figure 1, cloned in pENTR-DTOPO, to yield plasmid pE/DT-P4,) was selected as template for amplification of the left (LA) and right (RA) arms (Figure 2). PCR on pE/DT-P4 using the primers M13Rev and 992R5 produced the LA fragment flanked by the vector attL2 site, which provided overlap with the linearised pEYA (adjacent to the AscI site). Similarly, PCR on pE/DT-P4 with primers M13Fwd and 992F3BAR produced the RA fragment flanked by the vector attL1 site, which provided overlap with the linearised pEYA (adjacent to the NotI site). 992F3Bar is a 50 mer oligonucleotide which contains a 30 base overlap with the Bar cassette (Figure 3). The glyceraldehyde-3-phosphate dehydrogenase (gdPA) promoter from A. nidulans has previously been shown to drive heterologous expression in Beauveria, and was isolated from plasmid pAN7.1 by using the primers PGPDA992F and PGPDABAR. PGPDA992F is a 50 mer oligonucleotide in which the last 20 bases are complementary to the 3’ end of the gdPA promoter and the first 30 bases comprise a tail that overlaps with the 3’ end of LA. PGPDABAR has a 30 base tail that is complementary to the 5’ end (promoter) of the Bar cassette. A Bar cassette containing the basta-resistance coding region driven by the A. nidulans trpC promoter was amplified from pTAex3-BARorf3 using the primers BarFwd and BarRev. Transformation of yeast with the LA, gdPA promoter, Bar cassette, RA and pEYA vector fragments assembled the plasmid pEYdmbSKO/Sil, which was used to transform B. bassiana 992.05 protoplasts.

Sixty-two basta-resistant B. bassiana 992.05 transformants were subcloned on solid medium containing increasing concentrations of basta. 17 colonies resistant to basta at high concentrations (150 μg.mL⁻¹) were grown in 100 mL TPM for 10 days, then metabolites were extracted and analysed by LCMS.

Construction of pTAYAarg-tenSdmbS1 (tenS PKS - dmbS NRPS).

pTAYAarg-dmbS was digested with AgeI and the 13125 bp fragment containing the vector and dmbSNRPS was isolated (Figure 4). The tenSPKS was isolated in two overlapping fragments from pTAex3arg-tenS. The beginning fragment (tenSPKSBg, 6.3 kb), was isolated by digesting with Stul, and the end fragment (tenSPKSNd, 3.5 kb), was isolated by digesting with MfeI. Finally a linker region was amplified from pTAYAarg-dmbS using tenSPKSLSK-F, a forward primer that contained a 30 base tail homologous to the end of the tenSPKS, and REV99115, a reverse primer in the dmbSNRPS. All three restriction fragments plus the linker region were used to transform S. cerevisiae, where recombination occurred at the homologous overlapping regions. Restriction digestion of the resultant pTAYAarg-tenSdmbS1 with BglII confirmed the presence of all the fragments, and sequencing over the pks-nrps junction confirmed that the swap had occurred in the expected position and maintained the integrity of the coding region.

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**Figure 2.** Reconstruction of dmbS in a fungal expression vector. P6 was transferred to pTAYAGSarg by LR recombination. P2 was amplified from pTAYA992Bg. P3 was amplified from pZeroBunt-F3. P4 was amplified from pENTR-D-Topo-P4. P5 was amplified from pZeroBunt-P5.

**Figure 3.** Construction of pEYdmbSKO/Sil.
Figure 3. *tenS-dmbS* swap strategy: A, pTAYAarg·dmbS 13125 bp *A*geI fragment containing the NRPS module and the vector; B, 6329 bp *Stu*I fragment of pTAex3arg·tenS; C, 3544 bp *Mfe*I fragment of pTAex3arg·tenS containing the rest of the *tenSPKS*; D, PCR product amplified from *dmbS* template using a forward primer containing a 30 base tail homologous to the *tenSPKS*. Recombination occurred at the regions indicated with the dashed lines (Diagram not to scale).

**Construction and deployment of pTAex3bar·dmbC.**

The expression plasmid pTAex3bar·dmbC was produced by LR recombination between pENTR/D-TOPO-dmbC and the fungal expression vector pTAex3Targbar,9 and used with pTAYA-dmbS to transform *A. oryzae* M-2-3 protoplasts. Basta-resistant colonies growing on a minimal medium lacking arginine (~ twenty four per transformation) were picked and subcloned onto solid medium containing an increased concentration of basta (150 µg mL⁻¹).
Nucleotide sequence analysis.

### Table of Nucleotide Sequences

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*All figures inclusive; top two hits; complimentary sequence.*

### Comparison of TENS and DMBS Catalytic Domains Boundaries and Active Site Residues

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$^a$ approximate figures; $^b$ all active site motifs identical.
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Translation of tenS (2547) EIRTWFKLQLRVDVFVMKILGSTVQLSALAAKLARQDAKQMGRTA
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Translation of dmbS (3376) FMSLVQVFICALANGGTVIVHABRPVELLMAHKVSLTIATPSZ
References