

Insights into the Biosynthesis of Hormaomycin, An Exceptionally Complex Bacterial Signaling Metabolite

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SUMMARY

Hormaomycin produced by *Streptomyces griseoflavus* is a structurally highly modified depsipeptide that contains several unique building blocks with cyclopropyl, nitro, and chlorine moieties. Within the genus *Streptomyces*, it acts as a bacterial hormone that induces morphological differentiation and the production of bioactive secondary metabolites. In addition, hormaomycin is an extremely potent narrow-spectrum antibiotic. In this study, we shed light on hormaomycin biosynthesis by a combination of feeding studies, isolation of the biosynthetic nonribosomal peptide synthetase (NRPS) gene cluster, and in vivo and in vitro functional analysis of enzymes. In addition, several nonnatural hormaomycin congeners were generated by feeding-induced metabolic rerouting. The NRPS contains numerous highly repetitive regions that suggest an evolutionary scenario for this unusual bacterial hormone, providing new opportunities for evolution-inspired metabolic engineering of novel nonribosomal peptides.

INTRODUCTION

Streptomycetes, an exceptionally rich bacterial source of natural products, employ a complex network of signaling factors to regulate their secondary metabolism (Bibb, 2005; Rokem et al., 2007). While most of the identified signals are intracellular proteins, a small number of low-molecular weight compounds have been identified that diffuse away from the producer and trigger biosynthetic and morphological events at more remote locations in the bacterial community (Horinouchi and Beppu, 1994; Igarashi et al., 2001; Kondo et al., 1987; Recio et al., 2004; Takano, 2006). These substances (Figure 1) exhibit diverse structures, the most unusual being displayed by hormaomycin (**1**) (Reinscheid et al., 2006; Rössner et al., 1990; Zlatopolskiy et al., 2004), also known as takaokamycin (Omura et al., 1984).

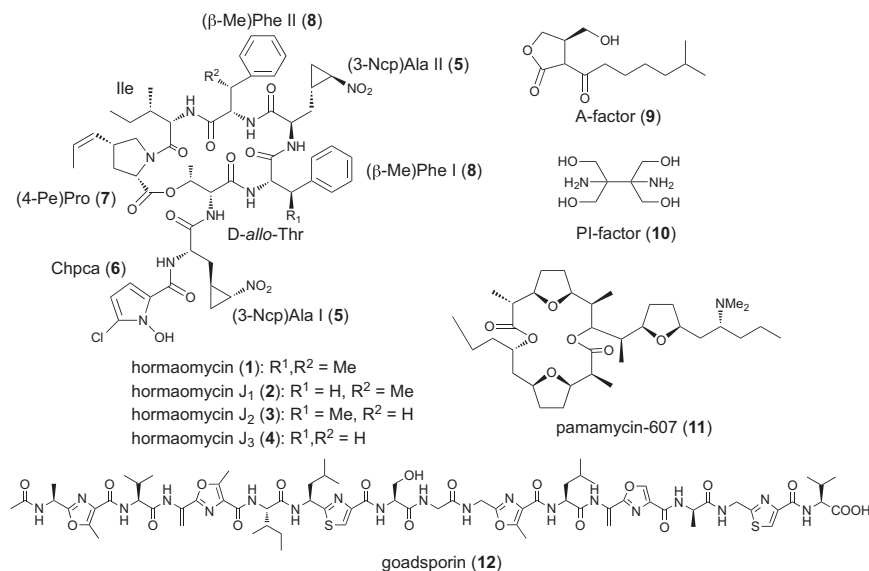
At nanomolar concentrations, this depsipeptide stimulates the production of antibiotics and the formation of aerial mycelium in various actinomycetes (Andres et al., 1989, 1990). In addition to the interspecies hormonal activity, **1** exhibits extremely potent narrow-spectrum antibiotic activities against coryneform actinomycetes (lowest measured MIC value 88 pM) (Andres et al., 1990). To date, the molecular mechanisms underlying these diverse activities are unknown.

The structure of **1**, which has been confirmed by total synthesis (Zlatopolskiy and de Meijere, 2004), is unique among signaling metabolites. It contains several unprecedented residues with chlorine, nitro, and cyclopropyl moieties in addition to a single proteinogenic amino acid, L-Ile. To date, limited information on the biosynthesis of only one residue exists. Feeding studies established that the 3-(*trans*-2'-nitrocyclopropyl)alanine ([3-Ncp]Ala [**5**]) unit is derived from L-lysine (Brandl et al., 2004) via functionalization of the γ -position and oxidation of the ω -amino group, but the individual enzymatic steps leading to this amino acid are unknown. Feeding studies also revealed that various analogs of amino acids can be readily incorporated into **1** (Kozhushkov et al., 2005; Zlatopolskiy et al., 2006) and demonstrated that this approach can result in dramatic changes of the pharmacological profile (Zlatopolskiy et al., 2006). In particular, hormaomycin D₂ containing a 5-nitronorvaline residue at one (3-Ncp)-Ala site exhibits a high antifungal activity against *Candida albicans*, while **1** itself is completely inactive. These data suggest an inherent catalytic flexibility of the biosynthetic machinery that could be exploited by metabolic engineering and mutasynthesis to generate novel bioactive peptides. To provide the foundation for such studies and obtain deeper insights into the biosynthesis of **1**, we report here feeding experiments, precursor-directed biosynthesis studies, isolation of the biosynthetic gene cluster and in vivo and in vitro functional experiments on several genes that reveal new insights into the assembly of this unique peptide.

RESULTS AND DISCUSSION

Identification of the Biosynthetic Precursors

To obtain information about the biosynthesis of residues other than (3-Ncp)Ala (**5**), incubation experiments with candidate



precursors were conducted. Pyrrole-2-carboxylic acid units related to Chpca (**6**) are found in several other natural products, such as aminocoumarins, phenalinolactone, pyoluteorin, and prodiginins (Walsh et al., 2006). They are often derived from proline via covalent attachment to a peptidyl carrier protein (PCP) and dehydrogenase-catalyzed aromatization. Therefore, a liquid culture of *S. griseoflavus* was supplemented with 2.2 mM L-[1-¹³C]Pro. After isolation of hormaomycin, its ¹³C-NMR spectrum showed 15.3% specific enrichment at the carbonyl signal of the Chpca (**6**) moiety, thus suggesting a biosynthesis from Pro (see Table S1 available online). In contrast, no enrichment of the (4-Pe)Pro (**7**) signals was observed, which argued against proline being a precursor of this amino acid. The biosynthetic origin of (β-Me)Phe (**8**) was probed by feeding stable isotope-labeled L-Phe and L-Met. The presence of 3 mM L-[1-¹³C]Phe in the cultivation medium resulted in strong isotope enrichments at the corresponding positions of the two **8** residues (45.3% for (β-Me)Phe I, δ = 169.7 ppm, and 42.0% for (β-Me)Phe II, δ = 170.8 ppm). Even higher enrichments resulted from feeding with 1 mM L-[methyl-¹³C]Met, which can serve as a methyl donor in S-adenosylmethionine (SAM)-dependent biological methylations. Here, 61.6% and 70.7% of the label were incorporated into the methyl groups of (β-Me)Phe I (δ = 13.3 ppm) and (β-Me)Phe II (δ = 17.7 ppm), respectively. The (β-Me)Phe units should therefore be derived from Phe or a related compound, which is methylated by a SAM-dependent methyltransferase.

In the feeding studies using labeled Met, we observed an additional enhanced carbon signal at δ = 13.2 ppm, which belongs to the terminal methyl group of (4-Pe)Pro (**7**). A similar incorporation pattern was previously reported for structurally related moieties in lincomycin (Brahme et al., 1984), and pyrrolo[1,4]benzodiazepines (Hurley, 1980). These originate from L-Tyr via a poorly characterized pathway involving cleavage of the oxidized intermediate L-DOPA (**13**). To test whether a similar route provides the **7** unit in hormaomycin biosynthesis, L-[1-¹³C]Tyr was administered to cultures of the producer strain

Figure 1. Selected Representatives of Major Structural Families of Bacterial Hormones

Hormaomycins J₁–J₃ are artificial analogs of the natural product hormaomycin (**1**) that were generated in this study. See also Figure S2, Tables S1 and S2 and Supplemental Experimental Methods.

at 2.8 mM concentration. This resulted in an isotope enrichment at the amide carbon of **7** (63.0% at δ = 61.4 ppm). In addition, hormaomycin isolated from another culture containing 2.8 mM L-[5,6,8,9-²H₄]Tyr showed an incorporation of three of the four deuterium labels into **7**, as judged from a depletion of the corresponding proton NMR signals. These data are in agreement with the loss of one deuterium by hydroxylation of this precursor to **13**.

Finally, feeding of [1-¹³C]-Ile (2 mM) resulted in an enhanced signals for C-1 of the Ile moiety (43.2%), while feeding of DL-[1-¹³C]-Thr (3 mM) resulted in a high enrichment of C-1 of the D-allo-Thr (88.6%) and additionally the Ile (11.5%) unit in **1**.

Isolation of the Hormaomycin Biosynthetic Gene Cluster

As a basis for molecular studies, a pWEB-based cosmid library of *S. griseoflavus* W-384 genomic DNA was prepared. One thousand clones were subjected to several rounds of screening by Southern hybridization. Since the structure of **1** suggested biosynthesis by a nonribosomal peptide synthetase (NRPS) (Sieber and Marahiel, 2005), we initially screened the library with a heterologous NRPS gene probe derived from the friulimycin gene cluster of *Actinoplanes friuliensis* (Müller et al., 2007). This analysis yielded cosmids covering three distinct genomic regions, as judged from the restriction patterns, indicating the presence of more than one NRPS cluster. To differentiate between these loci, an additional probe derived from the halogenase gene *cloN3* of the chlorobiocin pathway (Pojer et al., 2002) was used to target genes involved in the formation of the chlorinated building block Chpca (**6**). Hybridizing bands were obtained for only one of the cosmid groups, of which the two overlapping cosmids pHR6G10 and pHR1F4 were sequenced. The analysis revealed a 48,409 bp region of 23 clustered ORFs, designated as *hrm* genes, as candidates for hormaomycin (**1**) biosynthesis (Figure 2 and Table 1). The gene cluster consists of two giant NRPS genes surrounded by smaller ORFs related to genes involved in precursor biosynthesis, regulation and transport. Adjacent in the upstream and downstream directions we found close homologs to the genes SCO1544–SCO1553 from *Streptomyces coelicolor*. Except for the presence of the *hrm* system between the SCO1551 and SCO1552 counterparts, the genes are perfectly syntenous in both bacteria, suggesting that the *hrm* cluster has been inserted between the SCO1551 and SCO1552 orthologs in the **1** producer. The boundaries of the *hrm* system are therefore most likely represented by *hrmA* and *hrmW*.

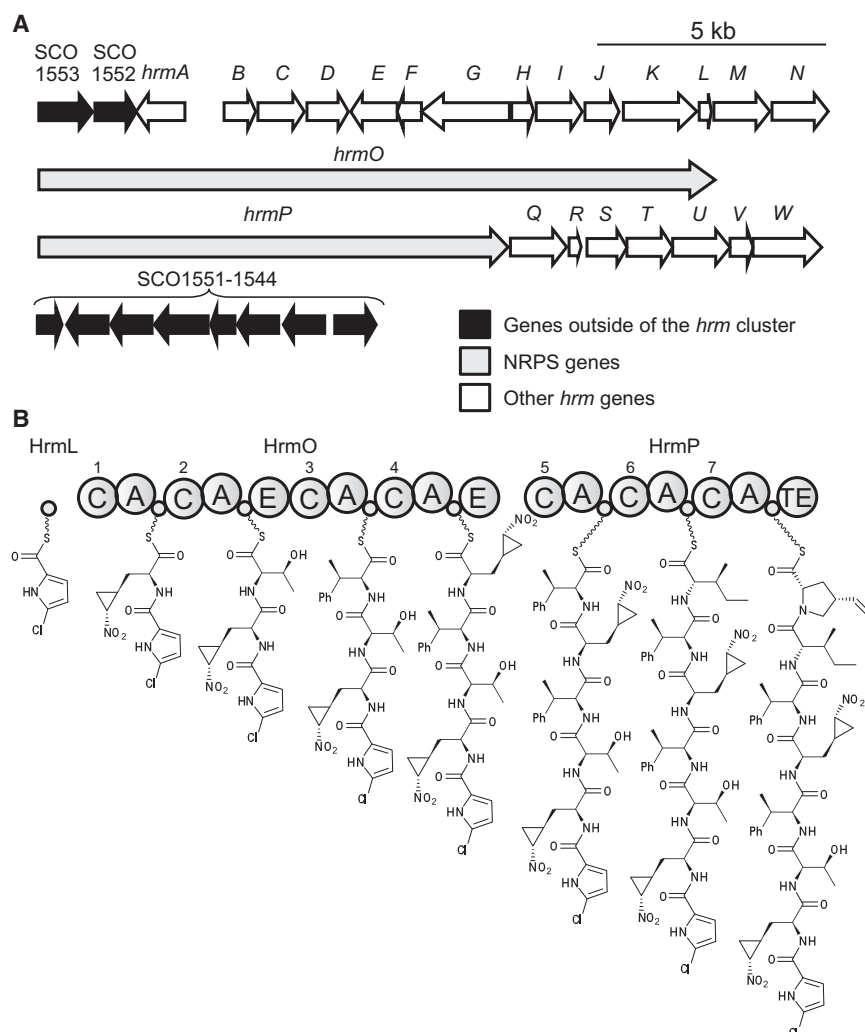


Figure 2. Genes and Enzymes Involved in Hormaomycin Biosynthesis

(A) Map of the *hrm* biosynthetic gene cluster. The SCO labels above the genes shown in black refer to close homologs present in the *Streptomyces coelicolor* genome.

(B) Biosynthetic model for peptide assembly. The small circles represent PCP domains. Numbers refer to the start of individual modules.

A striking feature of *hrmO* and *hrmP*, which severely complicated sequencing, was the high similarity between numerous DNA regions (Figure 3A). Particularly high nucleotide identities exceeding 90% exist between modules expected to incorporate identical amino acids, i.e., between modules 1 and 4 of *hrmO* (predicted: [3-Ncp]Ala[5]) and between module 3 of *hrmO* and module 1 or *hrmP* (predicted: [β-Me]Phe [8]). Remarkably, however, even some A domains that activate different amino acids are closely related to each other. Particularly striking examples are the domain pairs specific for 5 and 8, which exhibit 91% nucleotide identity except for an approximately 420 bp region immediately downstream of a conserved translated YTSGS motif (Figure 3B). Intriguingly, this region almost precisely corresponds to the amino acid recognition pocket previously identified in structural studies (Conti et al., 1997). These features provide direct insights into the evolution of NRPS systems, suggesting a scenario in which new NRPS function-

The presence of eight building blocks in **1** is perfectly mirrored by the NRPS architecture. In total, we identified eight homologs of peptidyl carrier proteins (PCPs), which covalently bind the building blocks during peptide biosynthesis. One of these is encoded by the free-standing gene *hrmL*, and seven are integrated into the NRPS modules of the large proteins HrmO and HrmP. A bioinformatic analysis of the recognition motifs of the amino acid-activating adenylation domains (A domains), which can provide information on their substrate specificities (Challis et al., 2000; Stachelhaus et al., 1999), suggested Thr being incorporated by module 2 and Val or Ile by module 6, in agreement with the structure of **1** (Table 2). In contrast, the motifs of the remaining modules, which recognize unusual amino acids, are to our knowledge novel. In addition, HrmK resembles a free standing A domain with a nonribosomal code that is 80% identical to the code of the Proactivating enzyme PltF, suggesting a possible role in starter unit biosynthesis. Also in line with the peptide structure, epimerase domains that normally generate D-configured amino acids are present on modules 2 and 4. According to their location, these should generate the D-*allo*-Thr and D-(3-Ncp)Ala residues.

ality can already arise by recombinatorial exchange of only short DNA stretches covering the specificity region, in addition to the previously observed exchanges of entire modules (Rounge et al., 2008), A domains (Fewer et al., 2007; Ishida et al., 2009), or multiple regions (Tooming-Klunderud et al., 2008).

To examine the function of the NRPS, the adenylation domain region of *hrmP1*, which was suspected to be specific for β-Me-Phe (**8**), was amplified by PCR and cloned into the expression vector pHis8. After overexpression of the octahistidine-tagged protein in *Escherichia coli* BL21(DE3), soluble enzyme was obtained and purified with Ni-NTA affinity chromatography (Figure 4A). The desalted 58 kDa protein was analyzed using a recently published nonradioactive adenylation assay based on pyrophosphate isotope exchange in [γ-¹⁸O₄]-ATP (Phelan et al., 2009). After incubating the protein with various amino acid test substrates, analysis of the products by MALDI-TOF MS showed the highest exchange rate with β-Me-Phe, as expected from the position of the A domain in the NRPS (Figure 4B; Figure S1). Since the sequences of the HrmP1 and HrmO3 A domains are virtually identical, the latter should exhibit the same activity.

Table 1. Deduced Proteins Encoded on the *hrm* Region and Their Putative Functions

Protein	Amino Acids	Proposed Function	Sequence Similarity (protein, origin)	Similarity/Identity	Accession Number
HrmA	321	AraC-type transcriptional regulator	SCO0287, <i>Streptomyces coelicolor</i>	84/75	CAB54172
HrmB	197	Putative regulator	NovE, <i>Streptomyces spheroides</i>	73/60	AAF67498
HrmC	358	Methyltransferase	LmbW, <i>Streptomyces lincolnensis</i>	72/57	CAA55769
HrmD	298	F420-dependent oxidoreductase	SCO3591, <i>S. coelicolor</i>	63/52	CAA22223
HrmE	326	Tyrosine hydroxylase	LmbB2, <i>S. lincolnensis</i>	55/47	CAA55748
HrmF	185	DOPA-cleaving oxidoreductase	TomH, <i>Streptomyces achromogenes</i>	69/61	ACN39014
HrmG	599	γ -Glutamyltranspeptidase	LmbA, <i>S. lincolnensis</i>	73/64	CAA55746
HrmH	132	Putative regulator	YjgH, <i>Kineococcus radiotolerans</i>	80/60	YP_001362405
HrmI	350	Unknown	PSPTOT1_5436, <i>Pseudomonas syringae</i> pv. <i>tomato</i>	72/57	ZP_03398257
HrmJ	227	Unknown	PSPTOT1_5436, <i>P. syringae</i> pv. <i>tomato</i>	70/51	ZP_03398257
HrmK	527	Acyl-CoA synthetase	CLOSCI_02396, <i>Clostridium scindens</i>	57/39	ZP_02432136
HrmL	91	Type II peptidyl carrier protein	VinL, <i>S. halstedii</i>	59/40	BAD08369
HrmM	388	Acyl-CoA dehydrogenase	MoeH5, <i>Streptomyces clavuligerus</i>	51/33	YP_002190358
HrmN	389	Acyl-CoA dehydrogenase	Strop_4265, <i>Salinispora tropica</i>	49/36	YP_001161071
HrmO	5252	NRPS (C A PCP C A PCP E C A PCP C A PCP E)	RHA1_ro00141, <i>Rhodococcus</i> sp. RHA	40-60% domain identity	YP_700135
HrmP	3471	NRPS (C A PCP C A PCP C A PCP TE)	SACE_4287, <i>Saccharopolyspora erythraea</i>	40-60% domain identity	YP_001106481
HrmQ	448	Halogenase	Pyr29, <i>Actinosporangium vitaminophilum</i>	78/65	ABO15865
HrmR	72	MbtH homolog	SCO3218, <i>S. coelicolor</i>	81/58	CAB38589
HrmS	329	Methyltransferase	MppJ, <i>Streptomyces hygroscopicus</i>	67/52	AAU34201
HrmT	301	Diaminopimelate epimerase	DAPF2 (SAV3161), <i>S. avermitilis</i>	63/47	BAB69347
HrmU	427	Integral membrane antitransporter	CZA382.28, <i>Amycolatopsis orientalis</i>	68/56	CAB45049
HrmV	224	ABC transporter ATP binding protein	A20C1_11161, marine actinobacterium PHSC20C1	56/38	ZP_01130568
HrmW	563	Integral membrane protein	JNB_10189, <i>Janibacter</i> sp. HTCC2649	38/26	ZP_00994281

Chpca Biosynthesis

In agreement with the feeding studies, the *hrm* system contains the clustered genes *hrmKLM*, homologs of which are found in various gene clusters for pyrrole carboxylic acid derivatives (Walsh et al., 2006). The nonribosomal code of HrmK indicates that it attaches Pro to a PCP, for which HrmL is a good candidate (Figure 5A). HrmM is closely related to dehydrogenases catalyzing the four-electron oxidation of prolyl-PCP to generate 2-pyrrolyl-PCP (Garneau et al., 2005; Garneau-Tsodikova et al., 2006). The adjacently encoded HrmN is also distantly related to these enzymes and might also participate in proline

aromatization. Chlorination is performed by HrmQ, which is highly similar to Pyr29 and PltA, enzymes in the pyrrolomycin and pyoluteorin pathway that chlorinate pyrrole moieties (Dorrestein et al., 2005; Zhang and Parry, 2007). Insights into its function came from recent combinatorial expression of *hrmQ* with genes of the clorobiocin gene cluster, which led to production of hybrid aminocoumarins with an additional chlorine atom at position 5 of the pyrrole unit, thus suggesting its role in Chpca (6) biosynthesis (Heide et al., 2008). A gene responsible for the *N*-hydroxylation was not directly obvious from the sequence data.

Table 2. Analysis of the Specificity-Conferring Residues in the *hrm* A Domains

Domain	Nonribosomal Code	Closest Related Code (A domain)	Specificity of Related Domain
HrmK	DLFYAAKVCK	DLLYAALVCK (PltF)	Pro
HrmO1/O4	DPIVVGGVAK	n.h. ^a	—
HrmO2	DFWNIGMVHK	DFWNIGMVHK (SypC5)	Thr
HrmO3/P1	DAWTTAAVAK	n.h.	—
HrmP2	DGYFWGVTFK	DAYFWGVTFK (AcmB2) DGFFFGVVFK (BacC1)	Val Ile
HrmP3	DVQFSAHGAK	n.h.	—

^a No significant hit in database searches.

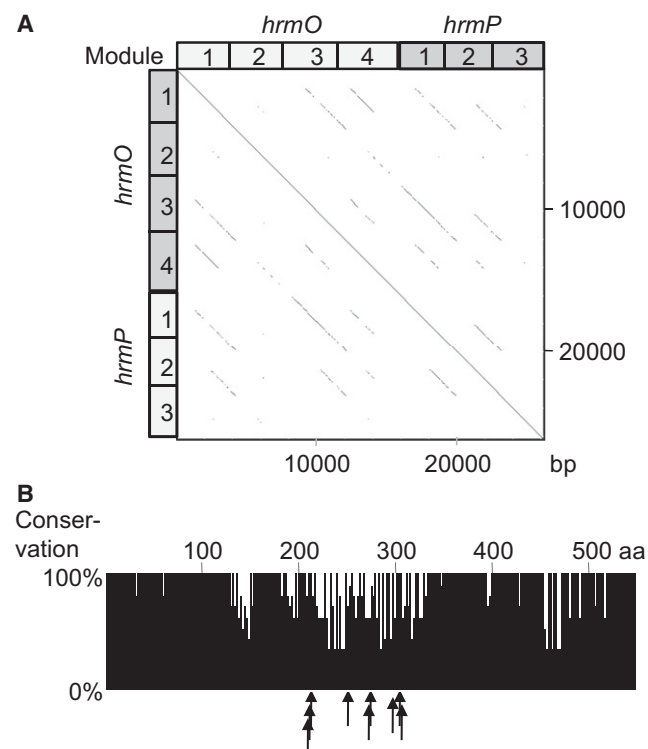


Figure 3. Similarity Analysis of Hormaomycin NRPS Components
(A) Dot plot analysis of the NRPS genes *hrmO* and *hrmP* (lines show region with at least 90% nucleotide identity).
(B) Amino acid conservation plot of the A domains of modules HrmO1 (specific for [3-Ncp]Ala) and HrmP1 (specific for [3-Me]Phe). The arrows mark the specificity-conferring residues that constitute the nonribosomal code (Challis et al., 2000; Stachelhaus et al., 1999).

Since pyrrole-2-carboxylic acid (**14**), tethered to the PCP HrmL, is a likely precursor of **1**, we tested whether peptide yields can be increased by addition of **14** to the cultivation medium. After initial growth of a culture for 24 hr, **14** was added over a period of 10 hr to a final concentration of 2 mM. This treatment resulted in the production of up to 45 mg L⁻¹ **1**, which is the 4-fold yield of a typical culture without added precursor. As this result suggested an efficient incorporation into **1**, we next examined whether novel hormaomycins can be obtained by feeding nonnatural analogs of **14**. However, after addition of furfural, thiophene-2-carboxylic, tetrahydrofuran-2-carboxylic, picolinic, pipercolinic or 3-chlorosalicylic acid, only authentic hormaomycin was detected with isolated yields being identical to untreated cultures. The loading and/or extension enzymes of the hormaomycin assembly line therefore seem to exhibit a high selectivity for the starter unit.

(β -Me)Phe Biosynthesis

According to the feeding studies, the methyl group of (β -Me)Phe (**8**) is introduced by a SAM-dependent methyltransferase. An analysis of the gene cluster revealed two methyltransferase gene candidates *hrmC* and *hrmS* present on cosmids pHR6G10 and pHR1F4, respectively. Amino acids carrying a β -methylation are components of several nonribosomal peptides. **8** or

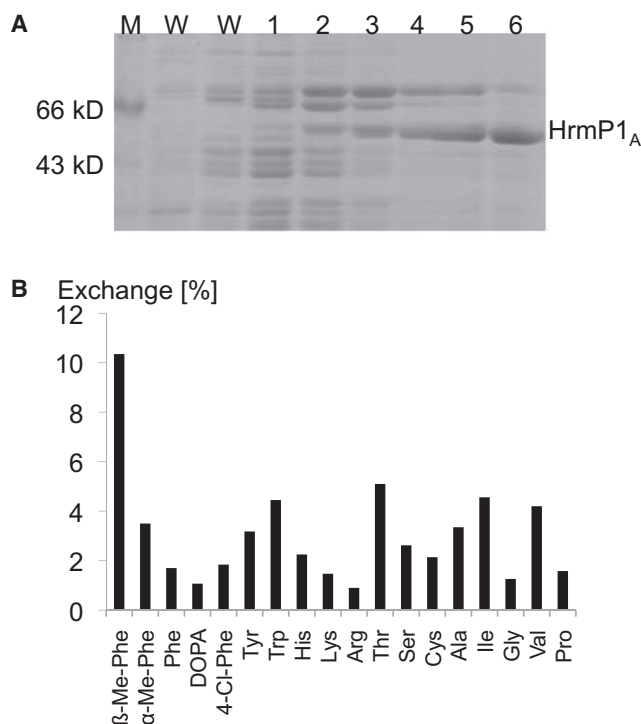


Figure 4. Expression and Analysis of the A Domain of Module HrmP1 (HrmP1A)
(A) SDS-PAGE analysis of protein fractions. M, marker; W, wash fractions; 1–6, elution fractions at 50, 100, 150, 200, 250, and 300 mM imidazole.
(B) Degree of exchange in the [γ -¹⁸O₄]-ATP assay with various test substrates, as measured by MALDI-TOF MS.
See also Figure S1.

derivatives are also incorporated in the sponge-derived metabolite theonegramide (Bewley and Faulkner, 1994) and the mannopeptimycin antibiotics (Magarvey et al., 2006). The mannopeptimycin gene cluster contains the close *hrmS* homolog *mppJ*, the deletion of which led to the production of a linear peptide carrying an unmethylated Phe residue (Magarvey et al., 2006). This suggests that HrmS is involved in the biosynthesis of **8** (Figure 5B). Studies on the lipopeptide antibiotics CDA, daptomycin and A54145 demonstrated that their common 3-methylglutamate residue is formed by methylation of α -ketoglutarate and subsequent amination (Mahlert et al., 2007). The latter reaction is catalyzed by IlvE, an aminotransferase from primary metabolism. As the hormaomycin gene cluster lacks a recognizable aminotransferase gene, amination by a housekeeping enzyme is also likely for (β -Me)Phe biosynthesis, indicating that *Streptomyces* spp. contain one or several promiscuous aminotransferases for the generation of a substantial number of nonproteinogenic amino acids. To test whether residues other than **8** can be incorporated at the two hormaomycin sites, we fed various analogs of this amino acid to liquid cultures of *S. griseoflavus*. Addition of L-Tyr or phenylglycine did not result in a change of the metabolic profile. After feeding of racemic *o*-fluorophenylalanine or its *meta* isomer, 7.7 and 17.8 mg, respectively, of isomeric mixtures of hormaomycins were isolated that could not be separated further by

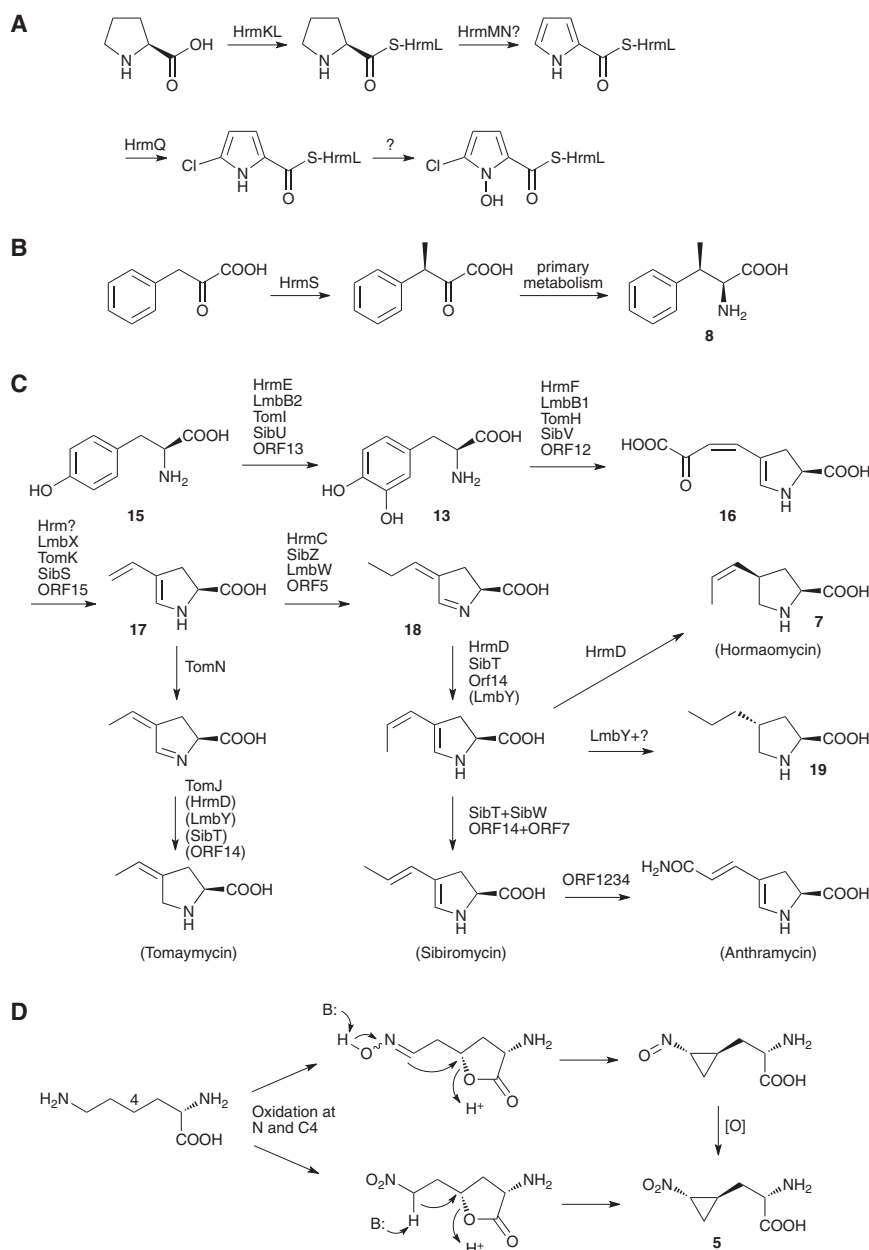


Figure 5. Biosynthetic Models for Amino Acid Pathways in Hormaomycin Biosynthesis

(A) Proposed biosynthesis of Chpca (**6**).

(B) Proposed biosynthesis of (β-Me)Phe (**8**).

(C) Possible scenario for the biosynthesis of (4-Pe)Pro (**7**). Enzyme homologs from the lincomycin (*lmb*), anthramycin (ORFxy), sibiromycin (*sib*), and tomaymycin (*tom*) pathways are also shown. Proteins in parentheses were proposed to convert variants of the substrates shown.

(D) Proposed biosynthesis of (3-Ncp)Ala (**5**).

Metabolites with the same mass were also detected after incubations with *p*-chloro-, *p*-bromo, and *p*-nitro-Phe. To characterize the compound(s), peptides from a fermentation with *p*-Cl[−]Phe were purified by HPLC. This yielded, in addition to authentic hormaomycin, the three new derivatives hormaomycin J₁-J₃ (**2-4**) at amounts of 1–2 mg. According to HRMS data, hormaomycin J₁ (**2**) and J₂ (**3**) both had the molecular formula C₅₄H₆₇N₁₀O₁₄Cl (*m/z* = 1137.44182 and 1137.44160, respectively, for [M+Na]⁺, calcd. 1137.44190), which indicated the absence of a methylene group. In agreement, the NMR spectra were almost identical to those of hormaomycin (Figure S2 and Table S2). Major differences for **2** were missing resonances at δ_H = 1.39 and 3.68 and δ_C = 13.0, which correspond to the methyl group of (β-Me)Phe I. The proton spectrum also shows a modified coupling pattern of the amino acid α-proton (δ_H = 4.47), which results from the presence of two methylene instead of one methine proton at the β-position (δ_H = 2.80 and 3.37). The residue replacing (β-Me)Phe I is therefore Phe, establishing a structure for **2** as shown in Figure 1. In analogy, NMR spectra for **3** lack the methyl signal of

chromatography. MS analysis by negative ion electrospray ionization (ESI) revealed in both mixtures the presence of two new compounds with the ions $m/z = 1145$ and 1163 $[M-H]^-$ corresponding to the molecular formulae $C_{55}H_{68}N_{10}O_{14}FCl$ and $C_{55}H_{67}N_{10}O_{14}F_2Cl$. This indicated that hormaomycin analogs carrying one and two fluorinated residues had been generated. A third feeding experiment with *para*-fluorinated Phe resulted in the isolation of 11 mg hormaomycins. By positive-ion ESI-MS, a new ion at $m/z = 1168$ $[M+Na]^+$ corresponding to a single incorporated residue was detected, but not the ion of the doubly modified analog. However, in contrast to the studies with *o*- and *m*-fluorinated analogs, another new ion at $m/z = 1137$ $[M+Na]^+$ was present, which was in agreement with a compound carrying two carbons and four hydrogens less than hormaomycin.

(β -Me)Phe II ($\delta_{\text{H}} = 1.29$) and exhibit a new coupling of an amino acid α -proton at $\delta_{\text{H}} = 4.61$ – 4.66 to two β -methylene protons at $\delta_{\text{H}} = 2.88$ and 2.96 , identifying this compound as a Phe analog with an altered (β -Me)Phe II position. Compound **4** has the molecular formula $\text{C}_{53}\text{H}_{65}\text{N}_{10}\text{O}_{14}\text{Cl}$ ($m/z = 1123.42646$ for $[\text{M}+\text{Na}]^+$, 1123.42625 calcd.), which is two carbons and four hydrogens less than for **1**. The NMR spectra was of poor quality due to the low available amounts, but clearly lacked signals for the methyl group of both (β -Me)Phe units and contain two additional methylene coupling systems similar to those present in **2** and **3**. Therefore, all (β -Me)Phe (**8**) residues in **4** should be replaced by Phe. The fact that *nor*- and *bisnor*-hormaomycins were produced instead of the expected analogs bearing halogen or nitro was unexpected. One possible explanation is that the

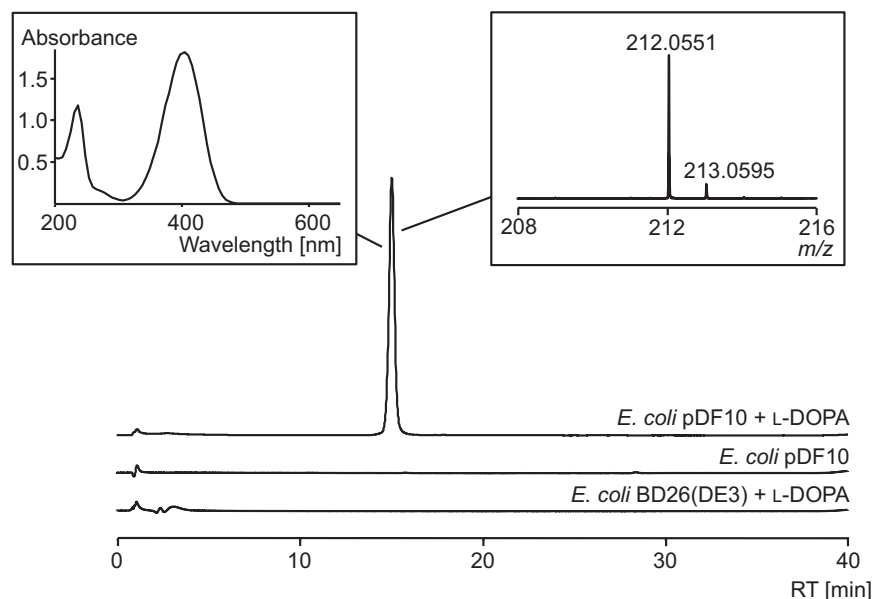


Figure 6. HPLC-MS Analysis of L-DOPA (9) Cleavage by HrmF

The boxes show the UV and HRMS spectrum of the cleavage product. HPLC traces at 413 nm are shown for the HrmF product mixture and two negative controls. *E. coli* pDF10 carries the *hrmF* expression plasmid, *E. coli* BL21(DE3) is the empty control strain. See also Figure S3.

dase HrmG, which should be involved in biosynthesis of the F_{420} cofactor, no other homologs in the related pathways were found. In particular, among the gene clusters generating (4-Pe)Pro-related moieties, the *hrm* system is the only one that lacks an *lmbX*-homolog, which has been proposed to be responsible for cleavage of the common precursor **16** (Li et al., 2009a, 2009b). *LmbX* was recently demonstrated to be essential in the biosynthesis of the lincomycin propylproline (**19**) unit (Ulanova et al., 2010).

added substances block **8** biosynthesis by inhibiting the methyltransferase HrmS or the aminotransferase. The A domains of the **8**-specific modules might then accept and incorporate Phe as an alternative substrate.

(4-Pe)Pro Biosynthesis

Our feeding studies suggested that the (4-Pe)Pro (**7**) moiety is derived from L-Tyr (**15**) via a pathway related to those found in lincomycin and pyrrolo[1,4]benzodiazepine (anthramycin, tomaymycin and sibiromycin) biosynthesis. In agreement, the *hrm* cluster contains a set of genes *hrmCDEFG* that are similar to counterparts in the gene clusters of these compounds (Figure 5C) (Hu et al., 2007; Li et al., 2009a, 2009b; Peschke et al., 1995). The only characterized related proteins are *LmbB2* and *LmbB1* of the lincomycin pathway. *LmbB1*, a homolog of HrmF, is a dioxygenase that performs an extradiol cleavage of L-DOPA (**13**) to the pyrroline intermediate **16** (Colabroy et al., 2008; Neusser et al., 1998). Both enzymes belong to the same superfamily as the ring-cleaving dioxygenases but exhibit no closer similarity to the classical extradiol cleavage enzymes (Bugg and Winfield, 1998). It was also shown that *LmbB1* and the HrmE homolog *LmbB2* jointly convert **15** to the same product (Neusser et al., 1998). *LmbB2* and HrmE could therefore be L-tyrosine hydroxylases. The other steps are poorly understood. HrmC exhibits the highest similarity to putative SAM-dependent methyltransferases encoded by the lincomycin, anthramycin, and sibiromycin clusters, which likely attach the terminal methyl function. Such a role for HrmC is also in agreement with our feeding studies using L-[methyl- ^{13}C]Met. HrmD resembles *LmbY*, a putative coenzyme F_{420} -dependent oxidoreductase. A mutant strain of the lincomycin producer impaired in formation of the F_{420} chromophore accumulated the diene **18**, suggesting that *LmbY* might reduce the diene system (Kuo et al., 1992). Since **18** already carries the terminal methyl group, cleavage and methylation should occur earlier in the pathway. Surprisingly, with the exception of the putative γ -glutamyl transpepti-

We initially suspected that a part of the hormaomycin pathway is encoded on an additional, unclustered section of the *S. griseoflavus* genome. To search for *hrm* genes that might have been missed, the genomic DNA of the hormaomycin producer was tested with degenerate primers derived from an alignment of *lmbX* with all known homologs (*sibS*, *tomK*, and anthramycin ORF15). However, no PCR product was observed. Likewise, extensive attempts to detect an *lmbX*-like gene by Southern hybridization using an *lmbX*-derived probe together with either genomic DNA or the arrayed library were unsuccessful. In the later course of our studies, a draft genome sequence of *S. griseoflavus* Tü4000, which is identical to the hormaomycin producer W-384 studied in this work (M. Fischbach, personal communication), became available in GenBank. Inspection of the genome revealed the presence of a locus highly similar to the *hrm* cluster. A major difference was found in the NRPS region, which consisted of only a single gene encoding five modules instead of two genes for seven modules. This architecture might be an artifact caused by the automated assembly of the highly repetitive NRPS sequences or the result of natural recombination. In agreement with the PCR and Southern studies, the draft genome lacked an *lmbX* homolog. The data therefore indicate that such an enzyme is not needed for (4-Pe)Pro biosynthesis and that the terminal two-carbon unit of **16** is cleaved off by another enzyme. To test the possibility that HrmF might perform this reaction in addition to L-DOPA (**13**) cleavage, it was heterologously expressed in *E. coli* (Figure S3). After cloning the gene into the vector pHis8, cultures of *E. coli* carrying the plasmid turned bright yellow after incubation with **13**, while the color change was not observed in control experiments in the absence of *hrmF* or **13** (Figure S3A). Analysis of the yellow product by HPLC-high resolution MS (HRMS) (Figure 6; Figure S3B) revealed the presence of a compound with the same UV spectrum as the previously reported *LmbB1* cleavage product (Neusser et al., 1998). Moreover, the MS spectrum showed two ions at $m/z = 212.0551$ and 234.0382 that were

attributed to the $[M+H]^+$ and $[M+Na]^+$ forms, respectively, of a compound with the molecular formula $C_9H_9NO_5$. Since this composition precisely matches to **16** and there was no indication for the formation of a downstream cleavage product, such as **17**, a role of HrmF in further cleavage of **16** is unlikely. To further characterize the HrmF-catalyzed reaction in vitro, the His₈-tagged protein was prepared (Figure S3C) and assayed in vitro with **13** in the presence of ascorbic acid. For a kinetic analysis, formation of the yellow cleavage product at different concentration of **13** was monitored at 414 nm (Figure S3D). The data support a K_M of 3.35 μ M and a k_{cat} of 57.8 min⁻¹, which correspond to a significantly higher catalytic efficiency than displayed by the homolog LmbB1 ($K_M = 38 \mu$ M, $k_{cat} = 4.2 \text{ min}^{-1}$) (Colabroy et al., 2008).

(3-Ncp)Ala Biosynthesis

Given the putative gene assignments already made, surprisingly few genes remain that might be attributed to (3-Ncp)Ala (**5**) production. Incorporation experiments suggested that this amino acid is generated from L-lysine via 4-hydroxylysine or its lactone, leading to the proposal that cyclopropanation is achieved by intramolecular nucleophilic substitution at the activated position 4 (Figure 5D) (Brandl et al., 2004). The only protein with a predictable role in lysine metabolism is HrmT, which displays high similarity to diaminopimelate epimerases catalyzing the penultimate step in lysine biosynthesis (Scapin and Blanchard, 1998). HrmT could therefore play a role in diverting lysine precursors from primary metabolism to hormaomycin biosynthesis. Of the remaining unassigned genes, *hrmR* is highly similar to the *mbtH* gene family, members of which are associated with many NRPS pathways and can positively influence amino acid activation (Drake et al., 2007; Felnagle et al., 2010; Lautru et al., 2007; Stegmann et al., 2006; Wolpert et al., 2007). A role in **5** formation is therefore unlikely. This leaves only two genes, *hrmI* and *hrmJ*, as candidates. Bioinformatic analyses provided no insights into their possible function, since only a few uncharacterized relatives exist. No obvious homologs of enzymes that were previously shown to be involved in cyclopropanation, such as α -ketoglutarate/Fe²⁺-dependent halogenases or a CmaC-related protein (Gu et al., 2009; Kelly et al., 2007; Vaillancourt et al., 2005) were found encoded in the *hrm* cluster or elsewhere in the genome. Functional studies on *hrmI* and *hrmJ* are currently under way to examine their role in hormaomycin biosynthesis.

SIGNIFICANCE

The bacterial depsipeptide hormaomycin from *Streptomyces griseoflavus* W-384 is unique among pro- and eukaryotic hormones both in terms of its complex, highly unusual structure and its dual activity as an inducing agent and potent narrow-spectrum antibiotic. Isolation of the biosynthetic gene cluster reveals a complex evolutionary history of the pathway consisting of DNA duplication and recombination events that resulted in switches of adenylation domain selectivity. Extensive feeding and enzymatic studies were conducted that yielded several novel hormaomycins and provide insights into the formation of most of the amino acids. The unique 3-propenylproline moiety is

generated from L-tyrosine via a pathway involving cleavage of L-DOPA, while the unprecedented chlorinated pyrrole derivative Chpca is generated from proline. The study provides the foundation for engineering experiments to generate further hormaomycin analogs that may aid in the identification and exploitation of bacterial signaling pathways and the as-yet unknown molecular target in antibiosis.

EXPERIMENTAL PROCEDURES

General

One- and two-dimensional ¹H- and ¹³C-NMR spectra were recorded on a Varian Inova-600, Varian Inova-500, Varian Mercury-300, Varian Unity-300 and Bruker AMX 300 spectrometer in CDCl₃ using the solvent signals as the reference. MALDI-TOF-MS spectra were recorded on a Bruker autoflex II TOF/TOF time of flight mass spectrometer. Electrospray high-resolution mass spectra and collision-induced dissociation (CID) MSⁿ spectra were recorded on a Bruker micrOTOF-Q time-of-flight mass spectrometer, and Bruker Apex-Q III and Bruker APEX IV Fourier-transform ion cyclotron-resonance (FT-ICR) mass spectrometers with 7.05 T magnets. Instruments were equipped with an Apollo electrospray ion source. Analyte solutions in acetonitrile were introduced into the ion source with a syringe pump (Cole-Parmer Instruments, Series 74900) at flow rates of 3 to 4 μ l min⁻¹ or with an Agilent HPLC pump (1200 series) at a flow rate of 50 μ l/min. Thin layer chromatography was performed on silica gel 60 F₂₅₄ plates (layer thickness 0.2 mm, Merck). Analytical HPLC separation was performed on a PerfectChrom 100 C18 reverse-phase analytic column (250 \times 4 mm, 5 μ m) with a flow rate 1 ml min⁻¹. The compounds were detected with a Jasco MD-2015 PDA detector at the wavelengths 280 or 413 nm.

Bacterial Strains and Culture Conditions

E. coli BL21(DE3) served as the host strain for heterologous expression experiments. *S. griseoflavus* W-384 was grown on M2+ plates or in NM6 or NM10 liquid medium (Brandl et al., 2004). *E. coli* strain XL1 blue served as host for routine subcloning. For intergeneric conjugation *E. coli* ET12567 containing the RP4 derivative pUZ8002 was used (Kieser et al., 2000). *E. coli* strains were grown in LB medium supplemented with ampicillin (100 μ g ml⁻¹), or apramycin (50 μ g ml⁻¹), or streptomycin (50 μ g ml⁻¹), or kanamycin (50 μ g ml⁻¹) for selection. For overexpression, 1 liter cultures of *E. coli* BL21(DE3) containing the expression plasmid were grown to an OD₆₀₀ of 0.6 (37°C, 200rpm), IPTG was then added to a final concentration of 1 mM and cells were grown for approximately 20 hr at 16°C.

Plasmids and General DNA Procedures

DNA isolation, plasmid preparation, restriction digests, gel electrophoresis, and ligation reactions were conducted according to standard methods (Kieser et al., 2000; Sambrook and Russel, 2001). pBluescript II SK(-) was the routine vector for subcloning and preparation of DNA templates for sequencing. For overexpression and purification, *hrm* Genes were cloned into the expression vector pHIS8 (Jez et al., 2000).

Purification of Proteins

The cells were harvested by centrifugation (6000 rpm, 30 min) at 4°C and resuspended in lysis buffer (100 mM Tris-HCl [pH 7.5]) containing 0.5 M NaCl and 10 mM imidazole. The cell suspension was kept on ice and sonicated in 10 s intervals. The cell debris was removed by centrifugation at 11,000 rpm for 30 min at 4°C. The supernatant was incubated with 1.5 ml Ni-NTA agarose (QIAGEN) for 1 hr at 4°C and washed with 5 ml lysis buffer. His-tagged proteins were eluted in 1 ml fractions with lysis buffer containing rising imidazole concentrations (50–300 mM). Protein concentrations were determined by Bradford (RotiNanquant, Roth) and protein containing fractions were pooled and desalted using HiTrap Columns (GE).

Isolation and Sequencing of the *hrm* Genes

A library of *Streptomyces griseoflavus* W-384 DNA in *E. coli* EPI305 was constructed in the cosmid vector pWEB (Epicenter). 2000 clones were picked into 96-well microtiter plates. A 1200 bp fragment encoding an A domain of the

frilulicin gene cluster was excised with EcoRI out of the plasmid pK19 (kind gift of D. Schwartz, Esslingen). A ^{32}P -labeled hybridization probe prepared from this fragment served to identify positive cosmids. The positives were further screened with a ^{32}P -labeled probe prepared from the gene *cloN4* of the clorobioicin cluster (kind gift of L. Heide, Tübingen). The two positive cosmids pHR6G10 and pHR1F4 were sonicated, end-repaired by BAL-31 and Klenow fragment and size-fractionated by gel electrophoresis to obtain fragments of 1–2 kb lengths. These were ligated into the EcoRV site of pBluescript II SK(-) and end sequenced with the BigDye Terminator Ready Mix (Applied Biosystems) and an ABI3700 sequencer (Applied Biosystems). Remaining gaps were filled by targeted subcloning and the use of specific primers. For genome screening of *lmbX* homologs, degenerate primers hrmXfor (5'-CAR GAR TGY GGN DSN MGN-3') and hrmXrev (5'-NGW HGC RCA NGC NGG RTC-3') were used. The *lmbX* gene fragment was amplified by PCR from the genomic DNA of lincomycin producer *Streptomyces lincolnensis* NRRL 2936 using primers lmbXfor(5'-GGA ATT CAT GAT CGT GGT CCC-3') and lmbXrev(5'-CCC AAG CTT CTA CAC GGA AGC-3'). PCR products were ligated into pBluescript SK(+) to get pXC7 and end sequenced. The *lmbX* gene fragment was digested with HindIII and EcoRI from pXC7 and labeled by DIG-High Prime DNA Labeling Kit as a probe to detect a possible *lmbX* homolog in either the genomic DNA or the arrayed library by Southern blot.

Kinetic Characterization of HrmF

The gene *hrmF* was amplified by PCR using primers hrmFfor (5'-GGA TCC CCC GAG AAC CTC G-3') and hrmFrev (5'-AAG CTT TCA CCC CGC CGA CT-3'). PCR products were ligated into pBluescript SK(+) via TA cloning (Marchuk et al., 1991) to give pDF017. After restriction digest with BamHI and HindIII the genes were ligated into the same restriction sites of the expression vector pHis8 to give pDF024. The overexpressed protein was purified with Ni-NTA affinity chromatography. Desalted HrmF in 100 μM phosphate buffer (pH 8) was treated with L-DOPA in different concentrations. A typical assay, adapted from the literature (Colabroy et al., 2008), consisted of 25 μl enzyme (1.3 μM), 10 μM L-DOPA, and 10 μM ascorbic acid in 500 μl phosphate buffer (100 μM [pH 8]). The enzymatic activity was monitored by measuring the absorbance increase at 414 nm resulting from the formation of the yellow cleavage product ($\epsilon = 20.300 \pm 890 \text{ M}^{-1} \text{ cm}^{-1}$) for 30 min using a BioMate 3 photometer (Thermo). Experiments were performed in triplicate. For determination of kinetic parameters initial rates were plotted versus the substrate concentration and fitted to the Michaelis-Menten equation with the Hanes-Woolf Plot.

Mass-Exchange Based Adenylation Assay of the HrmP1 A-Domain

The A domain region of the module HrmP1 was amplified by PCR using primers hrmP1for (5'-AAA GAA TTC GGC CGG CTG GAC GTC GT-3') and hrmP1rev (5'-AAA AAG CTT TCA CTC CTC CTG CGC GGT ACG CG-3'). PCR products were ligated into pBluescript SK(+) with TA cloning. After restriction digest with EcoRI and HindIII, the insert was ligated into the same sites of the expression vector pHis8, and the resulting plasmid was transformed into *E. coli* BL21(DE3). The overexpressed protein was purified by Ni-NTA affinity chromatography. The desalted enzyme in Tris HCl (50 mM, pH 7.5) was incubated with labeled [γ - $^{18}\text{O}_4$]-ATP, various test substrates, MgCl_2 and pyrophosphate as previously described (Phelan et al., 2009). The mass exchange of 8 D was observed by MALDI-TOF-MS.

Hormaomycin J, (2)

^1H NMR data, see Table S2. ^{13}C NMR (150.8 MHz, CDCl_3): $\delta = 10.6$ (+, C-5, *Ile*), 13.3 [+ , C-3', (4-*Pe*)*Pro*], 14.8 (+, C-1', *Ile*), 17.0 (+, C-4, *a-Thr*), 17.3 [- , C-3', (3-*Ncp*)*Ala* *I*], 17.9 [+ , C-4, (β -*Me*)*Phe*], 20.0 [+ , C-1', (3-*Ncp*)*Ala* *I*], 21.6 [+ , C-1', (3-*Ncp*)*Ala* *II*], 25.1 (-, C-3, *Ile*), 33.4 [- , C-3, (3-*Ncp*)*Ala* *I*], 34.8 [- , C-3, (3-*Ncp*)*Ala* *II*], 35.6 [- , C-3, (4-*Pe*)*Pro*], 36.6 [+ , C-4, (4-*Pe*)*Pro*], 37.6 (+, C-3, *Ile*), 38.2 [- , C-3, *Phe*], 42.0 [+ , C-3, (β -*Me*)*Phe*], 51.4 [+ , C-2, (3-*Ncp*)*Ala* *II*], 51.6 [+ , C-2, (3-*Ncp*)*Ala* *I*], 52.8 [- , C-5, (4-*Pe*)*Pro*], 54.7 (+, C-2, *Ile*), 55.1 (+, C-2, *a-Thr*), 56.2 (+, C-2, *Phe*), 58.2 [+ , C-2', (3-*Ncp*)*Ala* *I*], 59.2 [+ , C-2', (3-*Ncp*)*Ala* *II*], 59.9 [+ , C-2, (β -*Me*)*Phe*], 61.4 [+ , C-2, (4-*Pe*)*Pro*], 69.2 (+, C-3, *a-Thr*), 103.8 (+, C-4, *Chpca*), 110.6 (+, C-3, *Chpca*), 120.1 (C_{quat} , C-2, *Chpca*), 120.5 (C_{quat} , C-5, *Chpca*), 127.2, 127.29, 127.33, 127.67, 127.73, 128.6, 128.8, 128.9 [+ , C-1', C-2', (4-*Pe*)*Pro*, Ar-C], 141.6, 141.8 (C_{quat} , Ar-C),

159.3 (C_{quat} , C-1, *Chpca*), 168.8, 169.5, 169.9, 170.9, 171.3, 171.6, 171.9 (C_{quat} , C-1). HRMS: 1137.44182, calcd. 1137.44190 for $[\text{M}+\text{Na}]^+$, 1132.48710, calcd. 1132.48650 for $[\text{M}+\text{NH}_4]^+$, 1115.45963, calcd. 1115.45995 for $[\text{M}+\text{H}]^+$. UV (MeOH) λ_{max} ($\lg \epsilon$) = 269 (4.00) nm; (MeOH, HCl) 267 (3.92) nm; (MeOH, NaOH) 273 (3.84) nm. CD (MeOH): λ_{max} ($[\theta]^{20}$) = 275 (+6400), 221 (-40700), 208 (+2500) nm. $[\alpha] = +4^\circ$ (c = 0.1, MeOH). IR (KBr): $\nu = 3416$ (br), 2940, 2353, 1649, 1534 1369, 1184, 1064 cm^{-1} .

Hormaomycin J2 (3)

^1H NMR data, see Table S2. Due to the small amounts of produced substance no ^{13}C NMR spectrum could be obtained. HRMS: 1137.44160, calcd. 1137.44190 for $[\text{M}+\text{Na}]^+$, 1132.48664, calcd. 1132.48650 for $[\text{M}+\text{NH}_4]^+$, 1115.45946, calcd. 1115.45995 for $[\text{M}+\text{H}]^+$. UV (MeOH) λ_{max} ($\lg \epsilon$) = 278 (4.07) nm; (MeOH, HCl) 271 (3.48) nm; (MeOH, NaOH) 283 (3.80) nm. CD (MeOH): λ_{max} ($[\theta]^{20}$) = 280 (+22800), 226 (-50300), 207 (+8000) nm. $[\alpha] = -8^\circ$ (c = 0.1, MeOH). IR (KBr): $\nu = 3423$, 2929, 1741 (sh), 1630, 1543, 1445, 1377, 1255, 1178, 1129, 1075, 1025 (sh) cm^{-1} .

Hormaomycin J3 (4)

HRMS: 1123.42646, calcd. 1123.42625 for $[\text{M}+\text{Na}]^+$, 1118.47005, calcd. 1118.47085 for $[\text{M}+\text{NH}_4]^+$, 1101.44438, calcd. 1101.44430 for $[\text{M}+\text{H}]^+$. UV (MeOH) λ_{max} ($\lg \epsilon$) = 279 (4.06) nm; (MeOH, HCl) 270 (3.89) nm; (MeOH, NaOH) 282 (3.97) nm. CD (MeOH): λ_{max} ($[\theta]^{20}$) = 281 (+10500), 223 (-49500), 207 (+4200) nm. $[\alpha] = -8^\circ$ (c = 0.1, MeOH). IR (KBr): $\nu = 3419$ (br), 2941, 2356, 1649, 1536, 1442, 1387, 1442, 1387, 1264, 1185, 1028 cm^{-1} . Due to the small amounts of substance produced, the NMR spectrum was of poor quality, and no data are given.

ACCESSION NUMBER

Sequencing data are accessible at GenBank under accession number HQS542230.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two tables, and three figures and can be found with this article online at doi:10.1016/j.chembiol.2010.12.018.

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REFERENCES

Andres, N., Wolf, H., Zähler, H., Rossner, E., Zeeck, A., König, W.A., and Sinnwell, V. (1989). Hormaomycin, a novel peptide lactone with morphogenetic activity on *Streptomyces*. *Helv. Chim. Acta* 72, 426–437.

- Andres, N., Wolf, H., and Zähler, H. (1990). Hormaomycin, a new peptide lactone antibiotic effective in inducing cytodifferentiation and antibiotic biosynthesis in some *Streptomyces* species. *Z. Naturforsch. (C)* 45, 850–855.
- Bewley, C.A., and Faulkner, D.J. (1994). Theonegramide, an antifungal glycopeptide from the Philippine lithistid sponge *Theonella swinhoei*. *J. Org. Chem.* 59, 4849–4852.
- Bibb, M.J. (2005). Regulation of secondary metabolism in streptomycetes. *Curr. Opin. Microbiol.* 8, 208–215.
- Brahme, N.M., Gonzalez, J.E., Rolls, J.P., Hessler, E.J., Mizersak, S., and Hurley, L.H. (1984). Biosynthesis of the lincomycins. 1. Studies using stable isotopes on the biosynthesis of the propyl-L-hygric acid moieties of lincomycin A and lincomycin B. *J. Am. Chem. Soc.* 106, 7873–7878.
- Brandl, M., Kozhushkov, S.I., Zlatopolskiy, B.D., Alvermann, P., Geers, B., Zeeck, A., and de Meijere, A. (2004). The biosynthesis of 3-(trans-2-nitrocyclopropyl)alanine, a constituent of the signal metabolite hormaomycin. *Eur. J. Org. Chem.*, 123–135.
- Bugg, T.D.H., and Winfield, C.J. (1998). Enzymatic cleavage of aromatic rings: mechanistic aspects of the catechol dioxygenases and later enzymes of bacterial oxidative cleavage pathways. *Nat. Prod. Rep.* 15, 513–530.
- Challis, G.L., Ravel, J., and Townsend, C.A. (2000). Predictive, structure-based model of amino acid recognition by nonribosomal peptide synthetase adenylation domains. *Chem. Biol.* 7, 211–224.
- Colabroy, K.L., Hackett, W.T., Markham, A.J., Rosenberg, J., Cohen, D.E., and Jacobson, A. (2008). Biochemical characterization of L-DOPA 2,3-dioxygenase, a single-domain type I extradiol dioxygenase from lincomycin biosynthesis. *Arch. Biochem. Biophys.* 479, 131–138.
- Conti, E., Stachelhaus, T., Marahiel, M.A., and Brick, P. (1997). Structural basis for the activation of phenylalanine in the non-ribosomal biosynthesis of gramicidin S. *EMBO J.* 16, 4174–4183.
- Dorrestein, P.C., Yeh, E., Garneau-Tsodikova, S., Kelleher, N.L., and Walsh, C.T. (2005). Dichlorination of a pyrrolyl-S-carrier protein by FADH(2)-dependent halogenase PltA during pyoluteorin biosynthesis. *Proc. Natl. Acad. Sci. USA* 102, 13843–13848.
- Drake, E.J., Cao, J., Qu, J., Shah, M.B., Straubinger, R.M., and Gulick, A.M. (2007). The 1.8 Å crystal structure of PA2412, an Mbth-like protein from the pyoverdine cluster of *Pseudomonas aeruginosa*. *J. Biol. Chem.* 282, 20425–20434.
- Felnagle, E.A., Barkei, J.J., Park, H., Podevels, A.M., McMahon, M.D., Drott, D.W., and Thomas, M.G. (2010). Mbth-like proteins as integral components of bacterial nonribosomal peptide synthetases. *Biochemistry* 49, 8815–8817.
- Fewer, D.P., Rouhiainen, L., Jokela, J., Wahlsten, M., Laakso, K., Wang, H., and Sivonen, K. (2007). Recurrent adenylation domain replacement in the microcystin synthetase gene cluster. *BMC Evol. Biol.* 7, 11.
- Garneau, S., Dorrestein, P.C., Kelleher, N.L., and Walsh, C.T. (2005). Characterization of the formation of the pyrrole moiety during clorobiocin and coumermycin A(1) biosynthesis. *Biochemistry* 44, 2770–2780.
- Garneau-Tsodikova, S., Dorrestein, P.C., Kelleher, N.L., and Walsh, C.T. (2006). Protein assembly line components in prodigiosin biosynthesis: characterization of PigA, G, H, I, J. *J. Am. Chem. Soc.* 128, 12600–12601.
- Gu, L., Wang, B., Kulkarni, A., Geders, T.W., Grindberg, R.V., Gerwick, L., Hakansson, K., Wipf, P., Smith, J.L., Gerwick, W.H., et al. (2009). Metamorphic enzyme assembly in polyketide diversification. *Nature* 459, 731–735.
- Heide, L., Westrich, L., Anderle, C., Gust, B., Kammerer, B., and Piel, J. (2008). Use of a halogenase of hormaomycin biosynthesis for formation of new clorobiocin analogues with 5-chloropyrrole moieties. *ChemBioChem* 9, 1992–1999.
- Horinouchi, S., and Beppu, T. (1994). A-factor as a microbial hormone that controls cellular differentiation and secondary metabolism in *Streptomyces griseus*. *Mol. Microbiol.* 12, 859–864.
- Hu, Y., Phelan, V., Ntai, I., Farnet, C.M., Zazopoulos, E., and Bachmann, B.O. (2007). Benzodiazepine biosynthesis in *Streptomyces refuineus*. *Chem. Biol.* 14, 691–701.
- Hurley, L.H. (1980). Elucidation and formulation of novel biosynthetic pathways leading to the pyrrolo[1,4]benzodiazepine antibiotics anthramycin, tomaymycin and sibiromycin. *Acc. Chem. Res.* 13, 263–269.
- Igarashi, Y., Kan, Y., Fujii, K., Fujita, T., Harada, K., Naoki, H., Tabata, H., Onaka, H., and Furumai, T. (2001). Goadsporin, a chemical substance which promotes secondary metabolism and morphogenesis in streptomycetes II. Structure determination. *J. Antibiot.* 54, 1045–1053.
- Ishida, K., Welker, M., Christiansen, G., Cadel-Six, S., Bouchier, C., Dittmann, E., Hertweck, C., and de Marsac, N.T. (2009). Plasticity and evolution of aeruginosin biosynthesis in cyanobacteria. *Appl. Environ. Microbiol.* 75, 2017–2026.
- Jez, J.M., Ferrer, J.L., Bowman, M.E., Dixon, R.A., and Noel, J.P. (2000). Dissection of malonyl-coenzyme A decarboxylation from polyketide formation in the reaction mechanism of a plant polyketide synthase. *Biochemistry* 39, 890–902.
- Kelly, W.L., Boyne, M.T., Yeh, E., Vosburg, D.A., Galonic, D.P., Kelleher, N.L., and Walsh, C.T. (2007). Characterization of the aminocarboxycyclopropane-forming enzyme CmaC. *Biochemistry* 46, 359–368.
- Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., and Hopwood, D.A. (2000). *Practical Streptomyces genetics* (Norwich, UK: The John Innes Foundation).
- Kondo, S., Yasui, K., Katayama, M., Marumo, S., Kondo, T., and Hattori, H. (1987). Structure of pamamycin-607, an aerial mycelium-inducing substance of *Streptomyces alboniger*. *Tetrahedron Lett.* 28, 5861–5864.
- Kozhushkov, S.I., Zlatopolskiy, B.D., Brandl, M., Alvermann, P., Radzom, M., Geers, B., de Meijere, A., and Zeeck, A. (2005). Hormaomycin analogues by precursor-directed biosynthesis - Synthesis of and feeding experiments with amino acids related to the unique 3-(trans-2'-nitrocyclopropyl)alanine constituent. *Eur. J. Org. Chem.*, 854–863.
- Kuo, M.S., Yurek, D.A., Coats, J.H., Chung, S.T., and Li, G.P. (1992). Isolation and identification of 3-propylidene- Δ_1 -pyrroline-5-carboxylic acid, a biosynthetic precursor of lincomycin. *J. Antibiot.* 45, 1773–1777.
- Lautru, S., Oves-Costales, D., Pernodet, J.L., and Challis, G.L. (2007). Mbth-like protein-mediated cross-talk between non-ribosomal peptide antibiotic and siderophore biosynthetic pathways in *Streptomyces coelicolor*. *Microbiology* 153, 1405–1412.
- Li, W., Chou, S.C., Khullar, A., and Gerrattana, B. (2009a). Cloning and characterization of the biosynthetic gene cluster for tomaymycin, an SJG-136 monomeric analog. *Appl. Environ. Microbiol.* 75, 2958–2963.
- Li, W., Khullar, A., Chou, S., Sacramo, A., and Gerrattana, B. (2009b). Biosynthesis of sibiromycin, a potent antitumor antibiotic. *Appl. Environ. Microbiol.* 75, 2869–2878.
- Magarvey, N.A., Haltii, B., He, M., Greenstein, M., and Hucul, J.A. (2006). Biosynthetic pathway for mannopeptimycins, lipoglycopeptide antibiotics active against drug-resistant gram-positive pathogens. *Antimicrob. Agents Chemother.* 50, 2167–2177.
- Mahlert, C., Kopp, F., Thirlway, J., Micklefield, J., and Marahiel, M. (2007). Stereospecific enzymatic transformation of α -ketoglutarate to (2S,3R)-3-methylglutamate during acidic lipopeptide biosynthesis. *J. Am. Chem. Soc.* 129, 12011–12018.
- Marchuk, D., Drumm, M., Saulino, A., and Collins, F.S. (1991). Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Nucleic Acids Res.* 19, 1154.
- Müller, C., Nolden, S., Gebhardt, P., Heinzlmann, E., Lange, C., Puk, O., Welzel, K., Wohlleben, W., and Schwartz, D. (2007). Sequencing and analysis of the biosynthetic gene cluster of the lipopeptide antibiotic friulimicin in *Actinoplanes friuliensis*. *Antimicrob. Agents Chemother.* 51, 1028–1037.
- Neusser, D., Schmidt, H., Spizek, J., Novotna, J., Peschke, U., Kaschabek, S., Tichy, P., and Piepersberg, W. (1998). The genes *lmbB1* and *lmbB2* of *Streptomyces lincolnensis* encode enzymes involved in the conversion of L-tyrosine to propylproline during the biosynthesis of the antibiotic lincomycin A. *Arch. Microbiol.* 169, 322–332.
- Omura, S., Mamada, H., Wang, N.J., Imamura, N., Oiwa, R., Iwai, Y., and Muto, N. (1984). Takaokamycin, a new peptide antibiotic produced by *Streptomyces* sp. *J. Antibiot. (Tokyo)* 37, 700–705.

- Peschke, U., Schmidt, H., Zhang, H.Z., and Piepersberg, W. (1995). Molecular characterization of the lincomycin production gene cluster of *Streptomyces lincolnensis* 78-11. *Mol. Microbiol.* **16**, 1137–1156.
- Phelan, V.V., Du, Y., McLean, J.A., and Bachmann, B.O. (2009). Adenylation enzyme characterization using γ - $^{18}\text{O}_4$ -ATP pyrophosphate exchange. *Chem. Biol.* **16**, 473–478.
- Pojer, F., Li, S.M., and Heide, L. (2002). Molecular cloning and sequence analysis of the clorobiocin biosynthetic gene cluster: new insights into the biosynthesis of aminocoumarin antibiotics. *Microbiology* **148**, 3901–3911.
- Recio, E., Colinas, A., Rumbero, A., Aparicio, J.F., and Martin, J.F. (2004). PI factor, a novel type quorum-sensing inducer elicits pimarin production in *Streptomyces natalensis*. *J. Biol. Chem.* **279**, 41586–41593.
- Reinscheid, U.M., Farjon, J., Radzom, M., Haberz, P., Zeeck, A., Blackledge, M., and Griesinger, C. (2006). Effect of the solvent on the conformation of a depsipeptide: NMR-derived solution structure of hormaomycin in DMSO from residual dipolar couplings in a novel DMSO-compatible alignment medium. *ChemBioChem* **7**, 287–296.
- Rokem, J.S., Lantz, A.E., and Nielsen, J. (2007). Systems biology of antibiotic production by microorganisms. *Nat. Prod. Rep.* **24**, 1262–1287.
- Rössner, E., Zeeck, A., and König, W.A. (1990). Elucidation of the structure of hormaomycin. *Angew. Chem. Int. Ed. Engl.* **29**, 64–65.
- Rounge, T.B., Rohrlack, T., Kristensen, T., and Jakobsen, K.S. (2008). Recombination and selectional forces in cyanopeptolin NRPS operons from highly similar, but geographically remote *Planktothrix* strains. *BMC Microbiol.* **8**, 10.
- Sambrook, J., and Russel, D.W. (2001). *Molecular cloning: a laboratory manual* (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).
- Scapin, G., and Blanchard, J.S. (1998). Enzymology of bacterial lysine biosynthesis. In *Advances in Enzymology*, Volume 72 (New York: Wiley), p. 279.
- Sieber, S.A., and Marahiel, M.A. (2005). Molecular mechanisms underlying nonribosomal peptide synthesis: approaches to new antibiotics. *Chem. Rev.* **105**, 715–738.
- Stachelhaus, T., Mootz, H.D., and Marahiel, M.A. (1999). The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases. *Chem. Biol.* **6**, 493–505.
- Stegmann, E., Rausch, C., Stockert, S., Burkert, D., and Wohleben, W. (2006). The small MbtH-like protein encoded by an internal gene of the balhimycin biosynthetic gene cluster is not required for glycopeptide production. *FEMS Microbiol. Lett.* **262**, 85–92.
- Takano, E. (2006). γ -Butyrolactones: *Streptomyces* signalling molecules regulating antibiotic production and differentiation. *Curr. Opin. Microbiol.* **9**, 287–294.
- Tooming-Klunderud, A., Mikalsen, B., Kristensen, T., and Jakobsen, K.S. (2008). The mosaic structure of the mcyABC operon in microcystis. *Microbiology* **154**, 1886–1899.
- Ulanova, D., Novotna, J., Smutna, Y., Kamenik, Z., Gazak, R., Sulc, M., Sedmera, P., Kadlcik, S., Plhachova, K., and Janata, J. (2010). Mutasynthesis of lincomycin derivatives with activity against drug-resistant staphylococci. *Antimicrob. Agents Chemother.* **54**, 927–930.
- Vaillancourt, F.H., Yeh, E., Vosburg, D.A., O'Connor, S.E., and Walsh, C.T. (2005). Cryptic chlorination by a non-haem iron enzyme during cyclopropyl amino acid biosynthesis. *Nature* **436**, 1191–1194.
- Walsh, C.T., Garneau-Tsodikova, S., and Howard-Jones, A.R. (2006). Biological formation of pyrroles: nature's logic and enzymatic machinery. *Nat. Prod. Rep.* **23**, 517–531.
- Wolpert, M., Gust, B., Kammerer, B., and Heide, L. (2007). Effects of deletions of *mbtH*-like genes on clorobiocin biosynthesis in *Streptomyces coelicolor*. *Microbiology* **153**, 1413–1423.
- Zhang, X., and Parry, R.J. (2007). Cloning and characterization of the pyrrolomycin biosynthetic gene clusters from *Actinosporangium vitaminophilum* ATCC 31673 and *Streptomyces* sp strain UC 11065. *Antimicrob. Agents Chemother.* **51**, 946–957.
- Zlatopolskiy, B.D., and de Meijere, A. (2004). First total synthesis of hormaomycin, a naturally occurring depsipeptide with interesting biological activities. *Chemistry* **10**, 4718–4727.
- Zlatopolskiy, B.D., Loscha, K., Alvermann, P., Kozhushkov, S.I., Nikolaev, S.V., Zeeck, A., and de Meijere, A. (2004). Final elucidation of the absolute configuration of the signal metabolite hormaomycin. *Chemistry* **10**, 4708–4717.
- Zlatopolskiy, B.D., Radzom, M., Zeeck, A., and de Meijere, A. (2006). Synthesis and precursor-directed biosynthesis of new hormaomycin analogues. *Eur. J. Org. Chem.*, 1525–1534.