

# Biosynthesis of Himastatin: Assembly Line and Characterization of Three Cytochrome P450 Enzymes Involved in the Post-tailoring Oxidative Steps\*\*

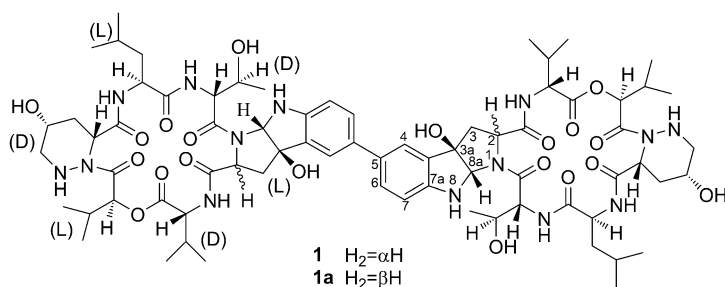
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Himastatin (**1**), a novel symmetrical dimeric cyclohexadepsipeptide antibiotic with the molecular formula  $C_{72}H_{104}N_{14}O_{20}$ , was first discovered in 1990 from a culture broth of *Streptomyces hygroscopicus* ATCC 53653 (later designated as *S. himastatinicus* sp. nov.) isolated from a soil sample collected from the State of Himachal Pradesh and in India.<sup>[1]</sup> It showed in vitro antibacterial activity against a number of Gram-positive bacteria and in vivo antitumor activity against localized P388 leukemia and B16 melanoma in mice.<sup>[1]</sup> In 1996 the structure of himastatin was proposed as **1a** (Scheme 1), which consists of a D-valine (D-Val), an L- $\alpha$ -hydroxyisovaleric acid (L-Hiv), a (3*R*,5*R*)-5-hydroxypiperazic acid (D-Pip), an L-leucine (L-Leu), a D-threonine (D-Thr), and a (2*R*,3*aR*,8*aR*)-3*a*-hydroxyhexahydropyrrolo[2,3*b*]indole 2-

carboxylic acid subunit in each monomer.<sup>[2]</sup> The striking structural features and potent biological activities of himastatin rendered it an ideal target for total synthesis. In 1998 Danishefsky et al.<sup>[3]</sup> completed the total synthesis of himastatin, with the biaryl central core assembled by a Stille coupling of aryl stannane with aryl iodide. However, the <sup>1</sup>H NMR spectral data of the synthesized himastatin (**1a**) did not match those of the natural compound. Careful examination of the NMR spectral data of the synthetic intermediates and natural degradation products of himastatin led them to revise the stereochemistry at C-2, suggesting that the 2,3,3*a*,8*a*-hexahydropyrrolo[2,3*b*]indole moiety in himastatin is derived from L-tryptophan (L-Trp) rather than from D-Trp. Hence, in the revised structure of himastatin (**1**), six components forming the depsipeptide monomer are arrayed in alternating D and L configurations (Scheme 1). Danishefsky et al. also demonstrated that isohimastatin (**1a**, C-2 epimer of **1**) and the monomer of **1** were inactive for antibacterial activity, and suggested that the alternating D and L substituents in each monomer and the biaryl linkage of two monomers are of critical importance for bioactivity.<sup>[4]</sup>

Compound **1** and a few other small-molecule depsipeptides/peptides, including cloptosin,<sup>[5]</sup> kutznerides,<sup>[6]</sup> and NW-G01,<sup>[7]</sup> share two rare structural characteristics, one being a D-Pip residue containing an unusual hydrazo linkage and the other being a 3*a*-hydroxy-hexahydropyrrolo[2,3*b*]indole 2-carboxylic acid moiety. Our interest to study the biosynthesis of **1** was inspired by its intriguing structural features, which hinted that it is biosynthesized by a nonribosomal peptide synthetase (NRPS) assembly line, followed by post-NRPS modifications including oxygenation of the L-Trp residue to a hexahydropyrroloindole subunit and subsequent symmetrical biaryl coupling. Many complex natural products are symmetric dimers formed through a biaryl linkage, usually biologically superior to those of their monomers.<sup>[8]</sup> Enzymes involved in biaryl natural product formation through oxidative phenol coupling have been reported;<sup>[9]</sup> however, the exact enzymatic reaction catalyzing this regioselective oxidative carbon-carbon coupling reaction is poorly understood. The aims of this study are to elucidate the biosynthetic pathway of **1**, and to delineate the enzymology of the exciting chemical transformations of the aforementioned unique structural characteristics.

Initially we attempted to clone the biosynthetic gene cluster of **1** from *S. himastatinicus* using degenerate primer-



Scheme 1. Structures of himastatin (**1**) and isohimastatin (**1a**).

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[\*\*] This work was financially supported by grants from the NSFC (20872152), MOST (2010CB833805), and the Knowledge Innovation Programs of the CAS (KZCX2-YW-JC202, KSCX2-YW-G-065, LYQY200805, and KZCX2-EW-G-12). J.J. is a scholar of the "100 Talents Program" of the Chinese Academy of Sciences (08L111001).

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/ange.201102305>.

PCR targeting of the NRPS adenylation (A) domains.<sup>[10]</sup> Sequencing of 20 cloned PCR products with expected size revealed five groups of A-domain fragments. A genomic library constructed with a SuperCos1 vector kit was screened using each of the five representative A-domain fragments. The genes corresponding to each of those fragments were inactivated by  $\lambda$ -RED-mediated recombination technology<sup>[11]</sup> and the resultant mutants were analyzed for **1** production. Unfortunately all the A-domain disrupted mutants still produced **1**. We then resorted to a genome scanning and bioinformatics analysis strategy to search for the putative **1** gene cluster. This endeavor led us to identify the biosynthetic gene cluster of **1**, which spans about 45 kb of genomic DNA and consists of 20 open reading frames (ORFs; *hmtA–T*) that are predicted to be involved in the biosynthesis of **1**. The gene cluster sequence has been deposited at the EMBL database with accession number FR823394. The gene cluster comprises four modular NRPS genes (*hmtFIKL*), three cytochrome P450 genes (*hmtNST*), one peptide monooxygenase gene (*hmtM*), two type II thioesterase genes (*hmtHJ*), one dehydrogenase/reductase gene (*hmtG*), six regulatory or resistant genes (*hmtABCDQR*), and three hypothetical genes (*hmtEOP*), as shown in Table 1 and Scheme 2A. Beyond *hmtA–T* are orf(–1) and orf(+1) encoding two methyltransferases that are not necessary for **1** biosynthesis.

The monomer backbone of **1** is proposed to be biosynthesized by an NRPS assembly line that consists of four NRPSs (HmtF, HmtI, HmtK, and HmtL), organized into seven modules (Scheme 2B). Module 1 contains three

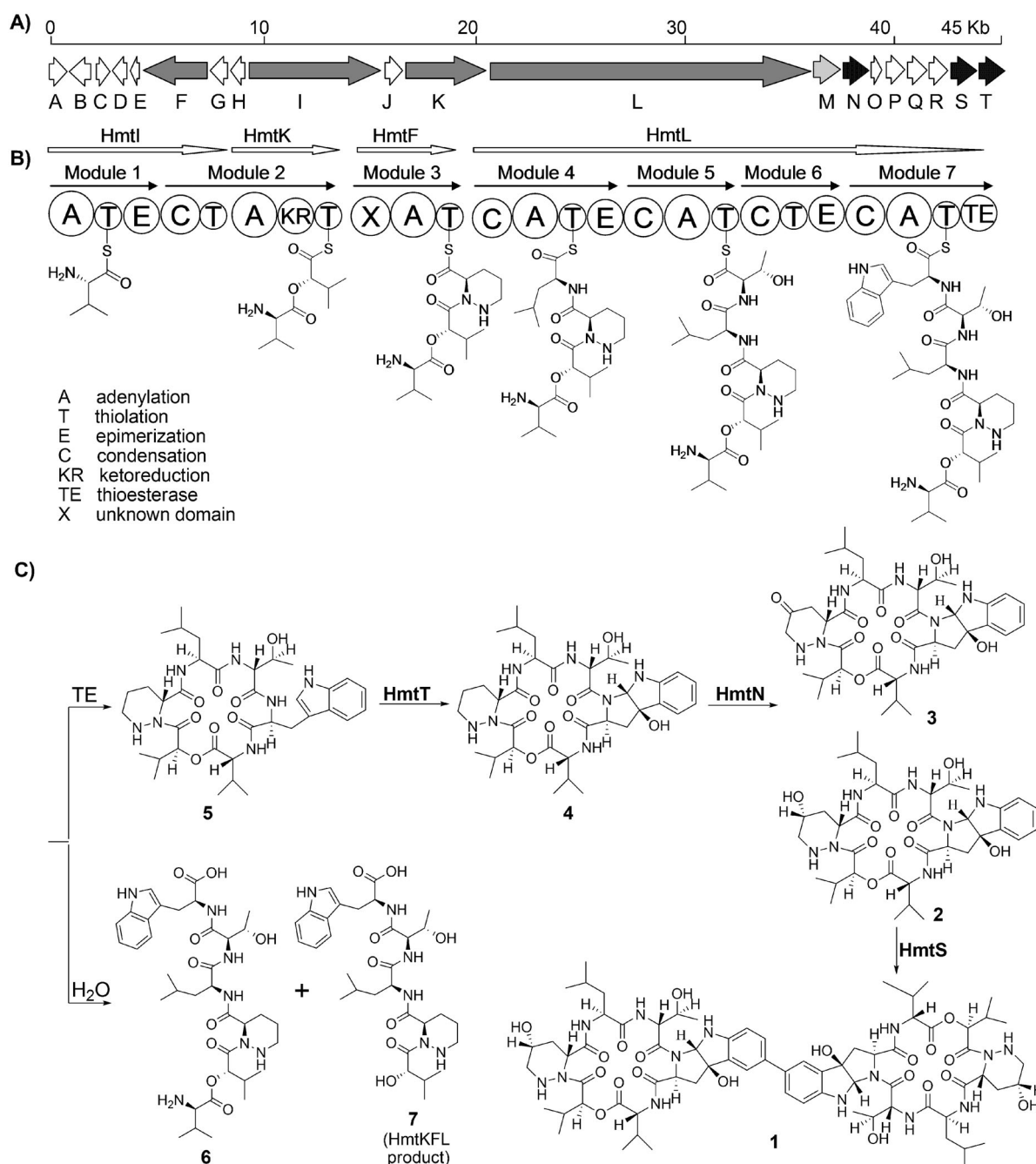
domains (A-T-E on HtmI) and is predicted to incorporate L-Val, which is then converted to D-Val by the epimerization (E) domain; the substrate specificity (DAYWWGGV) of this first A domain is most similar to those of other Val-activating A domains.<sup>[12]</sup> Module 2 contains five domains (C-T- on HtmI, connected to -A-KR-T on HtmK) and is likely to activate 2-keto-isovaleric acid (Kiv), which is then reduced in situ to L-Hiv by the embedded ketoreduction (KR) domain. The presence of KR domains in NRPS systems has been observed in the biosynthetic gene clusters of several other depsipeptide natural products including valinomycin,<sup>[13]</sup> cereulide,<sup>[14]</sup> hec-tochlorin,<sup>[15]</sup> kutznerides,<sup>[6c]</sup> and cryptophycins.<sup>[16]</sup> The condensation (C) domain on HmtI most likely catalyzes the formation of the D-Val-O-L-Hiv peptidyl intermediate via an oxoester linkage. Module 3 contains three domains (X-A-T on HtmF); the predicted substrate specificity (DFWNVGMV) of this A domain is Thr/Gln and X represents an unknown domain. Based on the structure feature of **1**, this module is presumed to be responsible for the incorporation of a Pip residue, the biosynthetic origin of which remains unclear.

Modules 4 (C-A-T-E), 5 (C-A-T), 6 (C-T-E), and 7 (C-A-T) all reside on HtmL sequentially; these modules are predicted to incorporate three remaining amino acid residues (L-Leu-D-Thr-L-Trp). Module 4 contains an E domain that is not required by its cognate amino acid substrate L-Leu, and thus may be responsible for the L-Pip to D-Pip conversion. Module 6 appears to be incomplete by lacking an A domain, but may serve as a way station for the upstream L-Thr

**Table 1:** Deduced functions of ORFs in the **1** biosynthetic gene cluster.

| Protein | Size <sup>[a]</sup> | Protein homologue and origin <sup>[b]</sup>                                             | Identity/<br>similarity | Proposed function                     |
|---------|---------------------|-----------------------------------------------------------------------------------------|-------------------------|---------------------------------------|
| Orf(–3) | 268                 | hypothetical protein (ZP_05800779); <i>Streptomyces flavogriseus</i> ATCC 33331         | 79/85                   | unknown function                      |
| Orf(–2) | 198                 | SACE_4216 (YP_001106410); <i>Saccharopolyspora erythraea</i> NRRL 2338                  | 73/82                   | TetR family transcriptional regulator |
| Orf(–1) | 275                 | PlaM2 (ABB69747); <i>Streptomyces</i> sp. Tu6071                                        | 57/70                   | SAM-dependent methyltransferase       |
| HtmA    | 247                 | transcriptional regulator (ADI09780); <i>Streptomyces bingchenggensis</i> BCW-1         | 85/91                   | MerR family transcriptional regulator |
| HmtB    | 327                 | acetylglutamate kinase (ADI05609); <i>Streptomyces bingchenggensis</i> BCW-1            | 69/81                   | acetylglutamate kinase                |
| HmtC    | 214                 | Orf4 (ABV56600); <i>Kutzneria</i> sp. 744                                               | 56/68                   | negative regulator                    |
| HmtD    | 227                 | Orf10 (ABV56606); <i>Kutzneria</i> sp. 744                                              | 56/72                   | putative regulator                    |
| HmtE    | 185                 | SeryN2_13909 (ZP_06563582); <i>Saccharopolyspora erythraea</i> NRRL 2338                | 54/69                   | hypothetical protein                  |
| HmtF    | 995                 | DhbF (EFF89267); <i>Streptomyces</i> sp. E14                                            | 53/64                   | NRPS (X-A-T)                          |
| HmtG    | 276                 | acyl-CoA dehydrogenase/reductase (ABD65959); <i>Streptomyces fungicidicus</i>           | 66/77                   | acyl-CoA dehydrogenase                |
| HmtH    | 232                 | SAV_3201 (NP_824377); <i>Streptomyces avermitilis</i> MA-468054/70                      | 54/70                   | thioesterase                          |
| HmtI    | 2058                | KtzE (ABV56585); <i>Kutzneria</i> sp. 744                                               | ≈ 60/71 <sup>[c]</sup>  | NRPS (A-T-E-C-T)                      |
| HmtJ    | 259                 | KtzF (ABV56586); <i>Kutzneria</i> sp. 744                                               | 67/75                   | thioesterase                          |
| HmtK    | 1261                | KtzG (ABV56587); <i>Kutzneria</i> sp. 744                                               | 62/72                   | NRPS (A-KR-T)                         |
| HmtL    | 5081                | KtzH (ABV565887); <i>Kutzneria</i> sp. 744 <sup>[c]</sup>                               | ≈ 58/70                 | NRPS (C-A-T-E-C-A-T-C-T-E-C-A-T-TE)   |
| HmtM    | 432                 | KtzI (ABV56589.1); <i>Kutzneria</i> sp. 744                                             | 79/66                   | peptide monooxygenase                 |
| HmtN    | 399                 | cytochrome P450-like enzyme (ZP_06563082); <i>Saccharopolyspora erythraea</i> NRRL 2338 | 57/72                   | P450-like enzyme                      |
| HmtO    | 173                 | SACE_3261 (ZP_06563582); <i>Saccharopolyspora erythraea</i> NRRL 2338                   | 54/63                   | hypothetical protein                  |
| HmtP    | 283                 | SACE_3262 (ZP_06563581); <i>Saccharopolyspora erythraea</i> NRRL 2338                   | 51/68                   | hypothetical protein                  |
| HmtQ    | 317                 | putative ABC transporter (ADI11450); <i>Streptomyces bingchenggensis</i> BCW-1          | 69/80                   | ABC transporter                       |
| HmtR    | 280                 | ABC-2 type transporter (ADI11451); <i>Streptomyces bingchenggensis</i> BCW-1            | 67/83                   | ABC-2 type transporter                |
| HmtS    | 397                 | KtzM (ABV56593); <i>Kutzneria</i> sp. 744                                               | 46/66                   | P450-like enzyme                      |
| HmtT    | 397                 | KtzM (ABV56593); <i>Kutzneria</i> sp. 744                                               | 63/79                   | P450-like enzyme                      |
| Orf(+1) | 336                 | KtzL (ABV56592); <i>Kutzneria</i> sp. 744                                               | 71/57                   | O-methyltransferase                   |
| Orf(+2) | 1048                | PokR1 (ACN64819); <i>Streptomyces diastatochromogenes</i>                               | 41/55                   | putative transcriptional regulator    |

[a] Numbers are in amino acids. [b] NCBI accession numbers are given in parentheses. [c] Module homology.



**Scheme 2.** Biosynthesis of **1**. A) Organization of the **1** biosynthetic gene cluster. B) NRPS domain organizations and a model for the **1** biosynthetic assembly line. C) Post-tailoring oxidative steps supported by isolated intermediates/shunt products **2–7** from  $\Delta hmtN$ ,  $\Delta hmtT$ , and  $\Delta hmtS$  mutants.

building block to be converted to D-Thr by the E domain. At the end of HmtL is a type I thioesterase (TE) domain, responsible for the macrocyclization and release of the hexadepsipeptide from the **1** assembly line to form the backbone of **1** monomer.

Overall, this seven-module assembly line roughly supports the D and L alternations of the structure **1**, but does not fully abide by the collinearity rule of classical NRPSs.<sup>[17]</sup> It is notable that bioinformatics analysis of the substrate specificity of all six A domains revealed that only the first A domain

on HmtI resembled other L-Val-activating A domains, in accordance with our failure to probe the **1** gene cluster using the degenerate primers targeting conserved region A3–A7 of the A domain. To confirm the involvements of these NRPSs in the biosynthetic assembly of **1**, we inactivated the A domain on HmtF and HmtK using the PCR-targeting system to yield  $\Delta hmtF$  and  $\Delta hmtK$  double-cross-over mutant strains, respectively. HPLC analyses of the fermentation broth of the mutants revealed that production of **1** is abolished in both mutants (see the Supporting Information).

The biosynthesis of **1** requires prior and post-NRPS oxidative modifications including the biosynthesis of a 3-hydroxy D-Pip residue, the formation of a tricyclic hexahydropyrroloindole moiety, and the final symmetrical biaryl coupling. In the **1** gene cluster, downstream of the NRPS genes are eight genes (*hmtM–T*) co-transcribed in the same direction. Among these genes, *hmtQ* and *hmtR* encode two ABC-type transporters, thereby suggesting their roles in transportation and resistance; *hmtO* and *hmtP* encode proteins with unknown functions. *hmtM* is predicted to encode a peptide monooxygenase, and *hmtN*, *hmtS*, and *hmtT* encode three cytochrome P450 monooxygenases. These three cytochrome P450 proteins shared significant homology to each other and to a large variety of P450s involved in secondary metabolites, most of the proteins of which have not yet been characterized (see the Supporting Information, Figure S1). To assign the exact roles of these genes, *hmtO*, *hmtP*, *hmtM*, *hmtN*, *hmtS*, and *hmtT* were individually inactivated by using the PCR-targeting method to obtain a double-crossover mutant for each gene. In addition, two dual-gene mutants,  $\Delta hmtMN$  and  $\Delta hmtST$ , were created using the same method.

HPLC–UV analyses of the fermentation extracts of  $\Delta hmtO$  and  $\Delta hmtP$  mutants (see the Supporting Information) revealed that the production of **1** remains unaffected, thus indicating that these two gene products with unknown functions are not necessary for **1** biosynthesis.

The  $\Delta hmtS$  mutant, upon HPLC–UV analysis of its fermentation extract (Figure 1, III), abolished the production of **1** and accumulated two new compounds **2** and **3** with UV absorptions ( $\lambda_{\text{max}} = 210, 290 \text{ nm}$ ) diagnostic of the tricyclic hexahydropyrroloindole moiety. Further liquid chromatography–mass spectrometry (LC–MS) analysis suggested that **2** has a molecular weight of 743.4, consistent with the **1**

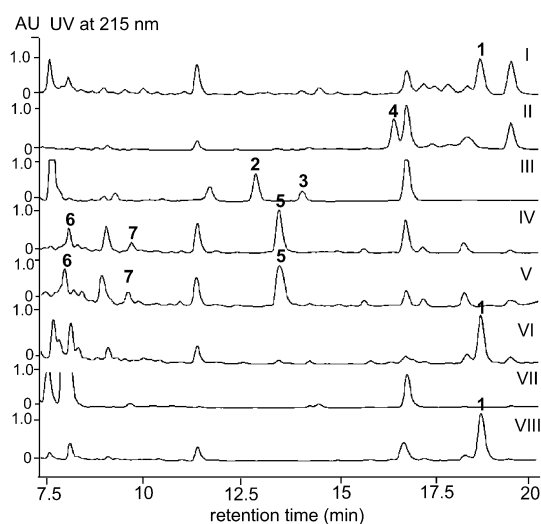
monomer, while **3** has a molecular weight of 741.3, two mass units smaller than that of **2**. Compounds **2** and **3** were subsequently purified from an 8-liter fermentation of the  $\Delta hmtS$  mutant. HRMS rendered the molecular formula of **2** and **3** as  $\text{C}_{36}\text{H}_{53}\text{N}_7\text{O}_{10}$  and  $\text{C}_{36}\text{H}_{51}\text{N}_7\text{O}_{10}$ , respectively.

Full sets of 1D ( $^1\text{H}$ ,  $^{13}\text{C}$ ) and 2D (HMQC, HMBC) NMR data of both **2** and **3** were acquired, which led to the full assignments of their respective  $^1\text{H}$  and  $^{13}\text{C}$  signals. Compounds **2** and **3** in each of the  $^1\text{H}$  NMR spectra clearly showed four aromatic protons assignable to H-4 to H-7 and in each  $^{13}\text{C}$  NMR spectrum showed readily discernable signals of C-3a and C-8a at circa 90 and 86 ppm, respectively, thus proving the existence of the tricyclic hexahydropyrroloindole moiety with no substitution at C-5 in both **2** and **3**. Inspection of other NMR spectral data of **2** and comparison with those of **1** demonstrates it exactly as **1** monomer. The NMR spectra of **3** are very similar to those of **2**, except for the  $\gamma$  position of the Pip subunit where a keto group replaced the hydroxy group in **2**, as evidenced by the HMBC correlations observed from  $\alpha\text{-H}$  ( $\delta = 5.46 \text{ ppm}$ ),  $\beta\text{-H}_2$  ( $\delta = 2.59, 3.24 \text{ ppm}$ ), and  $\delta\text{-H}_2$  ( $\delta = 3.50, 3.60 \text{ ppm}$ ) to  $\gamma\text{-C}$  ( $\delta = 203.4 \text{ ppm}$ ) in the D-Pip moiety. The production of **2**, the monomer of **1**, provided direct evidence that HmtS is responsible for the final symmetric biaryl coupling using a free small molecule as a substrate in the **1** biosynthetic pathway. Compound **3** might be an intermediate or shunt metabolite en route to **2** (Scheme 2C).

HPLC–UV analysis of the fermentation extract of  $\Delta hmtN$  mutant (Figure 1, II) revealed that it lost the production of **1** but produced a new analogue **4** showing similar characteristic UV absorption of the tricyclic hexahydropyrroloindole moiety. Compound **4** was subsequently purified and its molecular formula was determined to be  $\text{C}_{36}\text{H}_{53}\text{N}_7\text{O}_9$  by HRMS; it was 16 mass units smaller than **2**, probably indicating the lack of a hydroxy group. Comparison of the  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of **4** with those of **2** revealed that the C-3 hydroxylated methine signals of the D-Pip residue in **4** was missing; instead, an aliphatic  $\text{CH}_2$  signal appeared. Inspection of other 1D and 2D NMR (COSY, HMQC, HMBC) data of **4** confirmed other structural elements of **4** identical to **2**. The accumulation of **4** in  $\Delta hmtN$  mutant proves that HmtN is a post-tailoring hydroxylase, responsible for the incorporation of a  $\gamma$ -hydroxy group in the Pip residue of **4** to form the himastatin monomer **2** (Scheme 2C).

HPLC–UV analyses of the extracts of  $\Delta hmtT$  and  $\Delta hmtST$  mutants (Figure 1, IV and V) revealed that they both lost the production of **1** and showed three new compounds (**5–7**) with characteristic UV absorption of the Trp residue ( $\lambda_{\text{max}} = 220, 280 \text{ nm}$ ), strongly indicating the presence of an intact tryptophan residue that was not oxidized to the pyrroloindole moiety. Further LC–MS analysis suggested that **5–7** have molecular weights of 711.3, 729.6, and 630.5, respectively. A 15-liter fermentation of  $\Delta hmtT$  mutant and subsequent isolation resulted in the purified compounds **5–7**, the molecular formulae of which were determined as  $\text{C}_{36}\text{H}_{53}\text{N}_7\text{O}_8$ ,  $\text{C}_{36}\text{H}_{55}\text{N}_7\text{O}_9$ , and  $\text{C}_{31}\text{H}_{46}\text{N}_6\text{O}_8$ , respectively, by HRMS analyses.

To clarify the structures of these three compounds, full sets of 1D and 2D NMR (COSY, HMQC, and HMBC) data of **5–7** were acquired, thereby allowing the full assignment of



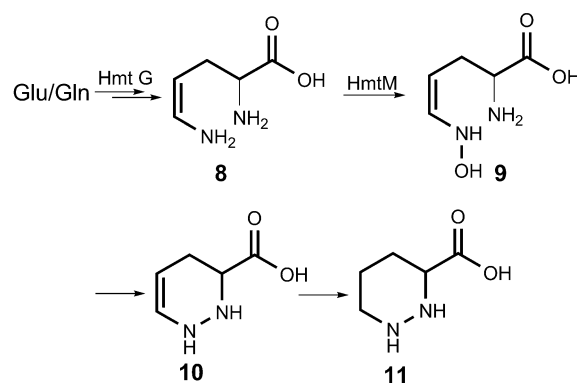
**Figure 1.** HPLC profiles of fermentation extracts of *S. himastatinicus* wild-type and mutant strains. I) Wild type, II)  $\Delta hmtN$  mutant, III)  $\Delta hmtS$  mutant, IV)  $\Delta hmtT$  mutant, V)  $\Delta hmtST$  mutant, VI)  $\Delta hmtT$  complementation mutant, VII)  $\Delta hmtM$  mutant, VIII)  $\Delta hmtM$  complementation mutant. See the Supporting Information for HPLC profiles of the other seven mutants.



their individual  $^1\text{H}$  and  $^{13}\text{C}$  signals. Careful examination of the 1D and 2D NMR data of **5** disclosed that it is the prematured cyclo-Val-Hiv-Pip-Leu-Thr-Trp hexadepsipeptide, but lacking a hydroxy substitution at C-3 of the Pip residue. The  $^1\text{H}$  NMR aromatic signals of H-2, H-4, H-5, H-6, and H-7, COSY correlations between H-4/H-5/H-6/H-7, and HMBC correlations from H-2 to C-7a and C-3a proved the presence of an intact tryptophan residue in **5**. In comparison with those of **2**, the  $^1\text{H}$  and  $^{13}\text{C}$  signals of the C-3 position in the Pip residue showed an aliphatic  $\text{CH}_2$  group in place of the hydroxylated methine group in **5**, which was further supported by COSY and HMBC correlations. Compound **6** was 18 mass units greater than **5**, and corresponded to the linear hydrolyzed product of **5**. Detailed analysis of the 1D and 2D NMR data of **6** confirmed this conclusion and revealed that Val is the N terminus and Trp is the C terminus of the molecule. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of compound **7** only showed signals for a decapeptide consisting of five residues, which is also implied by the MS data. Further 2D NMR data analyses revealed its structural identity with **6** but the N-terminal Val residue is missing (see the Supporting Information).

Complementation of  $\Delta hmtT$  mutant *in trans* restored **1** production (Figure 1, VI). Since *hmtT* is located at the downstream boundary of the gene cluster (the  $\Delta orf(+1)$  mutant still produced **1**), thereby excluding any polar effects the mutation may cause, the isolation of compounds **5–7** from  $\Delta hmtT$  mutant provided valuable clues as to key biosynthetic events of **1**. Firstly, compound **5** serves as the nascent macrocyclized NRPS product catalyzed by the TE domain in the terminal HmtL protein without any tailoring modifications. Secondly, compound **6** is the by-product arising from  $\text{H}_2\text{O}$  attack of the prematured hexadepsipeptide tethered in the terminal TE domain of the HmtL protein. Bioinformatics analysis of the NRPS assembly line provided little information regarding how these building blocks were incorporated into the molecule except for the Val residue; however, the structure of **6** unambiguously verified the incorporation sequence of each building block starting from the Val residue and ending with the Trp residue en route to **1**. Thirdly, compound **7** is a truncated product lacking the Val residue, the isolation of which indicated that the first module could possibly be bypassed and further substantiated the **1** biosynthetic assembly line. Finally, the structures of **5–7** all retained the intact Trp residue and Pip residue that lack the 3-hydroxy substitution, which indicates that HmtT acts first using **5** as a substrate to oxidize the Trp residue to a pyrroloindole moiety, possibly through epoxidation of the indole ring and subsequent cyclization, and that HmtN acts subsequently using **4** as a substrate to hydroxylate C-3 of the Pip residue to yield **2**. The  $\Delta hmtST$  mutant showed the same metabolite profile (Figure 1, V) as that of the  $\Delta hmtT$  mutant, thereby supporting the role of HmtS in the final step for biaryl coupling.

The remaining mystery in **1** biosynthesis is how the intriguing precursor—Pip residue—was biosynthesized. Feeding experiments in monamycin<sup>[18]</sup> and polyoxypeptin<sup>[19]</sup> suggested that glutamic acid and glutamine are precursors of the Pip residue in both molecules. We propose that this unusual N–N linkage-containing residue is biosynthesized by the routes depicted in Scheme 3; similar routes have also been



**Scheme 3.** Proposed biosynthetic pathway of piperazic acid residue.

proposed in the biosynthesis of piperazimycins<sup>[20]</sup> and kutznerides<sup>[6]</sup> but have not been experimentally verified. The peptide monooxygenase HtmM is a likely candidate to catalyze the proposed N-hydroxylation step (formation of **9**; Scheme 3). HPLC–UV analyses of the fermentation extracts of  $\Delta hmtM$  (Figure 1, VII) and  $\Delta hmtMN$  mutants (see the Supporting Information) showed that they both lost the production of **1** and did not produce any new analogues. Complementation of  $\Delta hmtM$  mutant *in trans* fully restored the **1** production (Figure 1, VIII), which suggests that *hmtM* is required in this precursor biosynthesis. *hmtG* encodes a dehydrogenase/reductase, disruption of which ( $\Delta hmtG$  mutant) fully abolished the production of **1** and no new intermediate was observed, thus indicating that HmtG is indispensable for **1** biosynthesis and may play a role in the transformations of Glu/Gln en route to **8** (Scheme 3).

In summary, we have sequenced and identified the biosynthetic pathway of himastatin (**1**), generated 12 gene inactivation mutants, and revealed unusual domain organizations of its assembly line. HmtT, a cytochrome P450 enzyme, has been discovered to regio- and stereoselectively catalyze the post-transformation of L-Trp residue to the pyrroloindole moiety. Such reactions are involved in stereospecific epoxidation of the C-2/C-3 double bond of the indole ring and subsequent regio- and stereospecific nucleophilic attack of the epoxy ring (C–N bond formation) to build the tricyclic hexahydropyrroloindole moiety. Enzyme catalysis of such a reaction has not been reported previously. HmtN, another cytochrome P450 enzyme, has been demonstrated to regio- and stereoselectively post-hydroxylate the D-Pip residue. HmtS, yet another cytochrome P450 enzyme, has also been verified to catalyze the regiospecific and symmetrical biaryl coupling. Such a reaction is involved in highly efficient regiospecific carbon–carbon bond formation under mild conditions, which, in chemical synthesis, always requires carbon activation. Enzymes catalyzing carbon–carbon bond formation have been reported in natural product biosynthesis, for example, cytochrome P450 158A2 has been reported to be able to catalyze the transformation of flaviinol to its dimers but the reaction yields four products.<sup>[21]</sup> OxyC was also reported to catalyze the C–C coupling in the glycopeptide antibiotics (vancomycin, balhimycin, and chloroeremomycin) biosynthesis but the reaction occurred with a PCP-bond

intermediate.<sup>[22]</sup> To the best of our knowledge, HmtS is the first identified enzyme that catalyzes the symmetrical and regioselective biaryl C–C coupling reaction using small complex molecules in nature.

Received: April 3, 2011

Published online: July 1, 2011

**Keywords:** biosynthesis · C–C coupling · cytochromes · natural products · peptides

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