

Iterative Assembly of Two Separate Polyketide Chains by the Same Single-Module Bacterial Polyketide Synthase in the Biosynthesis of HSAF**

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Abstract: Antifungal HSAF (heat-stable antifungal factor, dihydromaltophilin) is a polycyclic tetramate macrolactam from the biocontrol agent *Lysobacter enzymogenes*. Its biosynthetic gene cluster contains only a single-module polyketide synthase–nonribosomal peptide synthetase (PKS–NRPS), although two separate hexaketide chains are required to assemble the skeleton. To address the unusual biosynthetic mechanism, we expressed the biosynthetic genes in two “clean” strains of *Streptomyces* and showed the production of HSAF analogues and a polyene tetramate intermediate. We then expressed the PKS module in *Escherichia coli* and purified the enzyme. Upon incubation of the enzyme with acyl-coenzyme A and reduced nicotinamide adenine dinucleotide phosphate (NADPH), a polyene was detected in the tryptic acyl carrier protein (ACP). Finally, we incubated the polyene–PKS with the NRPS module in the presence of ornithine and adenosine triphosphate (ATP), and we detected the same polyene tetramate as that in *Streptomyces* transformed with the PKS–NRPS alone. Together, our results provide evidence for an unusual iterative biosynthetic mechanism for bacterial polyketide–peptide natural products.

Antifungal HSAF (**1**, heat-stable antifungal factor, dihydromaltophilin) was isolated from the biocontrol agent *Lyso-*
bacter enzymogenes (Figure 1).^[1–4] This bacterial metabolite

belongs to the polycyclic tetramate macrolactams (PTMs), which are emerging as a new class of natural products with distinct structural features.^[5,6] HSAF exhibits a potent antifungal activity and shows a novel mode of action.^[1–4] The HSAF biosynthetic gene cluster contains only a single-module hybrid polyketide synthase–nonribosomal peptide synthetase (PKS–NRPS), although the PTM scaffold is apparently derived from two separate hexaketide chains and an ornithine residue.^[1–4] This suggests that the same PKS module would act not only iteratively but also separately, in order to link the two hexaketide chains with the NRPS-activated ornithine to form the characteristic PTM scaffold. Recently, the Gulder group reported heterologous expression of the ikarugamycin (**4**) biosynthetic gene cluster in *Escherichia coli*,^[7] and the Zhang group reported the enzymatic mechanism for the formation of the inner 5-membered ring and demonstrated the polyketide origin of the ikarugamycin skeleton.^[8] Ikarugamycin is a *Streptomyces*-derived PTM, which has a 5,6,5 tricyclic system (Figure 1). Both the Gulder and Zhang groups showed that a three-gene cluster is sufficient for ikarugamycin biosynthesis. Despite the progress, this iterative polyketide biosynthetic mechanism has not been demonstrated by using purified PKS and NRPS. In addition, HSAF has a 5,5,6 tricyclic system, and its gene cluster contains at least six genes.^[3] Finally, unlike most PTM compounds, HSAF is produced by a Gram-negative bacterium, *L. enzymogenes*. Herein, we report the heterologous production of HSAF analogues in Gram-positive *Streptomyces* hosts, in which the native PKSs have been deleted. We also obtained evidence for the formation of a polyene tetramate intermediate in *Streptomyces* when only the single-module hybrid PKS–NRPS gene was expressed. Finally, we showed the in vitro production of the polyene tetramate by using the individually purified PKS and NRPS. The results provide direct evidence for an iterative polyketide biosynthetic mechanism that is probably general for the PTM-type hybrid polyketide–peptides.

First, we isolated a cosmid clone, Cos4'-1, from the genomic library of *L. enzymogenes* C3, which contains the entire HSAF biosynthetic gene cluster.^[2] The gene cluster was then transferred into vectors for expression in *Streptomyces* sp. However, the transformants failed to produce any detectable HSAF or analogues. Subsequently, we made two modifications in the experiments. One was to replace the putative promoter at the 5'-nontranslated region of the PKS–NRPS gene with the *ermE** promoter,^[9–11] which generates pSETHSAF3 (Figure S1A in the Supporting Information).

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[**] HSAF: heat-stable antifungal factor, dihydromaltophilin. We thank Dr. Zhongjun Qin for providing *Streptomyces coelicolor* strain ZM12. This work was supported in part by the NSFC (31329005 and 3120032), the NIH (R01AI097260), Nebraska Research Initiatives, and the Program for Changjiang Scholars and Innovative Research Team in University (IRT13028).

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/anie.201403500>.

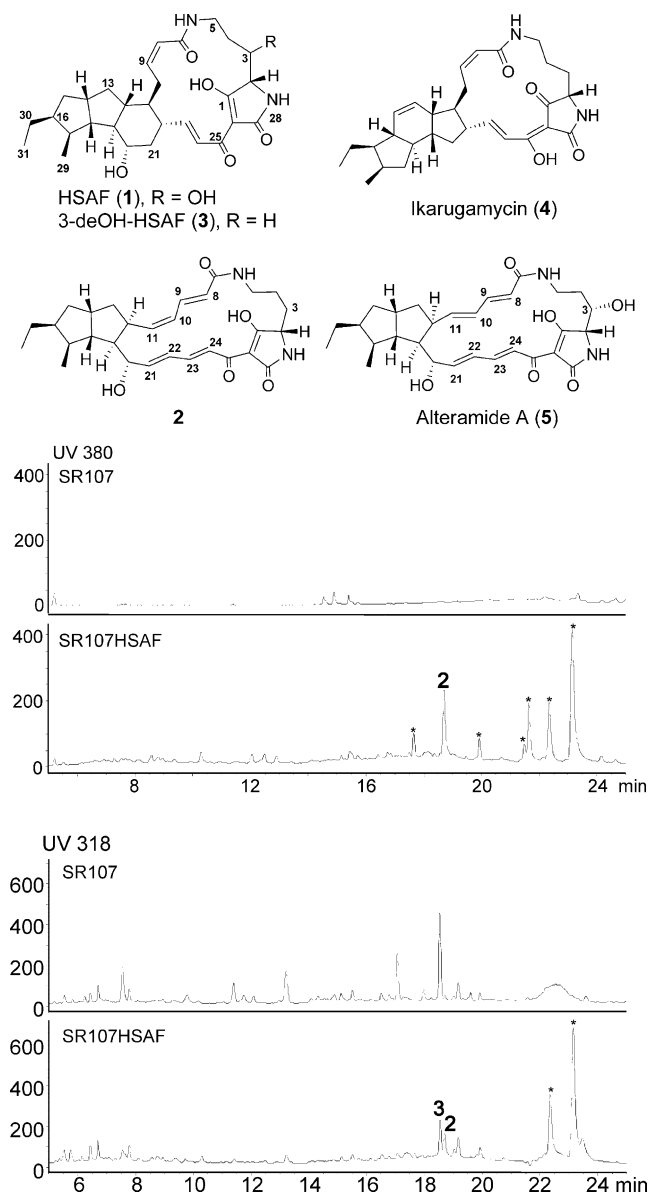


Figure 1. Chemical structures of HSAF and other PTM analogues (top) and HPLC analysis of the HSAF analogues produced in *Streptomyces* (middle and bottom). SR107: metabolites from nontransformed *Streptomyces* sp. LZ35 strain SR107, in which four native PKS gene clusters had been deleted; SR107HSAF: metabolites from strain SR107HSAF1, which was transformed with the PKS-NRPS biosynthetic gene cluster under control of the *ermE** promoter. The first two HPLC profiles show the metabolites detected at 380 nm, and the bottom two show the metabolites detected at 318 nm. The asterisks indicate peaks that were absent in the control.

The other modification was to use an engineered host: strain SR107 was derived from *Streptomyces* sp. LZ35 through deletion of the four native PKS gene clusters.^[12,13] This host was expected to provide a relatively “clean” background for the heterologous production of HSAF. We introduced pSETHSAF3 into strain SR107 to generate strain SR107HSAF1 and analyzed the metabolites in the transformant by using HPLC. Strain SR107HSAF1 produced approximately seven noticeable peaks that were absent in the

control strain SR107 (Figure 1). We first focused our attention on the peak at 18.8 min because it falls in the region that HSAF and analogues would appear.

Compound **2** was isolated (approximately 1 mg L⁻¹ titer) as a yellow powder. HR-ESI-MS gave a quasi molecular ion at *m/z* 495.2837 for [M+H]⁺ (calcd: 495.2853 for C₂₉H₃₈N₂O₅). The structure assignments were carried out by analysis of 1D and 2D NMR data (HSQC, HMBC, and ¹H-¹H COSY; Table S1 and Figure S14–S20 in the Supporting Information). The NMR comparison of **2** with alteramide A (**5**) indicated that the compounds are structurally similar,^[14] except for the absence of the hydroxy group at the C3 position and the *Z* geometry for the C10,C11 double bond in **2** (Figure 1 and Table S1 in the Supporting Information). The relative configuration of **2** was established by proton couplings and NOE correlations. The large coupling constants (*J* ≈ 15.0 Hz) between the olefinic protons (H8/H9, H21/H22, and H23/H24) led to assignment of the *E* configuration for the three double bonds, whereas the *Z* configuration of the C10,C11 double bond was deduced from the small coupling constant (*J* = 11.2 Hz) between the H10 and H11 atoms. The relative stereochemistry of the bicycle unit, which is identical to that of alteramide A (**5**),^[14] was determined from the NOESY experiment. Interestingly, the configuration at the C12 position in **2** and **5** is opposite to that in **1**. Compounds **2** and **5** may result from an alternative stereospecific cycloaddition that leads to the “wrong” configuration at the C12 position. This consequently may prevent the formation of the 6-membered ring in **1**, because the *L. enzymogenes* PKS-NRPS mutant was not able to convert **5** into **1**.

The rest of the major peaks (indicated by asterisks in Figure 1) detected at 380 nm appeared to be unstable, and we were not able to obtain the NMR data. To see if any other isolable HSAF analogue was produced in the transformant, we checked the metabolites under other wavelengths. At 318 nm, two peaks were detected in the HSAF region: one at 18.8 min (compound **2**) and the other at 18.6 min (compound **3**; Figure 1). These two compounds were not produced by the control strain SR107. (At this wavelength, a main peak at 18.6 min was also detected in the control, but it showed a different UV/Vis spectrum to that of compound **3**.) Compound **3** was then isolated (approximately 0.4 mg L⁻¹ titer) for structure determination. It was a white powder, with a quasi molecular ion at *m/z* 497.3021 for [M+H]⁺ (calcd: 497.3015 for C₂₉H₄₀N₂O₅) as determined by HR-ESI-MS. Comparison of the ¹H NMR spectrum of **3** to that of the previously reported 3-deOH-HSAF readily established the structure of **3** as 3-deOH-HSAF (Figure 1 and Figure S21–23 in the Supporting Information).^[15]

The production of compounds **2** and **3** in a *Streptomyces* strain supports the notion that the single-module hybrid PKS-NRPS in pSETHSAF3 is probably sufficient for the assembly of the PTM scaffold. To further prove this point, we transferred pSETHSAF3 into a second *Streptomyces* host that has a completely “clean” background. Strain ZM12 was derived from *S. coelicolor*, probably the best-studied model *Streptomyces*, through deletion of all ten native PKS and NRPS gene clusters present in its genome.^[16] HPLC showed that this strain produced very few metabolites (Figure S2A in

the Supporting Information). However, upon introduction of pSETHSAF3, both compounds **2** and **3** were produced as the main metabolites in strain ZM12, in addition to a number of minor peaks. No other PKS-NRPS is present in strain ZM12, so our data clearly demonstrated that the biosynthesis of HSAF only requires a single-module hybrid PKS-NRPS. The results also imply that the five domains (ketosynthase (KS)/acyltransferase (AT)/dehydratase (DH)/ketoreductase (KR)/acyl carrier protein (ACP)) of this PKS module act two separate times to assemble two separate hexaketide chains.

Compounds **2** and **3** lack the 3-hydroxy group of HSAF (**1**), which suggests that the sterol desaturase (SD) gene (see Figure S1 in the Supporting Information for the cluster) was not functional in the expression construct pSETHSAF3, in which the *ermE** promoter was placed in front of the PKS-NRPS gene (Figure S1A in the Supporting Information). The SD gene encodes the 3-hydroxylase converting 3-deOH-HSAF (**3**) into **1**.^[15] We subsequently generated a second expression construct, pSETHSAF4, in which the *ermE** promoter was placed in front of the SD gene (Figure S1B in the Supporting Information). The construct was introduced into *Streptomyces* sp. SR107 to generate the transformant strain SR107HSAF2. However, a careful search of the metabolites in this strain did not find any HSAF-like compound (Figure S2B in the Supporting Information). The reason for this is unclear at this moment; one possibility is that the insertion of the *ermE** cassette in front of the SD gene might not lead to transcription of the PKS-NRPS gene and downstream tailoring genes because the SD gene and the other genes in the cluster do not appear to share the same promoter (Figure S1 in the Supporting Information).^[15]

To further demonstrate that the single-module hybrid PKS-NRPS is able to assemble two separate polyketide chains and then link them to ornithine, we generated a third *Streptomyces* expression construct pSETHSAF5 that contains only the PKS-NRPS gene under the control of the *ermE** promoter (Figure S1C in the Supporting Information). The construct was introduced into *Streptomyces* sp. SR107 to generate strain SR107PKS/NRPS. HPLC analysis showed that the strain produced three new peaks that were absent in the control strain. The peaks showed absorption λ_{max} values at around 350–450 nm, which suggests the presence of conjugation systems in all of the compounds (Figure S3 in the Supporting Information). The peaks were individually collected and analyzed by LC-MS/MS. Among them, the peak (for compound **6**) with a retention time of 20.8 min gave a quasi molecular ion at m/z 475.26 (calcd: 475.26 for $[M+H]^+$ for the polyene tetramate **6**; Figure 2). MS/MS analysis showed fragments of m/z 173.10 and 147.08, which are consistent with the polyene structure. The same polyene was also observed in the recent heterologous expression of the ikarugamycin PKS/NRPS.^[8] Together, the data support the idea that the single-module hybrid PKS-NRPS is able to assemble two separate polyketide chains and then link them with an ornithine residue to generate the polyene tetramate **6**.

To obtain direct evidence for this iterative single-module PKS, we expressed the PKS in *E. coli* and purified the 199.8 kDa protein (Figure S4–6 in the Supporting Information). To test its activity, we converted the PKS into its holo-

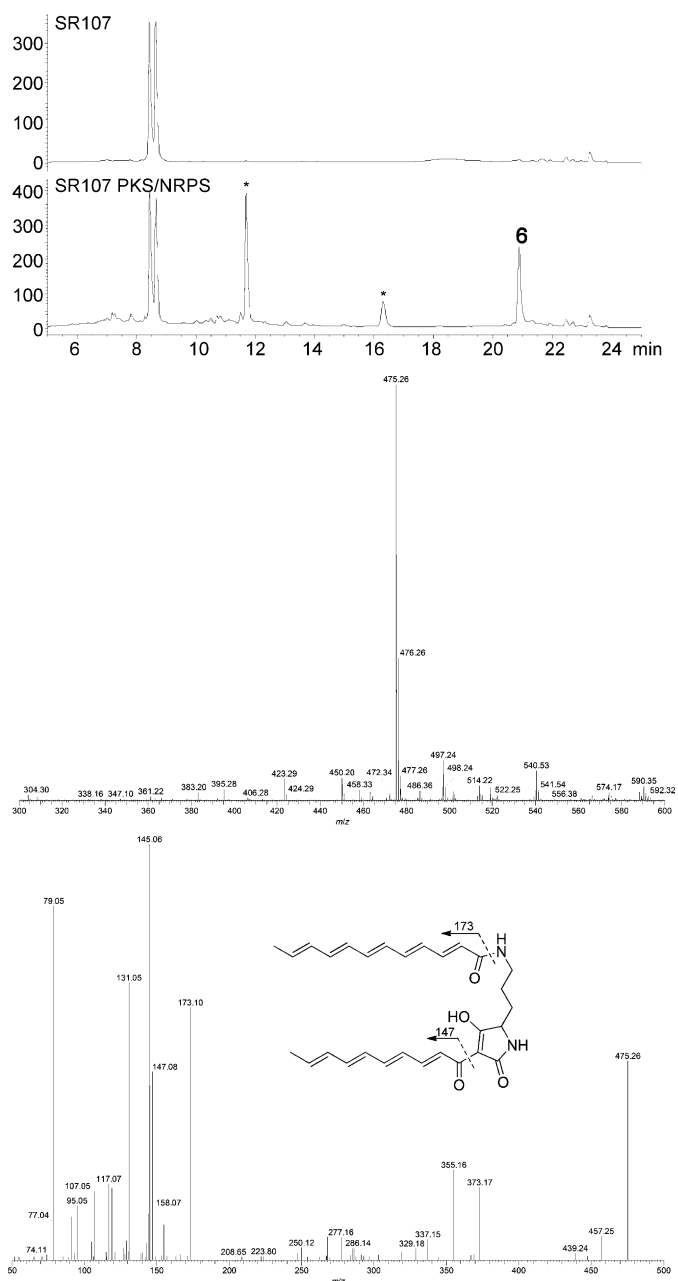


Figure 2. Production of the polyene tetramate **6** in *Streptomyces* transformed with the PKS-NRPS only. HPLC analysis (top); full ESI-MS spectrum of **6** (center) and MS/MS analysis of **6** (bottom). SR107: metabolites from nontransformed *Streptomyces* sp. LZ35 strain SR107; SR107PKS/NRPS: metabolites from the strain transformed with only the PKS-NRPS gene under control of the *ermE** promoter. The metabolites were detected at 380 nm. The asterisks indicate peaks that were absent in the control.

form by incubating it with coenzyme A (CoA) and Svp, a promiscuous 4'-phosphopantetheinyl transferase (PPTase).^[17] Due to the huge size of this protein, we treated the PKS with trypsin after the reaction and followed the mass change of the tryptic fragment within the ACP domain of the PKS, to which the 4'-phosphopantetheinyl (PPT) moiety and biosynthetic intermediates are covalently linked.^[18,19] Specifically, the trypsin digestion^[20] is predicted to release a 26-

residue fragment, VKPEQIDADASLNALGLDSLLA-MELR (the active site serine residue is underlined), within the ACP domain.

First, we wanted to confirm that this 26-residue fragment was indeed released from the PKS that was heterologously expressed in *E. coli*. Q-TOF-MS showed that the tryptic fragment of the apo-PKS had m/z 928.1374 for $[M+3H]^{3+}$ (calcd: 928.1604; Figure 3), and tandem MS-MS showed that this tryptic fragment had the predicted amino acid sequence (Figure S7 in the Supporting Information). After the correct tryptic PKS fragment was identified, we analyzed the holo-PKS and detected a tryptic fragment with m/z 1041.5487 for $[M+3H]^{3+}$ (calcd: 1041.5221) and m/z 781.4270 for $[M+4H]^{4+}$ (calcd: 781.3936; Figure 3; Table S2 and Figure S8 in the Supporting Information). The holo-PKS was also confirmed by using a phosphopantetheine ejection assay,^[21] which detected m/z 357.2021 (calcd 357.0891) for the predicted PPT-ejected product (Figure S8 in the Supporting Information). The data showed that the *E. coli* produced PKS was as expected, and the enzyme was active.

Next, we sought potential biosynthetic intermediates that were covalently attached to the ACP domain of the PKS. Upon incubation of the holo-PKS with malonyl-CoA, acetyl-CoA, and reduced nicotinamide adenine dinucleotide phosphate (NADPH), the tryptic fragment released from the PKS showed m/z 1113.4847 for $[M+3H]^{3+}$ (calcd: 1113.5533) and m/z 835.4001 for $[M+4H]^{4+}$ (calcd: 835.4170; Figure 3; Table S2 and Figure S9 in the Supporting Information). This mass change of the ACP fragment is coincident with a hexaketide polyene intermediate attached to the PPT moiety of the PKS (Figure 3). We also varied reaction conditions and searched for other potential biosynthetic intermediates with a varied carbon chain. However, the hexaketide polyene was the only one detected. The results are consistent with those obtained from the heterologous production of HSAF analogues/intermediates in *Streptomyces*. Interestingly, the in vitro data suggest that the PKS used malonyl-CoA as both the starter and the extender in the polyketide chain synthesis because a carboxylate appeared to be present in the intermediate (Figure 3; Figure S9 in the Supporting Information). To verify this, we generated another PKS expression construct, in which the active site cysteine in the KS domain of this PKS was mutated to alanine (C176A; protein accession number ABL86391; Figure S10 in the Supporting Information). The point-mutated PKS (mPKS) gene was heterologously expressed in *E. coli*, and the enzyme was purified and confirmed to contain the C176A mutation by Q-TOF-MS detection of the tryptic fragment containing the KS active site, GPSLSIDTAASSSLVAVHLACHSLRR (underlined residue indicates the C176A mutation), with m/z 883.16 for $[M+3H]^{3+}$ (calcd: 883.45; Figure S11 in the Supporting Information). All of the domains, except the KS domain, are still active in this PKS, so the synthase is expected to transfer an acyl-CoA (malonyl- or acetyl-CoA) to the ACP but to be unable to elongate the polyketide chain. Therefore, the acyl group from the starter acyl-CoA will be "stuck" on the ACP domain. Indeed, the ACP domain of the mPKS was 4'-phosphopantetheinylated by Svp, as evidenced by the tryptic fragment of m/z 1041.21 for $[M+3H]^{3+}$ (calcd:

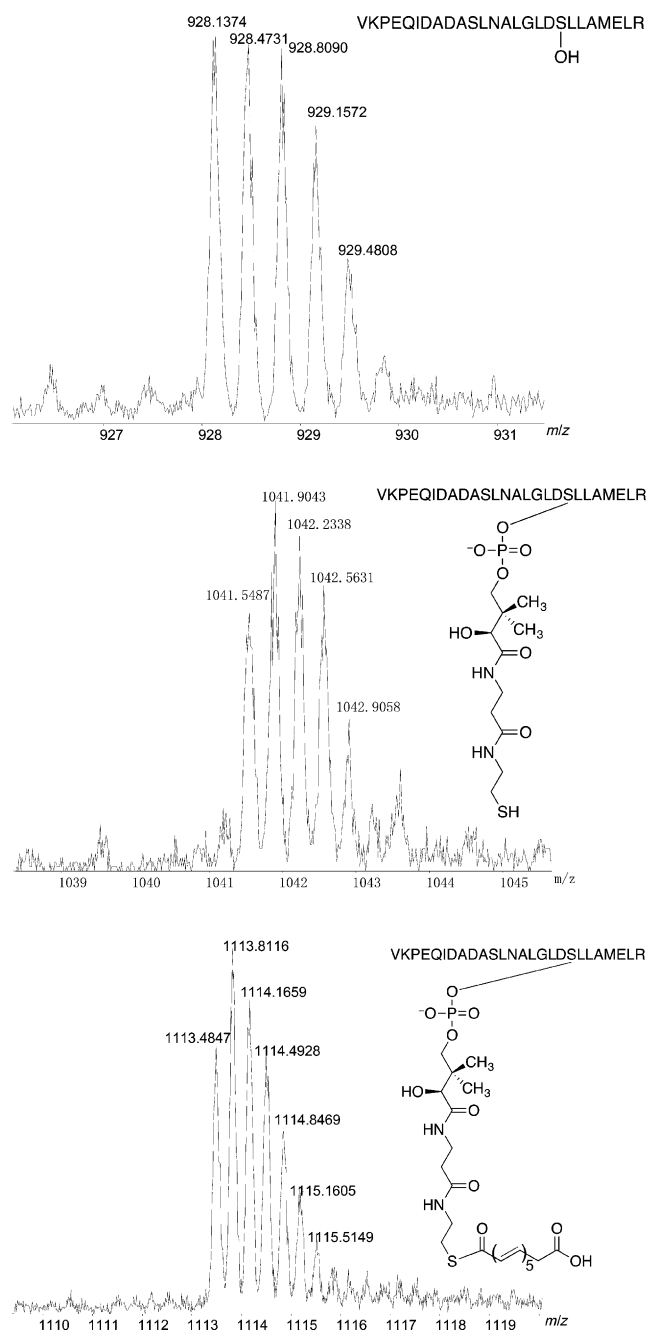


Figure 3. Q-TOF-MS detection of the 26-residue tryptic PKS fragment that harbors the serine active site of the ACP domain. Top: the tryptic fragment from the PKS produced in *E. coli*, with m/z 928.1374 for $[M+3H]^{3+}$ (calcd: 928.1604); center: the tryptic fragment from the 4'-phosphopantetheinylated PKS, with m/z 1041.5487 for $[M+3H]^{3+}$ (calcd: 1041.5221); bottom: the tryptic fragment from acylated PKS, with m/z 1113.4847 for $[M+3H]^{3+}$ (calcd: 1113.5533). This shows the hexaketide polyene synthesized by the single-module PKS.

1041.52; Figure S11 in the Supporting Information). Upon incubation of the holo-mPKS with both acetyl-CoA and malonyl-CoA in the presence of NADPH, we analyzed the acylated ACP fragment. The tryptic fragment showed m/z 1069.89 for $[M+3H]^{3+}$ (calcd: 1070.19 for malonyl-S-ACP or 1055.52 for acetyl-S-ACP) and m/z 802.69 for $[M+4H]^{4+}$

(calcd: 802.87 for malonyl-S-ACP or 791.86 for acetyl-S-ACP; Figure S12 in the Supporting Information). The results showed that the C176A mutated PKS was active in transferring the malonyl group from malonyl-CoA but not the acetyl group from acetyl-CoA to the ACP domain (Figure S12 in the Supporting Information). The result demonstrated that the PKS prefers malonyl-CoA over acetyl-CoA as the starter. It also explains why a carboxylate was observed in the ACP-bound polyketide intermediate (Figure 3).

Finally, we attempted to reconstitute the activity of the HSAF PKS-NRPS *in vitro* by using the individually purified enzymes. The NRPS module (condensation (C)/adenylation (A)/peptidyl carrier protein (PCP)/thioesterase (TE); 148.6 kDa) was separately expressed and purified.^[3] Prior to reconstitution of the activity, the purified NRPS was converted into the holo form by incubation with CoA and Svp, followed by incubation with L-ornithine and adenosine triphosphate (ATP) to form L-ornithine-S-NRPS.^[3] A TE domain is present in this module, so we expect the product(s) to be released into the reaction medium.^[3,4,22] We incubated the polyene-S-PKS with the L-ornithine-S-NRPS and used LC-MS to search for released products from the reconstitution reaction (Figure 4). Total ion chromatography of a selected mass range detected a distinct peak (15.48 min under these LC-MS conditions; see the Supporting Information) that was absent in the control reaction. In HR-ESI-MS analysis, this peak gave m/z 475.2572, which is coincident with the expected $[M+H]^+$ mass for the polyene tetramate **6** (Figure S13 in the Supporting Information). The result is also in agreement with the *in vivo* data, in which the production of compound **6** was observed in *Streptomyces* containing the PKS-NRPS gene alone (Figure 2). The product of the reconstituted PKS and NRPS did not contain the carboxylate moiety from the starter malonyl-CoA, which suggests that a decarboxylation must have taken place after the PKS-linked hexaketide polyene was transferred to the NRPS. Together, the *in vivo* heterologous production data (with the whole gene cluster and PKS-NRPS only) and the *in vitro* enzyme assays (with PKS alone and the PKS-NRPS reconstitution) support the conclusion that the PKS module acts iteratively and,

together the NPRS module, is sufficient for the synthesis of the PTM framework.

Based on the results, we propose a mechanism for the formation of the PTM scaffold (Figure 5). First, the five-domain PKS module iteratively catalyzes the formation of a polyene hexaketide. The geometry of the double bonds is unknown but is most likely to be *trans*. This hexaketide is transferred to the four-domain NRPS module, which activates L-ornithine and catalyzes the first amide bond formation, probably between the δ -amino group of L-ornithine and the thioester carbonyl group of the hexaketide. This leads to an NRPS-bound polyene-ornithine intermediate. In the meantime, the PKS module continues to assemble a second hexaketide, which is subsequently transferred to the NRPS through the formation of a second amide bond, probably between the α -amino group of L-ornithine and the second hexaketide chain. The timing of this transfer is right after the fifth cycle of polyketide chain elongation, in which the newly formed β -keto group of the second hexaketide chain has not been processed by the KR domain and DH domain. All PTM natural products contain this keto group in the final structure (at the C25 position in the HSAF structure; Figure 1).^[3,5,6,23] The determining factor for this timing is not known, but it is probably related to the redox enzymes that are proposed to cooperate with the PKS-NRPS in forming the PTM scaffold.^[7,8,15,23] The transfer of the second hexaketide chain leads to an NRPS-bound polyene-ornithine-polyene intermediate. Finally, the intermediate is released from the NRPS by formation of the tetramate moiety, which occurs through attack of the nucleophilic α -carbon atom of the second hexaketide on the thioester carbonyl carbon atom of L-ornithine. The presence of the β -keto group on the second hexaketide makes the α -carbon atom into a good nucleophile, which promotes the tetramate formation and product release. This perhaps contributes to the timing of the transfer of this hexaketide to NRPS. If the β -keto group were fully processed, the second polyene chain would not undergo tetramate formation, which results in an "unproductive process". *In vivo*, the polyene tetramate **6** is ultimately converted into the PTM scaffold by tailoring enzymes.^[8,23] Our previous study showed that this NRPS prefers a 12-carbon chain for forming

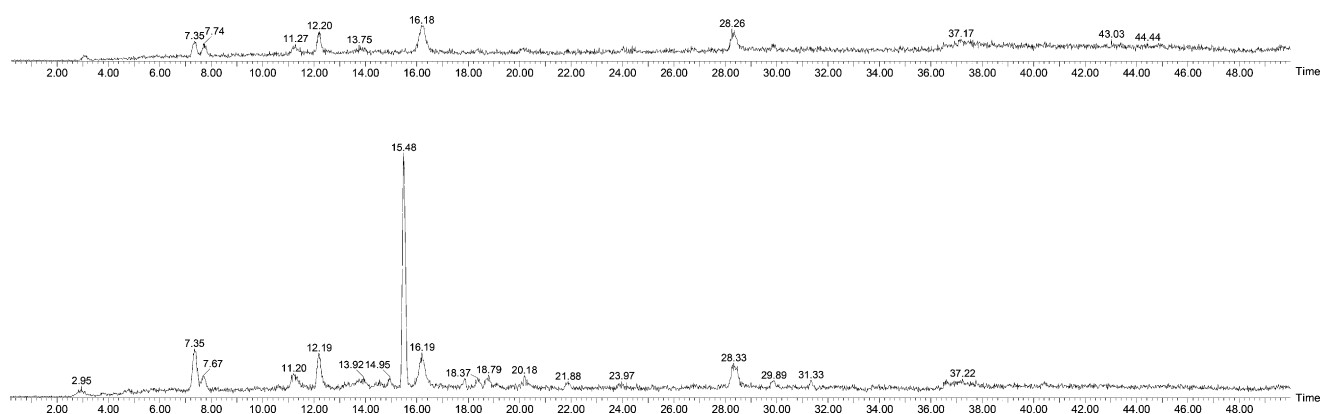


Figure 4. Analysis of the *in vitro* reconstituted PKS-NRPS. Total ion chromatography of the mass (m/z 475.2597) for compound **6** is shown for the control reaction (top) and the PKS-NRPS reaction (bottom). The peak at 15.48 min in the PKS-NRPS reaction gave m/z 475.2572 in HR-ESI-MS analysis.

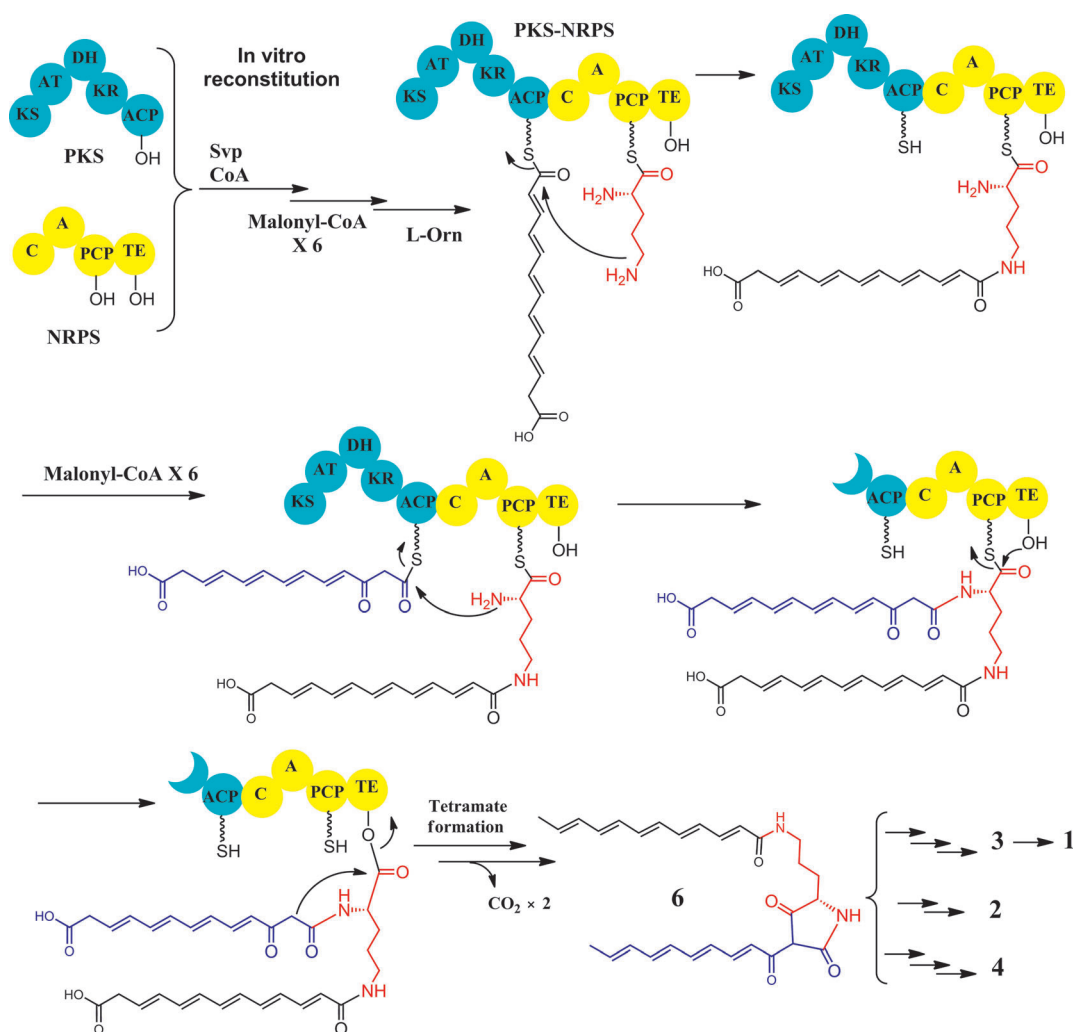


Figure 5. A proposed biosynthetic mechanism for the iterative bacterial PKS-NRPS in the assembly of the scaffold of HSAF and analogues.

tetramate acyl–ornithine–acyl products in vitro.^[3] Thus, the specificity of the domain (most likely the C domain) responsible for transferring the polyketide chain from the PKS to the NRPS is the key determinant for the macrolactam ring size observed in the characteristic PTM framework.

HSAF is the main antifungal factor that the biocontrol agent *Lysobacter enzymogenes* uses to fight against fungal diseases. *Lysobacter* are ubiquitous in the environment but largely remain untapped for bioactive natural products.^[23] HSAF has a potent activity against a broad spectrum of fungi by using a new mode of action. Its chemical structure is distinct from any existing fungicide or antifungal drug. Most interestingly, its biosynthesis involves an iterative PKS mechanism. The hybrid PKS-NRPS has a typical modular organization, including KS-AT-DH-KR-ACP for the PKS module and C-A-PCP-TE for the NRPS module. There are no obvious remnants of an inactive enoylreductase (ER^o) domain or a methyltransferase (CMeT) domain, as seen in several iterative fungal PKS-NRPS systems with similar organization, such as the PKSs for tenellin, lovastatin, and compactin.^[24–27] Although this type of iterative PKS-NRPS is commonly seen in fungi, it has not been common in bacteria

until recent years. Furthermore, PTM-type gene clusters have been identified from numerous bacterial genomes,^[5,6] and the biochemical investigation of these systems has only started recently.^[3,7,8,28] Herein, our studies of HSAF provide direct evidence for this iterative biosynthetic mechanism for bacterial polyketide–peptide natural products. The evidence comes from both the in vivo approach (heterologous production of HSAF analogues and the polyene tetramate intermediate in Gram-positive bacterial hosts with a clean background) and the in vitro approach (expression of the approximately 200 kDa PKS and the approximately 150 kDa NRPS separately in *E. coli*, purification of the giant enzymes, and reconstitution of the biosynthetic activity). In light of the huge number of uninvestigated PTM-type gene clusters in databases, our studies present herein will facilitate the future exploitation of new PTM products.

Experimental Section

Details of experimental procedures, construction of expression vectors, production of HSAF in heterologous hosts, PKS and NRPS gene expression in *E. coli*, protein purification, enzyme reactions and

enzyme activity assays, and spectroscopic data are included in the Supporting Information.

Received: March 20, 2014

Published online: May 30, 2014

Keywords: biosynthesis · enzymes · macrocycles · natural products · polyketides

- [1] S. Li, L. Du, G. Yuen, S. D. Harris, *Mol. Biol. Cell* **2006**, *17*, 1218–1227.
- [2] F. Yu, K. Zaleta-Rivera, X. Zhu, J. Huffman, J. C. Millet, S. D. Harris, G. Yuen, X. C. Li, L. Du, *Antimicrob. Agents Chemother.* **2007**, *51*, 64–72.
- [3] L. Lou, G. Qian, Y. Xie, J. Hang, H. Chen, K. Zaleta-Rivera, Y. Li, Y. Shen, P. H. Dussault, F. Liu, L. Du, *J. Am. Chem. Soc.* **2011**, *133*, 643–645.
- [4] L. Lou, H. Chen, R. L. Cerny, Y. Li, Y. Shen, L. Du, *Biochemistry* **2012**, *51*, 4–6.
- [5] J. A. Blodgett, D. C. Oh, S. Cao, C. R. Currie, R. Kolter, J. Clardy, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 11692–11697.
- [6] S. Cao, J. A. Blodgett, J. Clardy, *Org. Lett.* **2010**, *12*, 4652–4654.
- [7] J. Antosch, F. Schaefer, T. A. M. Gulder, *Angew. Chem.* **2014**, *126*, 3055–3058; *Angew. Chem. Int. Ed.* **2014**, *53*, 3011–3014.
- [8] G. Zhang, W. Zhang, Q. Zhang, T. Shi, L. Ma, Y. Zhu, S. Li, H. Zhang, Y. Zhao, R. Shi, C. Zhang, *Angew. Chem. Int.* **2014**, DOI: 10.1002/ange.201402078; *Angew. Chem. Int. Ed.* **2014**, DOI: 10.1002/anie.201402078.
- [9] M. J. Bibb, G. R. Janssen, J. M. Ward, *Gene* **1985**, *38*, 215–216.
- [10] C. J. Wilkinson, Z. A. Hughes-Thomas, C. J. Martin, I. Bohm, T. Mironenko, M. Deacon, M. Wheatcroft, G. Wirtz, J. Staunton, P. F. Leadlay, *J. Mol. Microbiol. Biotechnol.* **2002**, *4*, 417–426.
- [11] K. A. Datsenko, B. L. Wanner, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 6640–6645.
- [12] Y. Jiang, H. Wang, C. Lu, Y. Ding, Y. Li, Y. Shen, *ChemBioChem* **2013**, *14*, 1468–1475.
- [13] G. S. Zhao, S. R. Li, Y. Y. Wang, H. L. Hao, Y. M. Shen, C. H. Lu, *Drug Des. Dev. Ther.* **2013**, *7*, 185–188.
- [14] H. Shigemori, M. A. Bae, K. Yazawa, T. Sasaki, J. Kobayashi, *J. Org. Chem.* **1992**, *57*, 4317–4320.
- [15] Y. Li, J. Huffman, Y. Li, L. Du, Y. Shen, *MedChemComm* **2012**, *9*, 982–986.
- [16] M. Zhou, X. Jing, P. Xie, W. Chen, T. Wang, H. Xia, Z. Qin, *FEMS Microbiol. Lett.* **2012**, *333*, 169–179.
- [17] C. Sánchez, L. Du, D. J. Edwards, M. D. Toney, B. Shen, *Chem. Biol.* **2001**, *8*, 725–738.
- [18] T. S. Hitchman, J. Crosby, K. J. Byrom, R. J. Cox, T. J. Simpson, *Chem. Biol.* **1998**, *5*, 35–47.
- [19] J. Crosby, K. J. Byrom, T. S. Hitchman, R. J. Cox, M. P. Crump, I. S. Findlow, M. J. Bibb, T. J. Simpson, *FEBS Lett.* **1998**, *433*, 132–138.
- [20] J. P. Kayser, J. L. Vallet, R. L. Cerny, *J. Biomol. Tech.* **2004**, *15*, 285–295.
- [21] P. C. Dorrestein, S. B. Bumpus, C. T. Calderone, S. Garneau-Tsodikova, Z. D. Aron, P. D. Straight, R. Kolter, C. T. Walsh, N. L. Kelleher, *Biochemistry* **2006**, *45*, 12756–12766.
- [22] L. Du, L. Lou, *Nat. Prod. Rep.* **2010**, *27*, 255–278.
- [23] Y. Xie, S. Wright, Y. Shen, L. Du, *Nat. Prod. Rep.* **2012**, *29*, 1277–1287.
- [24] A. A. Yakasai, J. Davison, Z. Wasil, L. M. Halo, C. P. Butts, C. M. Lazarus, A. M. Bailey, T. J. Simpson, R. J. Cox, *J. Am. Chem. Soc.* **2011**, *133*, 10990–10998.
- [25] J. Kennedy, K. Auclair, S. G. Kendrew, C. Park, J. C. Vederas, C. R. Hutchinson, *Science* **1999**, *284*, 1368–1372.
- [26] S. M. Ma, J. W. Li, J. W. Choi, H. Zhou, K. K. Lee, V. A. Moorthie, X. Xie, J. T. Kealey, N. A. Da Silva, J. C. Vederas, Y. Tang, *Science* **2009**, *326*, 589–592.
- [27] Y. Abe, T. Suzuki, C. Ono, K. Iwamoto, M. Hosobuchi, H. Yoshikawa, *Mol. Genet. Genomics* **2002**, *267*, 636–646.
- [28] Y. Luo, H. Huang, J. Liang, M. Wang, L. Lu, Z. Shao, R. E. Cobb, H. Zhao, *Nat. Commun.* **2014**, *4*, in press.