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Biosynthesis of HSAF, a Tetramic Acid-Containing Macrolactam from *Lysobacter enzymogenes*

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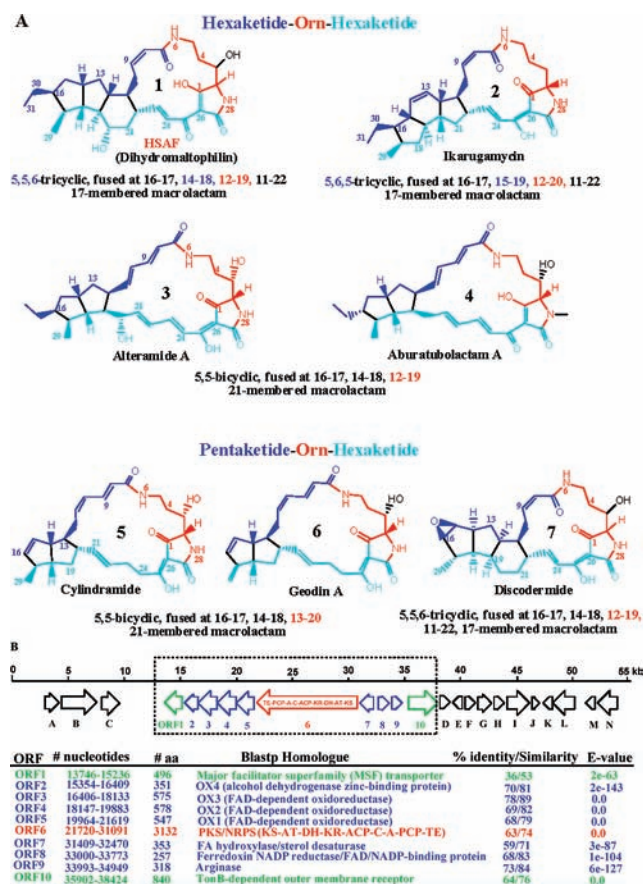
Supporting Information

ABSTRACT: HSAF was isolated from *Lysobacter enzymogenes,* a bacterium used in the biological control of fungal diseases of plants. Structurally, it is a tetramic acid-containing macrolactam fused to a tricyclic system. HSAF exhibits a novel mode of action by disrupting sphingolipids important to the polarized growth of filamentous fungi. Here we describe the HSAF biosynthetic gene cluster, which contains only a single-module polyketide synthase/nonribosomal peptide synthetase (PKS/NRPS), although the biosynthesis of HSAF apparently requires two separate polyketide chains that are linked together by one amino acid (ornithine) via two amide bonds. Flanking the PKS/NRPS are six genes that encoding a cascade of four tightly clustered redox enzymes on one side and a sterol desaturase/fatty acid hydroxylase and a ferredoxin reductase on the other side. The genetic data demonstrate that the four redox genes, in addition to the PKS/NRPS gene and the sterol desaturase/fatty acid hydroxylase gene, are required for HSAF production. The biochemical data show that the adenylation domain of the NRPS specifically activates L-ornithine and that the four-domain NRPS is able to catalyze the formation of a tetramic acid-containing product from acyl-*S*-ACP and ornithinyl-*S*-NRPS. These results reveal a previously unrecognized biosynthetic mechanism for hybrid PK/NRP in prokaryotic organisms.

We previously isolated a novel antifungal compound, HSAF (**1**), from *Lysobacter enzymogenes* C3, which is a bacterium used for the biological control of fungal diseases of plants.[1] HSAF exhibits potent inhibitory activities against a wide range of fungal species and shows a novel mode of action by disrupting the biosynthesis of fungal sphingolipids.[2] Sphingolipids represent an attractive new target for the development of novel antifungal drugs because their structure in fungal cells is distinct from that in mammalian cells.[3] HSAF is a tetramic acid (2,4-pyrrolidinedione)-containing macrolactam (Figure 1).[1] Tetramic acid is the key structural feature of many bioactive heterocycles that exhibit a wide range of biological activities, including antibiotic and anticancer activities.[4] The macrolactam structure is also found in many bioactive natural products. HSAF combines these two structural features. In addition, it contains a 5,5,6-tricyclic system fused to the macrolactam. Such structural features are shared by a group of natural products



ORF	# nucleotides	# aa	Blastp Homologue	% identity/Similarity	E-value
ORF1	13746-15236	496	Major facilitator superfamily (MSF) transporter	36/53	2e-63
ORF2	15354-16409	351	OX4 (alcohol dehydrogenase zinc-binding protein)	70/81	2e-143
ORF3	16406-18133	575	OX3 (FAD-dependent oxidoreductase)	78/89	0.0
ORF4	18147-19883	578	OX2 (FAD-dependent oxidoreductase)	69/82	0.0
ORF5	19964-21619	547	OX1 (FAD-dependent oxidoreductase)	68/79	0.0
ORF6	21720-31091	3132	PKS/NRPS(KS-AT-DH-KR-ACP-C-A-PCP-TE)	63/74	0.0
ORF7	31409-32470	353	FA hydroxylase/sterol desaturase	59/71	3e-87
ORF8	33000-33773	257	Ferredoxin NADP reductase/FAD/NADP-binding protein	68/83	1e-104
ORF9	33993-34949	318	Arginase	73/84	6e-127
ORF10	35902-38424	840	TonB-dependent outer membrane receptor	64/76	0.0

Figure 1. (A) Structure of HSAF and related tetramic acid-containing macrolactams. On the basis of their possible biosynthetic mechanisms, the compounds can be divided into two groups: the hexaketide–ornithine–hexaketide group and the pentaketide–ornithine–hexaketide group. Both groups can have a 17-membered macrolactam fused to a tricyclic system or a 21-membered macrolactam fused to a bicyclic system. (B) Map of the HSAF gene cluster (EF028635.2). The ORFs in the blocked region are annotated here; the annotation of the rest of the ORFs is included in Figure S6.

(**2**–**7**) that were isolated from marine invertebrates and/or associated microorganisms (Figure 1). No biosynthetic studies on these

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marine metabolites have been reported. Previously, we reported four HSAF biosynthetic genes, including a hybrid polyketide synthase/nonribosomal peptide synthetase (PKS/NRPS). Since then, we have been searching for additional PKS genes around this PKS/NRPS locus because the biosynthesis of HSAF apparently requires two separate polyketide chains. We now provide the complete sequence of the gene cluster based on the genome sequences. The most striking finding is that only a single PKS/NRPS is present in the gene cluster. We have also expressed the NRPS module and demonstrated in vitro that it specifically activates L-ornithine and catalyzes the formation of a tetramate product.

In *L. enzymogenes* C3, we previously identified three genes downstream from the PKS/NRPS. Gene disruption data showed that only the gene encoding sterol desaturase/fatty acid hydroxylase is required for HSAF production.[1] Because biosynthetic genes for PK/NRP in microorganisms are almost always clustered together, we searched the region upstream from the PKS/NRPS, where we found four genes (OX1 to OX4, EF028635.2) that encode a cascade of NADP/FAD-dependent oxidoreductases. OX1, OX2, and OX3 are similar to each other, while OX4 is distinct (Figure 1). The OX genes appear to form an operon with the PKS/NRPS because they are either translationally coupled or have only a small intergenic region between them. To test their relevance to HSAF biosynthesis, we disrupted each of the OX genes. The disruption mutants of OX1, OX2, and OX3 did not produce HSAF, whereas the disruption mutants of OX4 produced a small amount of a metabolite with $[M + H]^+$ at m/z 511.2789, which is two mass units smaller than HSAF and coincident with the mass of maltophilin, a putative precursor of HSAF (Figure S1 in the Supporting Information). To eliminate the potential polar effect caused by insertion of the conjugal vector, we then generated four in-frame deletion mutants by deleting a small sequence (6−11 residues) at the highly conserved active sites of the OXs (Figures S2−S5). The results showed that the deletion mutants of OX1, OX2, and OX3 did not produce HSAF while the OX4 deletion mutants still produced the same putative precursor. Together, the results show that the OX genes are required for the biosynthesis of the final product.

The Liu group has been using *L. enzymogenes* OH11 as a biocontrol agent for plant fungal diseases in China.[5] We recently completed the draft genome sequence of strain OH11. The results showed that strain OH11 has all of the HSAF genes found in strain C3. Within the HSAF genes (20280 bp), the two strains have a 96% identity. At the amino acid sequence level, the individual open reading frames (ORFs) have an identity of 98−99%. With the genome sequence, we were able to search for “extra PKS” genes juxtaposed to the HSAF locus. Surprisingly, no such gene was found. The center of the gene cluster is the hybrid PKS/NRPS. Flanking the PKS/NRPS are genes encoding six redox enzymes, arginase, and putative transporters and receptors/regulators (Figure 1B and Figure S6). Beyond the 55 kb region shown in Figure 1B, we found only genes encoding rRNA, ribosome proteins, tRNA, and DNA-repairing proteins, which apparently are not related to HSAF biosynthesis. The HSAF biosynthetic gene cluster contains only a single-module PKS/NRPS, a cascade of redox enzymes, and several putative transporter/regulators. Recently, Clardy and co-workers also observed similar results in gene clusters mined from a number of *Streptomyces* genomes.[6] They found that this type of single-module hybrid PKS/NRPS is conserved among phylogenetically diverse bacterial species.

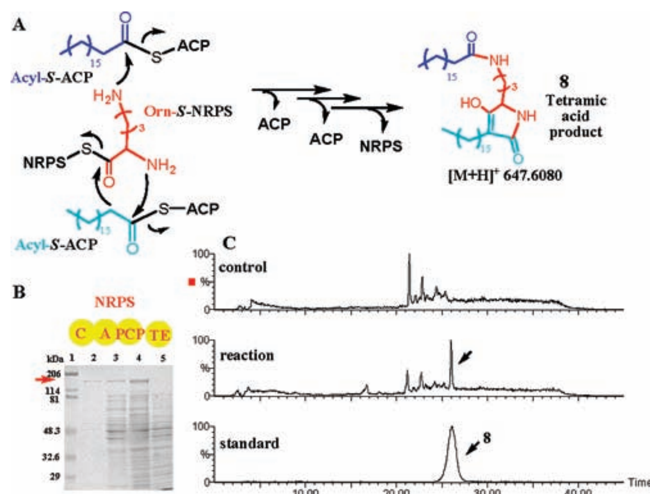
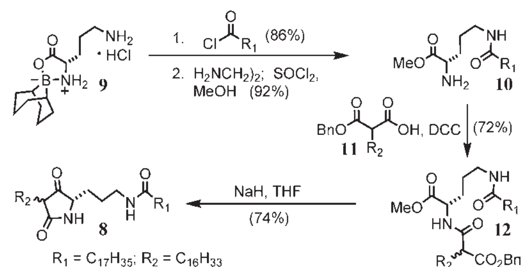


Figure 2. In vitro synthesis of tetramic acid-containing product **8** using the purified HSAF NRPS. (A) Schematic illustration of the reactions. The acyl-*S*-ACP substrate was prepared by incubation of ACP and stearoyl-CoA with Svp, and Orn-*S*-NRPS was prepared by incubation of NRPS and CoA with Svp followed by addition of L-ornithine and ATP . (B) SDS-PAGE gel showing the expression and purification of the four-domain NRPS. The expected size of the NRPS was 148.6 kDa. Lane 1, markers; lane 2, purified NRPS; lane 3, soluble fraction of protein extracts; lane 4, total protein extracts upon IPTG induction; lane 5, total protein extracts before IPTG induction. (C) LC−MS detection of the expected product **8**. The top spectrum is for a control reaction without the NRPS. The middle spectrum is for a complete reaction and shows the production of the expected product with $[M + H]^+$ at m/z 647.6080. The bottom spectrum is that of the chemically synthesized standard **8** (mixture of diastereomers).

To test whether the single NRPS module could accept two separate acyl chains to produce the characteristic tetramic acid-containing product, we conducted a series of in vitro experiments. We first expressed the adenylation domain of the PKS/NRPS in *Escherichia coli* and used the purified 66.4 kDa protein (Figure S7) for an ATP−PPi exchange assay. Among the 16 amino acids tested, the A domain specifically activates L-ornithine and, to a lesser extent, L-lysine (Figure S7). We then tested the in vitro activity of the entire NRPS module (Figure 2). The four-domain NRPS (C−A−PCP−TE) was expressed in *E. coli* as a His_6-tagged protein with an expected size of 148.6 kDa (Figure 2B). The promiscuous 4′-phosphopantetheine transferase Svp was used to prepare stearoyl-*S*-ACP and holo-NRPS.[7] The holo-NRPS was then loaded with L-ornithine in the presence of ATP, producing the ornithinyl-*S*-NRPS substrate (Figure 2A). When stearoyl-*S*-ACP and ornithinyl-S-NRPS were incubated together, we detected an $[M + H]^+$ ion at m/z 647.6080 in the reactions (Figure 2C), which is consistent with the predicted mass of the product **8**. This molecular ion was not present in the control reaction without the NRPS protein. The putative tetramic acid structure was correlated with an authentic sample prepared as illustrated in Scheme 1 (also see Scheme SI-1 in the Supporting Information). The boroxazolidinone adduct of L-ornithine (**9**) was selectively acylated at the terminal amino group with octadecanoyl chloride to furnish a monoamide.[8] Deprotection of the boroxazolidinone with ethylenediamine followed by reaction with thionyl chloride in methanol furnished methyl ester **10**. Conversion of the amino acid ester to the tetramic acid was accomplished through a modification of a published approach.[9] Condensation of the amino ester with

Scheme 1. Preparation of Tetramic Acid 8



2-benzyloxycarbonyloctacadecanoic acid (**11**), which was prepared in two steps from dibenzyl malonate, furnished the α,δ-bisamide **12** as an inseparable ∼1:1 mixture of diastereomers. Reaction with excess sodium hydride resulted in tandem Claisen condensation and deacylation to furnish tetramic acid **8** as a 1:1 mixture of epimers at C_3 of the 3,5-dioxopyrrolidine. The retention time and mass spectra of the synthetic tetramic acid sample exactly matched those of the enzymatically synthesized material (Figure 2C).

These results show that the two amide functionalities in HSAF are formed between two acyl chains and ornithine, one at the α-amino group and the other at the δ-amino group of ornithine. The results also suggest that the HSAF NRPS module is able to accept two polyketide acyl-*S*-ACPs, making two amide bonds on the same amino acid via two Claisen condensation reactions and forming the tetramic acid ring via a Dieckmann-type reaction.

Hybrid PKS/NRPS have been found in gene clusters for several tetramic acid-containing natural products, such as tenellin, aspyridone A, equisetin, and cyclopiazonic acid.[10] All these PKS/NRPS contain a reductase (R) domain in the end of the enzyme, which is responsible for the cyclization of tetramic acid and the release of the hybrid PK/NRP chain via a Dieckmann-type condensation. In HSAF PKS/NRPS, the TE domain is the most likely candidate for this activity. In spite of the functional similarity, the R domain's substrate is a thioester attached to the PCP domain, whereas the TE domain's substrate is an oxoester attached to TE itself.[11] Another interesting question in HSAF biosynthesis is whether the C domain catalyzes the formation of both amide bonds or the TE domain is also involved. We are currently working on the enzymes to answer this question.

Clardy and co-workers[6] found that all HSAF-type gene clusters contain a central PKS/NRPS flanked by genes encoding a cascade of redox enzymes. In HSAF, we found that all four OX genes, in addition to the genes for PKS/NRPS and sterol desaturase/fatty acid hydroxylase, are required for HSAF biosynthesis. These genes could be involved in the formation of the 5,5,6-tricyclic system and the conversion of maltophilin to HSAF. Although the disruption of the ferredoxin reductase gene and the arginase gene did not eliminate HSAF production in the mutants, these genes may play a pathway-specific role (as a reducing partner for the redox enzymes or in the synthesis of ornithine from arginine via the urea cycle). The MFS transporter and the TonB-dependent outer membrane receptor could play a role in resistance and regulation.

The iterative use of a single-module PKS/NRPS is common in fungi but rare in bacteria.[12] Only a few bacterial modular PKSs have been reported to act iteratively.[13] Our present results show that HSAF PKS/NRPS represents one of the first two examples in which a single-module PKS assembles two separate polyketide chains that are linked together via two amide bonds on the same amino acid and are subsequently folded/cyclized into a complex scaffold, probably by a cascade of redox enzymes. These results open a new area of biosynthetic research to study hybrid PK/NRP products with a very unusual scaffold and interesting biological activities.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Details of experimental procedures, HSAF gene cluster annotation, gene disruption/deletion, chemical synthesis, and protein expression and activity assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Yu, F.; Zaleta-Rivera, K.; Zhu, X.; Huffman, J.; Millet, J. C.; Harris, S. D.; Yuen, G.; Li, X. C.; Du, L. *Antimicrob. Agents Chemother.* **2007**, *51*, 64–72.

(2) Li, S.; Du, L.; Yuen, G.; Harris, S. D. *Mol. Biol. Cell* **2006**, *17*, 1218–1227.

(3) Thevissen, K.; Francois, I. E.; Aerts, A. M.; Cammue, B. P. *Curr. Drug Targets* **2005**, *6*, 923–928.

(4) Schobert, R.; Schlenk, A. *Bioorg. Med. Chem.* **2008**, *16*, 4203–4221.

(5) Qian, G. L.; Hu, B. S.; Jiang, Y. H.; Liu, F. Q. *Agric. Sci. China* **2009**, *8*, 68–75.

(6) Blodgett, J. A. V.; Oh, D. C.; Cao, S.; Currier, C. R.; Kolter, R.; Clardy, J. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 11692–11697.

(7) Sanchez, C.; Du, L.; Edwards, D. J.; Toney, M. D.; Shen, B. *Chem. Biol.* **2001**, *8*, 725–738.

(8) Dent, W. H.; Erickson, W. R.; Fields, S. C.; Parker, M. H.; Tromiczak, E. G. *Org. Lett.* **2002**, *4*, 1249–1251.

(9) Page, P. C. B.; Hamzah, A. S.; Leach, D. C.; Allin, S. M.; Andrews, D. M.; Rassias, G. A. *Org. Lett.* **2003**, *5*, 353–355.

(10) (a) Halo, L. M.; Marshall, J. W.; Yakasai, A. A.; Song, Z.; Butts, C. P.; Crump, M. P.; Heneghan, M.; Bailey, A. M.; Simpson, T. J.; Lazarus, C. M.; Cox, R. J. *ChemBioChem* **2008**, *9*, 585–594. (b) Bergmann, S.; Schumann, J.; Scherlach, K.; Lange, C.; Brakhage, A. A.; Hertweck, C. *Nat. Chem. Biol.* **2007**, *3*, 213–217. (c) Sims, J. W.; Schmidt, E. W. *J. Am. Chem. Soc.* **2008**, *130*, 11149–11155. (d) Liu, X.; Walsh, C. T. *Biochemistry* **2009**, *48*, 8746–8757.

(11) Du, L.; Lou, L. *Nat. Prod. Rep.* **2010**, *27*, 255–278.

(12) Cox, R. J. *Org. Biomol. Chem.* **2007**, *5*, 2010–2026.

(13) (a) Shao, L.; Qu, X. D.; Jia, X. Y.; Zhao, Q. F.; Tian, Z. H.; Wang, M.; Tang, G. L.; Liu, W. *Biochem. Biophys. Res. Commun.* **2006**, *345*, 133–139. (b) Wenzel, S. C.; Gross, F.; Zhang, Y.; Fu, J.; Stewart, A. F.; Muller, R. *Chem. Biol.* **2005**, *12*, 349–356.