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Biosynthesis of the Polycyclic System in the Antifungal HSAF and Analogues from *Lysobacter enzymogenes***

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Abstract

The biocontrol agent *Lysobacter enzymogenes* produces polycyclic tetramate macrolactams (PoTeM) including the antifungal HSAF. To elucidate the biosynthesis of the cyclic systems, we identified eleven HSAF precursors/analogues with zero, one, two, or three rings, by heterologous expression of the HSAF gene cluster. Through a series of combinatorial gene expression and deletion, we showed that OX3 is the “gatekeeper” responsible for the first 5-membered ring formation from lysobacterene A, OX1 and OX2 are for the second ring formation but with

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Conflict of interest

The authors declare no conflict of interest.

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Experimental Section

Details of experimental procedures and spectroscopic data are included in the Supporting Information.

different selectivity, and OX4 is for the 6-membered ring formation. In vitro, we showed that OX4 is an NADPH-dependent enzyme catalyzing the reductive cyclization of 3-dehydroxy alteramide C to form 3-dehydroxy HSAF. Thus, the multiplicity of OX genes is the basis for the structural diversity of the HSAF family, which is the only characterized PoTeM that involves four redox enzymes in the formation of the cyclic system.

Graphical abstract

Biosynthesis of 0, 1, 2, and 3 Rings of the 5-5-6 Tricyclic System in the Antifungal HSAF and Analogues, as Revealed by Combinatorial Heterologous Expression of the Biosynthetic Genes and *in vitro* Enzyme assays.

Four OX genes that encode NADPH-dependent redox enzymes dictate the structural diversity of the antifungal HSAF family of polycyclic tetramate macrolactams from the Gram-negative gliding bacterium *Lysobacter enzymogenes*, a potent biocontrol agent and prolific producer of bioactive products.

Keywords

Polycyclic tetramate macrolactam; HSAF; *Lysobacter enzymogenes*; Antifungal natural products; Combinatorial biosynthesis

HSAF is a polycyclic tetramate macrolactam (PoTeM) isolated from the biocontrol agent *Lysobacter enzymogenes* (Figure 1).^[1] HSAF has a distinct chemical structure and potent biological activity, and its mode of action is also attractive. HSAF inhibited filamentous fungi through interfering with sphingolipid biosynthesis, which is distinct from existing antifungal drugs.^[1a] In *Candida albicans*, the antifungal activity of HSAF and alteramide B was through β -tubulin-binding that induced ROS (reactive oxygen species)-mediated apoptosis.^[2] In addition to HSAF, we have identified six analogues from *L. enzymogenes*, including 3-dehydroxy HSAF (**1**), alteramide A, alteramide B, 3-dehydroxy alteramide A, lysobacteramide A, and lysobacteramide B (Figure 1).^[1] These compounds share a common structural scaffold and differ mainly at the fused cyclic system. We previously showed that a polyene-tetramate-polyene is the precursor for the polycycles found in HSAF and analogues. However, the mechanism by which the polyene-tetramate-polyene precursor is transformed into the various cyclic patterns, including HSAF's 5-5-6 tricyclic system that is embedded with nine chiral centers, was not clear.

We have shown that the polyene-tetramate-polyene scaffold is assembled from two separate hexaketide chains and an ornithine residue, which is biosynthesized by a single-module hybrid polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS).^[1] The Gulder group and the Zhang group studied the biosynthesis of ikarugamycin, a *Streptomyces*-

derived PoTeM containing a 5-6-5 tricyclic system (Figure 1).^[3] The formation of the inner 5-membered ring was shown to be catalyzed by a redox enzyme (IkaC), and evidence also showed that the formation of the outer 5-6 two rings was catalyzed by a single redox enzyme, IkaB. The Zhang group also showed that three redox enzyme genes, *ptmB2*, *ptmB1*, and *ptmC*, are responsible for the 5-5-6 tricycle formation of pactamides in *Streptomyces pactum* SCSIO 02999.^[4]

Unlike the biosynthesis of ikarugamycin and pactamides from *Streptomyces*, the biosynthesis of HSAF and analogues from *Lysobacter* requires a gene cluster containing four genes coding for redox enzymes, OX1-4, in addition to the genes for PKS-NRPS and 3-hydroxylation and ornithine metabolism.^[1] Coincidentally, *L. enzymogenes* produces a family of HSAF analogues containing a variety of cyclic systems, instead of a single polycycle as found in ikarugamycin and pactamides. Our previous study indicated that these four OX genes are involved in the cyclic system formation in HSAF.^[1] However, the exact role of each of the OX genes in formation of the multiple HSAF analogues remains to be determined. Moreover, although the polyene-tetramate-polyene is the common precursor for all HSAF analogues, the order of cyclization steps for this precursor to form the various cyclic systems was unclear. Because the polycyclic system is a hallmark of all PoTeM compounds that are emerging as a new family of natural products with distinct structural features and diverse biological activities,^[5] the characterization of HSAF OX1-4 is important in understanding the biosynthetic mechanism of this growing group of natural products.

To understand the function of the four redox genes, we generated a “clean” host, strain SR111, by deleting seven native PKS clusters and one native NRPS gene cluster in *Streptomyces* sp. LZ35 (Supporting Information). When the expression construct pIB-HSAF1 containing the HSAF biosynthetic gene cluster (less the genes for 3-hydroxylation) under the control of *ermE** promoter^[6] was introduced in SR111 (Table S1 and Figure S1), we found the resulted strain (SR111-HSAF1) produced ten compounds (**1-4** and **a-f**) that were not present in the host strain SR111 (Figures 2 and S2). Two of the compounds were 3-deOH HSAF (**1**) and 3-deOH alteramide B (**2**) (Figure 1), which are the “final” products of the biosynthetic pathway in the heterologous host because of the lack of the genes for 3-hydroxylation. Compound **3** is identical to the polyene-tetramate-polyene precursor previously generated from *Streptomyces* sp. strain SR107 that contains only the PKS-NRPS gene; this precursor is named lysobacterene A (Figure 1).^[1e] Interestingly, the most abundant compound (**4**) produced by strain SR111-HSAF1 does not appear to belong to any of the known HSAF analogues.

To determine the structure of this new product, we prepared ~10 mg pure compound **4** from 20 liters of solid culture. Spectroscopic analyses established **4** as a new member of the HSAF family (Supporting Information), which contains only one ring of the cyclic system, but retains the characteristic tetramate as well as a triene in both of the hexaketide chains that are originally formed by the PKS. Compound **4** is named lysobacterene B (Figure 1). The identification of lysobacterene B suggests that the outer most 5-membered ring is formed before the inner 5- and 6-membered rings in HSAF.

In addition to **1-4**, strain SR111-HSAF1 produced six other new compounds (**a**, **b**, **c**, **d**, **e**, and **f**) that were not present in the host. The UV-Vis spectrum of compounds **a**, **b**, and **c** is similar to that of lysobacterene A (**3**), the polyene-tetramate-polyene precursor that has no ring formed in polycyclic position (Figures 1 and S10). These UV-Vis spectra are clearly distinct from that of the compounds containing one, two, or three rings at the polycyclic position (**1**, **2** or **4**). The mass of compound **c** is identical to that of **3** (m/z 475.2588, for $[M + H]^+$), whereas compounds **a** and **b** have the same mass (m/z 479.2177, for $[M + H]^+$), which is four mass units bigger than **3** (Figure S11). Together, the data suggest that compound **c** is likely a polyene tetramate isomer of **3** and that compounds **a** may be an isomer of **b**, both with two double bonds reduced in the polyenes. Compounds **d**, **e**, and **f** have the same mass as lysobacterene B (**4**, m/z 477.2749, for $[M + H]^+$) (Figures S12-13). Besides, the UV-Vis spectrum of compound **e** is similar to that of compound **4**, suggesting that **e** may be an isomer of **4** with one ring formed in polycyclic position (Figures 1 and S12-13). For compounds **d** and **f**, the UV-Vis spectra did not show a similarity to that of the other compounds, although they have the same mass as **4**.

With the identification of compounds containing zero, one, two, or three rings in the polycyclic position of HSAF, we figured that some of them might be biosynthetic intermediates and the structural information could be used to guide the functional elucidation of the *OX* genes in the polycycle formation. Subsequently, we took an *in vivo* combinatorial approach, in which each of the *OX* genes was removed from strain SR111-HSAF1 by in-frame deletion and the products in the resulting strains were examined.

As a control, we first examined the products in strain SR111-HSAF2 that contained only the PKS-NRPS gene without any of the *OX* genes (Figure 2). As expected, this strain produced the zero ring-containing polyene-tetramate-polyene compounds (**3**, **a**, **b**), which is consistent with previously reported results.^[1e, 3c] When *OX1* gene was removed from strain SR111-HSAF1, the resultant strain (SR111-HSAF1 *OX1*) produced the one ring-containing compounds (**4** and **e**) as the main product and the zero ring-containing lysobacterene A (**3**) and the three ring-containing 3-dehydroxy HSAF (**1**) as minor products (Figures 2 and S2). The result suggests that *OX1* is not necessary for formation of the 3-ring system in HSAF. No analogue of **2** was observed in the *OX1* mutant, suggesting that *OX1* may be mainly involved in the two ring-containing shunt products such as 3-deOH alteramide A/B.

When *OX2* gene was deleted, the resultant strain (SR111-HSAF1 *OX2*) produced the one ring-containing **4** and the two ring-containing shunt product **2** (see Figure S2, detected at 265 nm for more prominent absorption), as well as zero ring compounds **3**, **a**, **b**, and **c**. The result suggests that *OX2* is not required for formation of the first ring (**4**) and the second ring of 3-deOH alteramide B (**2**), but it is required for formation of the complete three ring system in HSAF. When *OX3* gene was deleted, the products in the resultant strain (SR111-HSAF1 *OX3*) was the same as strain SR111-HSAF2, which contained only the PKS-NRPS gene without any of the *OX* genes (Figures 2 and S2). This is strong evidence supporting that *OX3* is responsible for the first-ring formation. Without *OX3*, none of the rings could be formed. Finally, when *OX4* gene was deleted, the resultant strain (SR111-HSAF1 *OX4*) produced all compounds of the parent strain SR111-HSAF1, except the three ring-

containing **1** (Figures 2 and S2). In addition, a new compound (**5**) was accumulated in this strain (Figure S2, HPLC at 265 nm for more prominent absorption). Compound **5** exhibited a similar UV-Vis spectrum to compound **2** and the same mass as **2** (m/z 495.2855, for $[M + H]^+$) (Figures S10-11), indicating compound **5** may be a new 3-deOH alteramide isomer.

Because it was observed only in *OX4* mutant, compound **5** may be the immediate precursor for *OX4* enzyme for the 6-membered ring formation. To obtain evidence, we need to determine the chemical structure of **5**. However, we were not able to obtain a sufficient amount of **5** from strain SR111-HSAF1 *OX4* due to a very low yield. To solve the problem, we generated three new mutant strains from *L. enzymogenes* strain C3 (Table S2, Figure S3). We figured if we blocked both the shunt products (by *OX1* deletion) and the 6-membered ring (by *OX4* deletion), the double mutant might be able to produce a higher yield of compound **5**. Indeed, both the single mutant C3 *OX1* and double mutant C3 *OX14* produced a significantly higher amount of **5** than that by SR111-HSAF1 *OX4* (Figure S3). In addition, C3 *OX1* produced 3-dehydroxy HSAF (**1**), as well as HSAF (3-OH form of **1**) and the 3-OH form of **5**, since the 3-hydroxylation genes were present in the *L. enzymogenes* C3-derived strains. C3 *OX14* produced **5** and its 3-OH form, but not **1** or HSAF. This is expected since this double mutant lacks the third-ring-forming gene (*OX4*). As a control, another double mutant C3 *OX12* did not produce any of the two-ring or three-ring containing products, due to the lack of the genes for the second-ring formation, but produced the first-ring-containing compounds (such as **4** and **e**) (Figure S3). In the end, we prepared 7 mg compound **5** from 3 liters of solid culture of C3 *OX14*. Spectroscopic analyses (Table S3, Figures S8, S21-S26) revealed that **5** is an analogue of alteramide A, [1f, 7] and differs from alteramide A in the absence of an hydroxyl group at C3. The configuration of double bonds was established to be 9*E*, 10*E*, 21*E*, and 23*E* as in alteramide A. The relative configuration of the 5/5-bicyclic unit in **5** is almost identical to that of alteramide A, except for C-12 (Figures S7-S8). Thus, **5** is a new compound and named 3-deOH alteramide C (Figure 1).

The sequences of *OX1*, *OX2*, and *OX3* are homologous to each other and encode NAD(P)H-dependent flavin oxidoreductases (Figure S14).^[1c] *OX4* is distinct from the other three *OX* enzymes and similar to NAD(P)H-dependent alcohol dehydrogenases/zinc-binding proteins. To further understand the role of these homologous *OX* genes, we generated another series of SR111 strains that hosted constructs with double or triple deletion of the *OX* genes (Figure S4, Table S4). The *OX1* and *OX2* double deletion strain (SR111-HSAF1 *OX12*) was designed to mimic the ikarugamycin biosynthetic gene cluster (Figure S14). This strain produced compounds with one ring (**4**, **d**, **e**, **f**) and zero ring (**3**, **a**, **b**, **c**) (Figure S4). The result is consistent with that from C3 *OX12* and supports that *OX3* is responsible for the first ring (as in **4**), but unlike ikarugamycin, *OX3*-*OX4* alone is not sufficient to convert **4** to compounds with two-ring (as in **2** or **5**) or three-ring (as in **1**). This is a clear difference from the ikarugamycin enzymes.^[3] We then generated another two double deletion strains, SR111-HSAF1 *OX14* and SR111-HSAF1 *OX24*, and two triple *OX* deletion strains, SR111-HSAF1 *OX124* and SR111-HSAF1 *OX234* (Figure S4). The metabolite profile of the mutants is summarized in Table S4. As expected, SR111-HSAF1 *OX14* produced the two-ring compound **5**, in addition to a product profile almost identical to SR111-

HSAF1 OX12. Notably, SR111-HSAF1 OX24 produced the expected two ring-containing compound **2**, in addition to zero ring compounds. The difference in these two strains, together with the single OX deletion results described above, clearly indicates that OX1 is involved in the second ring formation of the shunt products (such as 3-deOH alteramide B), but not the second ring of 3-deOH-HSAF (**1**) or lysobacteramide A,^[1f] which have different stereochemistry at the ring system. The product profile of SR111-HSAF1 OX124 was also close to that of SR111-HSAF1 OX12, only producing zero-ring and one-ring compounds (Figure S4). The product profile of SR111-HSAF1 OX234 was almost identical to that of strain SR111-HSAF2. Together, the results not only reiterate the role of OX3 but also supports that OX1 alone (without OX3) could not make any ring formation.

The isolation of products with a variety of cyclic systems provides a great opportunity for a direct *in vitro* test of the activity and specificity of the OX enzymes. To do this, we attempted to produce these enzymes by expressing the OX genes in heterologous hosts. We were able to produce all four proteins; however, OX1, OX2 and OX3 were insoluble after exhaustive attempts. OX4 was partly soluble (Figure S5), and we used the purified OX4 to test its activity using the two-ring compounds (**5** and **2**) as substrates. In the presence of NAD(P)H, OX4 was able to convert **5** to **1**, the penultimate product of the HSAF pathway and with the complete 5-5-6 tricyclic system (Figure 3). The result clearly demonstrated the function of OX4 as the enzyme for 6-membered ring formation in HSAF biosynthesis. When **2** was used as substrate, a new compound (**6**) was produced in the presence of OX4 and NADPH (NADH less effective) (Figure 3). To determine the structure of this new product, we carried out a preparative enzyme reaction (30 ml) using 8 mg **2** (prepared from 7 liters of solid culture of strain SR111-HSAF1), 30 mg purified OX4 enzyme, and 20 mg NADPH. The product **6** (4.3 mg) was subject to HRMS, 1D- and 2D-NMR analyses (Figures S9, S27-S31). The spectroscopic data of **6** was similar to those of 3-deOH-alteramide B, except the absence of C-21/22 and C-23/24 double bonds and the presence of the C22/23 double bond (Figure 1). We named this new compound 3-deOH alteramide D (**6**). The results show that **2** does not serve as a precursor for HSAF and OX4 only reduces one of the diene systems in **2**, instead of forming the 6-membered ring.

Together, the data show that OX3 is the “gatekeeper” that converts **3** to **4** and other putative one ring compounds (**7** and **8**), by forming the first 5-membered ring (Figure 4). This ring can have a variety of stereochemistry, apparently due to a relaxed selectivity of OX3 enzyme during the cyclization reaction. This flexibility of OX3 leads to 3-deOH alteramide B (**2**) from **7** or 3-deOH alteramide A (**10**) from **8**, by the action of OX1. It also leads to 3-deOH HSAF (**1**) from **4** via **5** and **11**, by the combined action of OX2 and OX4. It is only when the first ring has the “correct” stereochemistry (as in **4**) that OX2 and OX4 would process it to form the second ring (as in **5**) and the third ring (as in **11**, which is tautomerized to **1**) (Figure 4). Both *in vivo* and *in vitro* studies establish that OX4 catalyzes the reductive cyclization of the third ring in the tricyclic system, which is homologous to the function of IkaC for ikarugamycin and PtmC for pactamides.^[3-4] *In vitro*, OX4 is also able to reduce a double bond in the two-ring compound **2** that does not have the “correct” stereochemistry in the rings. This would lead to the putative compound **9**, which is tautomerized to the new compound **6** (Figure 4). Among the three homologous redox enzymes, OX3 and OX2

together are functionally equivalent of IkaB for ikarugamycin and PtmB2 and PtmB1 for pactamides in forming the first two rings, but the cyclic systems formed by OX3 and OX2 are different from that by IkaB or PtmB2/PtmB, particularly in the configurations and ring sizes. OX1 is distinct from any of the above and leads to different ring systems. OX3 and OX1 together make the alteramide A/B shunt products. Thus, it is the multiple OX enzymes that lead to the structural diversity of HSAF family.

There is a large number of PoTeM gene clusters in the databases that contain four *OX* genes as seen in HSAF gene cluster (Figure S14). So far, several PoTeM clusters have been linked to their products (HSAF, ikarugamycin, frontalamides, compound d, and pactamides, capsimycins, and clifednamides).^[1, 3–5, 8] In this work, we successfully identified two new HSAF biosynthetic intermediates, **4** and **5**, established the pathways leading to the tricyclic analogues and bicyclic analogues, and demonstrated the importance of stereochemistry in determining the pathways. HSAF represents the first biosynthetically characterized PoTeM that involves four redox enzymes in the tricycle formation. Besides, this is the only PoTeM family that has been investigated in a source other than *Streptomyces*. The understanding of the function of the multiple *OX* genes in HSAF should facilitate future efforts in generating new polycyclic system with various configurations, sizes, and numbers of the rings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. a Li S, Du L, Yuen G, Harris SD. *Mol Biol Cell*. 2006; 17:1218–1227. [PubMed: 16394102] b Yu F, Zaleta-Rivera K, Zhu X, Huffman J, Millet JC, Harris SD, Yuen G, Li XC, Du L. *Antimicrob Agents Chemother*. 2007; 51:64–72. [PubMed: 17074795] c Lou L, Qian G, Xie Y, Hang J, Chen H, Zaleta-Rivera K, Li Y, Shen Y, Dussault PH, Liu F, Du L. *J Am Chem Soc*. 2011; 133:643–645. [PubMed: 21171605] d Lou L, Chen H, Cerny RL, Li Y, Shen Y, Du L. *Biochemistry*. 2012; 51:4–6. [PubMed: 22182183] e Li Y, Chen H, Ding Y, Xie Y, Wang H, Cerny RL, Shen Y, Du L. *Angew Chem Int Ed Engl*. 2014; 53:7524–7530. [PubMed: 24890524] f Xu L, Wu P, Wright SJ, Du L, Wei X. *J Nat Prod*. 2015; 78:1841–1847. [PubMed: 26200218] g Li Y, Huffman J, Li Y, Du L, Shen Y. *MedChemComm*. 2012; 9:982–986.
2. a Ding Y, Li Z, Li Y, Lu C, Wang H, Shen Y, Du L. *RSC Adv*. 2016; 6:30895–30904. [PubMed: 27594989] b Ding Y, Li Y, Li Z, Zhang J, Lu C, Wang H, Shen Y, Du L. *Biochim Biophys Acta*. 2016; 1860:2097–2106. [PubMed: 27373684]
3. a Greunke C, Glöckle A, Antosch J, Gulder TAM. *Angew Chem Int Ed Engl*. 2017; 56:4351–4355. [PubMed: 28128495] b Antosch J, Schaefer F, Gulder TA. *Angew Chem Int Ed Engl*. 2014; 53:3011–3014. [PubMed: 24519911] c Zhang G, Zhang W, Zhang Q, Shi T, Ma L, Zhu Y, Li S, Zhang H, Zhao YL, Shi R, Zhang C. *Angew Chem Int Ed Engl*. 2014; 53:4840–4844. [PubMed: 24706593]
4. Saha S, Zhang W, Zhang G, Zhu Y, Chen Y, Liu W, Yuan C, Zhang Q, Zhang H, Zhang L, Zhang W, Zhang C. *Chem Sci*. 2017; 8:1607–1612. [PubMed: 28451290]
5. a Blodgett JA, Oh DC, Cao S, Currie CR, Kolter R, Clardy J. *Proc Natl Acad Sci U S A*. 2010; 107:11692–11697. [PubMed: 20547882] b Cao S, Blodgett JA, Clardy J. *Org Lett*. 2010; 12:4652–4654. [PubMed: 20843016]
6. a Bibb MJ, Janssen GR, Ward JM. *Gene*. 1985; 38:215–226. [PubMed: 2998943] b Wilkinson CJ, Hughes-Thomas ZA, Martin CJ, Bohm I, Mironenko T, Deacon M, Wheatcroft M, Wirtz G, Staunton J, Leadlay PF. *J Mol Microbiol Biotechnol*. 2002; 4:417–426. [PubMed: 12125822] c Datsenko KA, Wanner BL. *Proc Natl Acad Sci U S A*. 2000; 97:6640–6645. [PubMed: 10829079]

7. Hideyuki S, Myung Ae B, Kazunaga Y, Takuma S, Junichi K. J Org Chem. 1992; 57:4317–4320.
8. a Luo Y, Huang H, Liang J, Wang M, Lu L, Shao Z, Cobb RE, Zhao H. Nat Commun. 2013; 4:2894. [PubMed: 24305602] b Yu HL, Jiang SH, Bu XL, Wang JH, Weng JY, Yang XM, He KY, Zhang ZG, Ao P, Xu J, Xu MJ. Sci Rep. 2017; 7:40689. [PubMed: 28098172] c Qi Y, Ding E, Blodgett JAV. ACS Synth Biol. 2018; 7:357–362. [PubMed: 29249153]

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Figure 1. Chemical structure of HSAF analogues from *Lysobacter*, including the newly identified analogues (4, 5, and 6) described in this study. Ikarugamycin is included as a representative PoTeM from *Streptomyces*.

Figure 2.

Expression of pIB-HSAF constructs containing various portions of the HSAF biosynthetic gene cluster in strain SR111. HPLC showed the metabolite profile of the control strain (SR111), the gene cluster-containing strain (SR111-HSAF1, less genes for 3-hydroxylation), PKS-NRPS only strain (SR111-HSAF2), and strains with one of the four *OX* genes being deleted from strain SR111. The eleven metabolites (**1-5** and **a-f**) that were not present in SR111 are indicated. The organization of the gene cluster is also shown with a pair of primers, P3 and P5, used for confirming the various *OX*s mutants.

Figure 3.
In vitro activity assays of OX4 enzyme using compound **5** and compound **2** as substrate.

Figure 4.
A proposed biosynthesis for the tricyclic system in HSAF and analogues.