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Construction of a hybrid gene cluster to reveal coupled ring formation–hydroxylation in the biosynthesis of HSAF and analogues from *Lysobacter enzymogenes*^{†‡}

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HSAF and analogues are polycyclic tetramate macrolactams (PoTeMs) isolated from *Lysobacter enzymogenes*. Due to their antifungal activity, distinct chemical structure and new mode of action, PoTeMs have been the subject of several studies for their biosynthetic mechanism. However, polycycle formation is still not well understood. HSAF and several analogues (alteramides) carry a C20-hydroxyl, which is absent in most known PoTeMs such as combamides and pactamides. Previous studies indicated that two genes encoding NAD(P)H-dependent flavin enzymes (OX1/OX2) are responsible for the second five-membered ring formation in HSAF and alteramides. Intriguingly, the products of OX1/OX2 always carry the C20-OH. To test the hypothesis that the formation of the second five-membered ring is coupled with the C20-hydroxylation, we constructed a hybrid PoTeM gene cluster through removing OX1/OX2 in the HSAF cluster and functional complementation by CbmB, which also catalyzes the second five-membered ring formation in combamides but lacking the C20-OH. Two heterologous hosts carrying the hybrid cluster generated the same three PoTeMs, including lysobacterene B (**3**, the one-ring precursor of HSAF) and combamide D (**4**, a two-ring product lacking the C20-OH). The third product was not related to either of the clusters and was identified to be pactamide A (**5**) using mass spectrometry, 1D- and 2D-NMR, and ECD spectroscopy. The results demonstrate the feasibility of producing new PoTeM compounds through combinatorial biosynthesis. More importantly, this study provides the first experimental evidence to support that the second ring formation is coupled with the C20-hydroxylation in the biosynthesis of HSAF and analogues.

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In the effort to search for new antifungal compounds from nature, we previously isolated HSAF and analogues from *Lysobacter enzymogenes*.^{1–3} HSAF exhibits broad spectrum antifungal activity and its structure and mode of action are distinct from those of existing antifungal drugs and fungicides.^{4–6} HSAF belongs to the family of polycyclic tetramate macrolactams (PoTeMs) that have been isolated from diverse sources.⁷ These include ikarugamycin from *Streptomyces phaeochromogenes* sub-sp. *ikaruganensis*,⁸ frontalamides from *Streptomyces* sp. SPB78,⁷ combamides

from *Streptomyces* sp. S10,⁹ and from the marine-derived *Streptomyces pactum* SCSIO 02999 (ref. 10) (Fig. 1). The cyclic systems in the PoTeMs can vary from 5/5, 5/5/6, 5/4/6, 5/6/5, to 5/5/5/8 and are usually embedded with multiple chiral centers. Even within the same type of cyclic system, some PoTeMs carry a C20-hydroxyl group (such as HSAF and alteramides), while others do not carry this modification (such as combamides and pactamides) (Fig. 1).

The 5/5/6 tricycle of HSAF and 3-deOH HSAF (**1**) is embedded with 9 chiral centers, and four redox enzymes (OX1–4) are involved in the biosynthesis of the cyclic system of HSAF and analogues including alteramides A–D (Fig. 1).^{11–13} OX1 and OX2 are responsible for the second five-membered ring formation. Depending on the one-ring precursor's stereochemistry, OX1 forms two-ring products, 3-deOH alteramide B (**2**), 3-deOH alteramide A, or 3-deOH alteramide D, whereas OX2 forms another type of two-ring product, 3-deOH alteramide C, which is the precursor that has the “correct” stereochemistry for OX4 to form the six-membered ring in 3-deOH HSAF (**1**). Finally, upon the C3-hydroxylation by the

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Fig. 1 Chemical structure of selected polycyclic tetramate macrolactams (PoTeMs). The C20-hydroxyl group is highlighted to indicate the structural feature shared by HSAF and alteramides from *Lysobacter enzymogenes*.

fatty acid hydroxylase (sterol desaturase),¹⁴ the 3-deOH compounds are converted to the corresponding HSAF and analogues (Fig. 1).

Several previous studies have revealed that OX homologs are present in other PoTeMs.^{10,15–18} Different PoTeM gene clusters contain a different number of OX homologs, varying from 2 homologs (ikarugamycin, capsimycins, clifednamides, and combamides), 3 homologs (pactamides, “compounds a–d”, and frontalamides), to 4 OX genes (HSAF). Almost uniquely, among the PoTeM family, HSAF and alteramides carry a C20-hydroxyl group that is absent in most PoTeMs (except “compounds a and c” of the SGR810-815 gene cluster from *S. griseus*,¹⁸ and frontamides with a C20-keto group⁷). Intriguingly, the products of OX1/OX2 always carry this C20-hydroxyl,¹³ and we hypothesized that the second five-membered ring formation in HSAF and alteramides is coupled with the C20-hydroxylation.^{3,13} Here, we report experimental evidence to support this unique coupling between polyene cyclization and hydroxylation during the second ring formation in HSAF and analogues.

Results and discussion

To understand the C-20 hydroxylation during the 5/5/6 ring system formation in the HSAF and analogues, we constructed a hybrid PoTeM cluster using genes from both the HSAF cluster

and the combamide cluster (see the ESI†). The expression construct, pSET5035-HSAF_{hybrid}, contains the genes for PKS-NRPS, OX3, and OX4 from the HSAF cluster and the *cbmB* gene from the combamide cluster (Fig. 2). Our previous results have shown that OX2 is responsible for the second five-membered ring in 3-deOH HSAF and 3-deOH alteramide C, and OX1 for the second five-membered ring in 3-deOH alteramides A/B/D (Fig. 1).¹³ *CbmB*, a homologous enzyme of OX1/OX2, is also responsible for the second five-membered ring in combamides.⁹ The genes for PKS-NRPS, OX3 and OX4 were placed under the control of promoter *kasOp**, and the *cbmB* gene under the control of promoter *ermEp** (Fig. S1†), using a similar strategy as reported.⁹

The hybrid construct was introduced into two strains of *Streptomyces*, SR111 and S001, through conjugation with an intermediate *E. coli* strain that carried the expression construct. This resulted in two engineered strains, SR111-HSAF_{hybrid} and S001-HSAF_{hybrid}. The metabolites from the strains, together with SR111, S001 and SR111-HSAF1 (containing the whole HSAF cluster except the sterol desaturase gene for C3-OH)¹³ as controls, were analyzed by HPLC and LC-HRMS (Fig. 2). As expected, strain SR111-HSAF1 produced compounds 3-deOH HSAF (**1**), 3-deOH alteramide C (**2**), lysobacterene B (**3**), and other uncharacterized lysobacterene analogues, whereas the host alone, SR111 or S001, did not produce any of the PoTeM

Fig. 2 (A) Scheme for constructing the expression construct, HSAF_{hybrid}, that contains a hybrid PoTeM gene cluster. (B) HPLC analysis of the metabolites from two strains of *Streptomyces*, SR111 and S001, that contain the expression of construct HSAF_{hybrid}. Strain SR111, the host alone as the control; strain SR111-HSAF1, containing the HSAF gene cluster except the SD gene (for C3-hydroxylation); strain S001, the host alone as the control. Lysobacterene B and combamide D were included as reference compounds. *Uncharacterized 3 analogues.

compounds. The engineered strains, SR111-HSAF_{hybrid} and S001-HSAF_{hybrid}, exhibited a similar profile of metabolites in the PoTeM region by HPLC (Fig. 2). One compound (3) co-migrated with standard lysobacterene B. Mass spectrometry analysis of this compound gave a m/z of 477.2771 for $[M + H]^+$, which is identical to lysobacterene B; the UV-vis spectrum of this compound was also almost identical to that of lysobacterene B (Fig. S2†). Since the first five-membered ring-containing product is known to result from the action of PKS-NRPS and OX3, the result confirmed that the genes from the HSAF cluster in the engineered strains were functional.

In addition to 3, the engineered strains produced two compounds (4 and 5) that were absent in SR111-HSAF1 or

the hosts (SR111, S001) (Fig. 2). Compound 4 co-migrated with standard combamide D, a 5/5 two-ring-containing PoTeM that we recently identified.⁹ Mass spectrometry analysis of 4 gave a m/z of 479.2923 for $[M + H]^+$, which is identical to that of combamide D; the UV-vis spectrum of 4 was consistent with that of combamide D (Fig. S2†). The stereochemistry of the first five-membered ring in 4 was different from that in compounds 1–3. This result is also consistent with the observation that OX3 exhibits relaxed selectivity during the first ring formation.¹³ The result shows that CbmB in this engineered PoTeM cluster is functional. CbmB is not only able to form the second five-membered ring, but also to form it in the “correct” stereochemistry. More importantly, because the formation of the second ring in 4 by this engineered gene cluster did not lead to a C20–OH, in contrast to the OX1/OX2-catalyzed second ring formation in HSAF and alteramides, the result implies that OX1/OX2 is responsible for the coupled ring formation–hydroxylation.

Compound 5 was the major product in both engineered strains and is different from any of the known compounds we previously identified (Fig. 2). To determine the structure, we carried out the preparative fermentation (10 liters) of strain S001-HSAF_{hybrid} and isolated ~15 mg 5 (ESI†). Compound 5 gave a m/z of 481.3086 (calculated to be 481.3066) for $[M + H]^+$ in the high resolution ESIMS spectra and a molecular formula of C₂₉H₄₀N₂O₄ (Fig. S2†). Its UV-vis result was different from that for 3 or 4, suggesting that 5 is unlikely to be a one- or two-ring-containing PoTeM (Fig. S2†). The 1D- and 2D-NMR data of 5 (Table S2, Fig. S3, S7–S12†) establish the structure, which is identical to the 5/5/6 tricyclic pactamide A that was previously isolated from the marine-derived *Streptomyces pactum* SCSIO 02999.¹⁰ Moreover, the ECD spectrum of 5 was also consistent with that of pactamide A, indicating that the absolute configuration of 5 is the same as that of pactamide A (Fig. 1 and S3†). The production of 5 in the engineered strain confirmed that CbmB from the combamide gene cluster is able to form the second five-membered ring from the one-ring precursor, which would be synthesized by HSAF PKS/NRPS (for the polyene–ornithine–polyene scaffold, lysobacterene A) and OX3 (for the first five-membered ring), with the expected stereochemistry as seen in the original product (4). After the second ring formation by CbmB, OX4 in the engineered strains is able to form the six-membered ring of 5, with the same stereochemistry as 3-deOH HSAF (1). It also shows that OX4 can take the precursor without C20–OH as the substrate. Three heterologous redox enzymes, OX3, CbmB, and OX4, together are able to use the polyene precursor (lysobacterene B) as the substrate and to synthesize a PoTeM that is not a native product of either of the original gene clusters. The results also confirm that C20–OH is not formed without OX1/OX2. Together, the data support coupled ring formation–hydroxylation in the biosynthesis of HSAF and alteramides.

OX1/OX2 and CbmB are NAD(P)H-dependent flavin oxidoreductases responsible for the second five-membered ring

formation in several PoTeMs. It is intriguing that OX1/OX2 catalyzes coupled ring formation–hydroxylation, whereas the homologous CbmB catalyzes only ring formation. To find out the difference, we performed a phylogenetic analysis of the NAD(P)H-dependent flavin oxidoreductases (Fig. S4†). Coincidentally, OX1/OX2 for HSAF and alteramides,¹³ SGR–OX1 for “compounds a–c”,¹⁸ and OX1 for frontamides⁷ fall in the same clade. All the PoTeM compounds carry a C20–OH, and we thus named this group of oxidoreductases “20-OH clade”. There is a large number of homologs of this clade of PoTeM enzymes in databases, and we predict the enzymes to catalyze coupled ring formation–hydroxylation during the biosynthesis of these uncharacterized PoTeM compounds. In contrast, CbmB for combamides⁹ and PtmB1 for pactamides¹⁰ fall in a distinct clade (Fig. S4†). These PoTeM compounds do not carry a C20–OH, and we thus named this group of oxidoreductases “20-deOH clade” and predict the homologous enzymes in databases to catalyze only ring formation during the biosynthesis of PoTeMs.

Inspired by the sequence analysis, we carried out a series of point-mutagenesis processes to test if we could convert HSAF OX1 from the “20-OH clade” to the “20-deOH clade”. Multiple sequence alignments of the amino acid sequences of the enzymes in both clades revealed residues that are highly conserved in the “20-OH clade”, but not in the “20-deOH clade” (Fig. S5†). For example, all the “20-OH clade” enzymes contain an arginine at position 218, while all the “20-deOH clade” enzymes contain a proline at this position. In addition, lysine-116, proline-225, glycine-337, and serine-518 that are conserved in the “20-OH clade” enzymes correspond to threonine-116, histidine-225, lysine-337, and alanine-518 in CbmB of the “20-deOH clade”. Consequently, we generated two mutated OX1 genes, OX1-R218P and OX1-K116T/P225H/G337K/S518A, using the PCR coupled Gibson assembly strategy (Fig. S6†). Each of the point-mutated OX1 genes (under the control of *ermEp*^{*}) was recombined with the HSAF PKS–NRPS gene and OX3–OX4 (under the control of *kasOp*^{*}) (Fig. 3). The two constructs were transformed individually into strain S001 to produce the engineered strain S001-MT1 and S001-MT2. HPLC analysis showed that the control strain S001-HSAF_{hybrid} produced compounds 3–5 as expected. However, strains S001-MT1 and S001-MT2 only produced the one-ring-containing 3 and an analogue, but not the two- (4) or three-ring-containing (5) compounds (Fig. 3). The results suggest that the point-mutations in OX1 not only led to the absence of the C20-hydroxylation, but also the absence of the ring formation. Further studies are needed to understand the structural basis for the coupled ring formation and hydroxylation in OX1/OX2.

In conclusion, we have successfully constructed a hybrid PoTeM gene cluster using genes originating from two very different sources, the Gram-negative bacterium *Lysobacter enzymogenes* and the Gram-positive bacterium *Streptomyces* sp. S10. The hybrid gene cluster was introduced into heterologous *Streptomyces* hosts (strain SR111 and strain

Fig. 3 (A) Scheme for constructing an engineered HSAF gene cluster with a point-mutated OX1. (B) HPLC analysis of the metabolites from two strains of S001 that contain the OX1-mutated HSAF gene cluster. S001-HSAF_{hybrid}, strain S001 containing the functional hybrid PoTeM cluster as shown in Fig. 2, as the control; S001-MT1, strain S001 containing engineered HSAF gene cluster with one point-mutation (R218P) in OX1; S001-MT2, strain S001 containing engineered HSAF gene cluster with four point-mutations (K116T, P225H, G337K, and S518A) in OX1. *Uncharacterized 3 analogues.

S001) to generate two engineered strains. The isolation and analysis of the metabolites from the engineered strains showed that the hybrid gene cluster was functional in the new hosts. At least three PoTeMs were produced in the new hosts. The products included those that were known to be a HSAF precursor (lysobacterene B, 3) in *L. enzymogenes* and a product in the combamide pathway in *Streptomyces* sp. S10 (combamide D, 4). A third product (pactamide A, 5) was not known to be the product of either the HSAF pathway or the combamide pathway, but a PoTeM previously isolated from the marine-derived *Streptomyces pactum* SCSIO 02999.¹⁰ The results demonstrate the feasibility of producing new PoTeMs through combinatorial engineering of PoTeM biosynthetic genes from different clusters. Because there is a large number of PoTeM gene clusters in databases that remain to be explored,^{7,13} our work should encourage more researchers to exploit this rich source of bioactive natural products.

The mechanism for the polycycle formation in PoTeM biosynthesis is still not fully understood, despite the effort in the past decades. One of the most intriguing aspects in the polycycle formation is the possible coupling between the ring formation and hydroxylation, as proposed in HSAF biosynthesis.^{3,13} The NADPH hydride-initiated reductive cyclization could be coupled with the hydroxylation by FAD-OOH, resulting in the formation of the five-membered ring from two separate polyene chains. The *in vitro* demonstration of this coupling using purified enzymes has not been achieved due to the challenges in obtaining NAD(P)H-dependent flavin

Fig. 4 The biosynthetic steps during polycycle formation in HSAF and analogues, which are catalyzed by four redox enzymes, OX1–OX4. Combamide D is shown to illustrate the difference between CbmB for combamides and OX1/OX2 for HSAF and analogues during the second five-membered ring formation.

enzymes. In this work, we used an *in vivo* approach, through a careful design of a hybrid PoTeM gene cluster that contains genes for assembling a polyene–ornithine–polyene backbone and for cyclizing polyenes into desired cyclic systems. The data show that the functional replacement of OX1/OX2 by CbmB resulted in PoTeMs that lack the C20–OH group, and this result was demonstrated in two different heterologous hosts. To our knowledge, this is the first experimental evidence to support that the second ring formation is coupled with the C20-hydroxylation in the biosynthesis of HSAF and analogues (Fig. 4). We tried to convert the coupling enzyme OX1 to a non-coupling enzyme through point-mutagenesis of amino acids that are highly conserved in the “C20–OH clade” enzymes, but not in the “C20–deOH clade”. However, the resulting strains failed to produce PoTeMs with multiple rings. This suggests that the mechanism for this coupling is more complex than simple conservation of certain amino acids and that more studies are needed to figure out the structural and enzymatic basis for this reaction. We have proposed a mechanism for the second ring closure, which is NADPH-dependent reductive cyclization.¹³ Alternatively, the mechanism could be the initial epoxidation at C19–C20, followed by ring closure at C12–C19 along with epoxide opening, which would result in the C20–OH group. However, for compounds that do not have the C20–OH (such as pactamides and combamides), epoxidation would not be

needed for initiating the ring closure, and the reductive cyclization mechanism is more likely to occur.

Conflicts of interest

There are no conflicts of interest to declare.

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References

- 1 F. Yu, K. Zaleta-Rivera, X. Zhu, J. Huffman, J. C. Millet, S. D. Harris, G. Yuen, X. C. Li and L. Du, *Antimicrob. Agents Chemother.*, 2007, **51**, 64–72.
- 2 L. Xu, P. Wu, S. J. Wright, L. Du and X. Wei, *J. Nat. Prod.*, 2015, **78**, 1841–1847.
- 3 Y. Xie, S. Wright, Y. Shen and L. Du, *Nat. Prod. Rep.*, 2012, **19**, 1277–1287.
- 4 S. Li, L. Du, G. Yuen and S. D. Harris, *Mol. Biol. Cell.*, 2006, **17**, 1218–1227.
- 5 Y. Ding, Z. Li, Y. Li, C. Lu, H. Wang, Y. Shen and L. Du, *RSC Adv.*, 2016, **6**, 30895–30904.

- 6 Y. Ding, Y. Li, Z. Li, J. Zhang, C. Lu, H. Wang, Y. Shen and L. Du, *Biochim. Biophys. Acta*, 2016, **1860**, 2097–2106.
- 7 J. A. Blodgett, D. C. Oh, S. Cao, C. R. Currie, R. Kolter and J. Clardy, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 11692–11697.
- 8 K. Jomon, Y. Kuroda, M. Ajisaka and H. Sakai, *J. Antibiot.*, 1972, **25**, 271–280.
- 9 Y. Liu, H. Wang, R. Song, J. Chen, T. Li, Y. Li, L. Du and Y. Shen, *Org. Lett.*, 2018, **20**, 3504–3508.
- 10 S. Saha, W. Zhang, G. Zhang, Y. Zhu, Y. Chen, W. Liu, C. Yuan, Q. Zhang, H. Zhang, L. Zhang, W. Zhang and C. Zhang, *Chem. Sci.*, 2017, **8**, 1607–1612.
- 11 L. Lou, G. Qian, Y. Xie, J. Hang, H. Chen, K. Zaleta-Rivera, Y. Li, Y. Shen, P. H. Dussault, F. Liu and L. Du, *J. Am. Chem. Soc.*, 2011, **133**, 643–645.
- 12 Y. Li, H. Chen, Y. Ding, Y. Xie, H. Wang, R. L. Cerny, Y. Shen and L. Du, *Angew. Chem., Int. Ed.*, 2014, **53**, 7524–7530.
- 13 Y. Li, H. Wang, Y. Liu, Y. Jiao, S. Li, Y. Shen and L. Du, *Angew. Chem., Int. Ed.*, 2018, **57**, 6221–6225.
- 14 Y. Li, J. Huffman, Y. Li, L. Du and Y. Shen, *MedChemComm*, 2012, **9**, 982–986.
- 15 G. Zhang, W. Zhang, Q. Zhang, T. Shi, L. Ma, Y. Zhu, S. Li, H. Zhang, Y. L. Zhao, R. Shi and C. Zhang, *Angew. Chem., Int. Ed.*, 2014, **53**, 4840–4844.
- 16 J. Antosch, F. Schaefer and T. A. Gulder, *Angew. Chem., Int. Ed.*, 2014, **53**, 3011–3014.
- 17 Y. Qi, E. Ding and J. A. V. Blodgett, *ACS Synth. Biol.*, 2018, **7**, 357–362.
- 18 Y. Luo, H. Huang, J. Liang, M. Wang, L. Lu, Z. Shao, R. E. Cobb and H. Zhao, *Nat. Commun.*, 2013, **4**, 2894.