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# Re-investigation of the HExxH enzyme DarF reveals a dehydrogenation–epoxidation reaction

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**Darobactin A is a potent Gram-negative antibiotic, but the biosynthesis of its derivative, dehydrodarobactin A, remains unclear. Here, we re-investigated the HExxH enzyme DarF from *Pseudoalteromonas luteoviolacea* H33 through *in vitro* characterization and found that it catalyzes a dehydrogenation–epoxidation reaction and detected these darobactin A derivatives in native strains.**

## Introduction

Darobactin A is a ribosomally synthesized and post-translationally modified peptide (RiPP) antibiotic composed of two three-residue motif cyclophanes that target the essential outer membrane protein BamA of Gram-negative bacteria.[1,2] The two cyclophanes in darobactin A are installed by a single radical *S*-adenosylmethionine (rSAM) enzyme, DarE (Fig. 1), and the enzymatic mechanism of DarE is proposed through radical-mediated cross-linking.[3–5] The name daropeptide was proposed for the family of natural products formed by DarE or its homologue.[3] Interestingly, DarE and its homolog PasB exhibit substrate-controlled catalysis, generating C–C cross-link, ether linkage or Ser oxidation depending on the substrate.[6,7] Monoaryl and biaryl cross-linking on peptides catalyzed by rSAM, cytochrome P450, DUF3328, BURP and α-ketoglutarate (KG)-dependent proteins is a hot topic in the RiPP research field.[8–15]

Since the discovery of darobactin A, many predicted natural and unnatural analogues have been explored through native strain cultivation, heterologous expression and rational design to access more potent antibiotics.[16–23] Among these reported engineered variants, darobactin 22 with the core peptide sequence **WNWTKRW** outperformed darobactin A (**WNWSKSF**) and was the most potent candidate.[18,19] In addition, the total synthesis of darobactin A has also been achieved through Larock macrocyclization.[24,25] In 2023, darobactin A and its three derivatives, bromodarobactin A, dehydrodarobactin A and dehydrobromodarobactin A, were isolated from the marine bacterium *Pseudoalteromonas luteoviolacea* H33, and a flavin-dependent halogenase DarH was discovered, which is responsible for the bromination of bromodarobactins.[22] However, the biosynthesis of dehydrodarobactin A remains elusive (Fig. 1). We are interested in revealing the enzyme involved in the biosynthesis of dehydrodarobactin A.

## Results and discussion

At present, DarF is proposed to be a protease that degrades darobactin A for self-resistance.[21,22,26] Recently, three rSAM-fused HExxH enzymes, MscBH, SjiBH and ChlBH, containing an N-terminal rSAM domain and a C-terminal HExxH domain have been shown to catalyze cyclophane formation and β-hydroxylation on cyclophanes, respectively.[27] These three fused HExxH domains were characterized as αKG-dependent non-heme iron enzymes and named MscH, SjiH and ChlH, respectively. The recent characterization of HExxH enzymes (TIGR04267) prompted us to re-examine the *in vitro* activity of the non-fused HExxH enzyme DarF, using $Fe^{2+}$, αKG and ascorbate as cofactors instead of $Zn^{2+}$ tested previously.[21]

To investigate DarF activity through *in vitro* experiments, a modified full-length precursor peptide was used as a substrate and purified $His_6$-DarF was visualized by SDS-PAGE (Fig. S1). The modified full-length $His_6$-SUMO-DarA was prepared by coexpression of the $His_6$-SUMO-DarA + rSAM enzyme DarE in *Escherichia coli* NiCo21(DE3) and verified by detection of peaks

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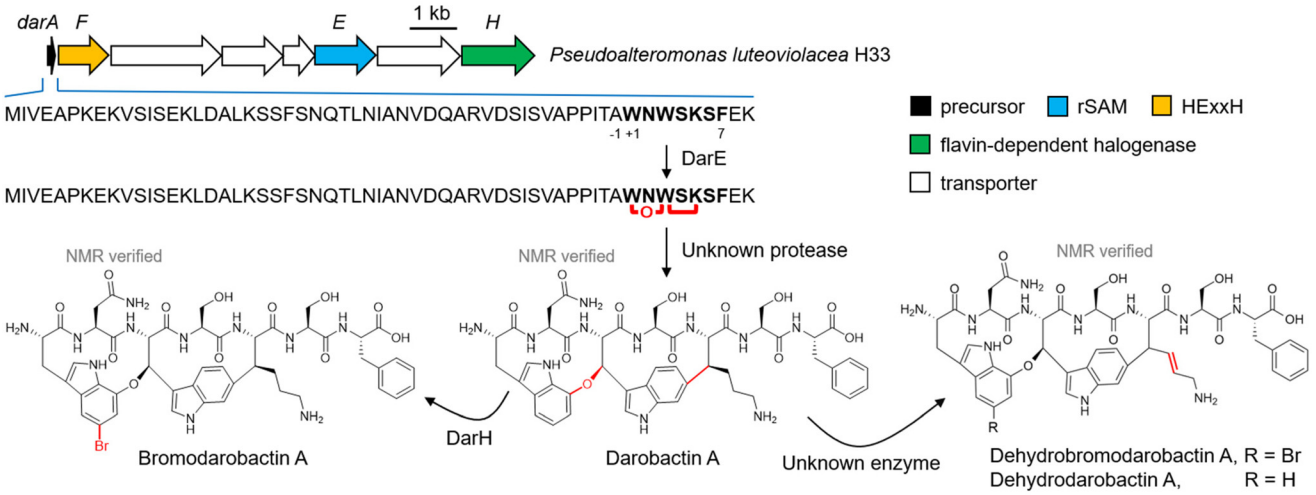


**Fig. 1** The biosynthesis of darobactin A and its three derivatives isolated from *Pseudoalteromonas luteoviolacea* H33. The core peptide on the precursor peptide sequences is shown as bold letters. Cross-link formation on the peptide sequences is shown as red connectors.

**1** (unmodified, 0 Da) and **2** (modified, +12 Da) (Fig. S2–S4). When modified full-length DarA was incubated with native enzyme $His_6$-DarF, we detected two additional peaks **3** and **4**, which were absent in boiled enzyme incubations (Fig. 2A and S5, S6). The tandem mass spectrometry (MS/MS) analysis localized the +10 and +26 Da mass gain to the core peptide in fragments **3** and **4**, respectively (Fig. 2A and S7, S8). Next, we determined that the DarF activity requires the cofactors αKG and $Fe^{2+}$ (Fig. 2B), similar to other HExxH enzymes MscH, SjiH and ChlH.[27] After that, we aimed to determine the product formation order. To this end, we performed time-course and enzyme concentration-dependent *in vitro* assays of $His_6$-DarF (Fig. S9 and S10). The results show that the modified full-length DarA is first converted to **3** and then to **4**.

Despite large-scale protein expression at 64 L to obtain modified full-length DarA for *in vitro* reactions with $His_6$-DarF, fragments **3** and **4** likely underwent degradation during chromatographic purification, making it impossible to obtain

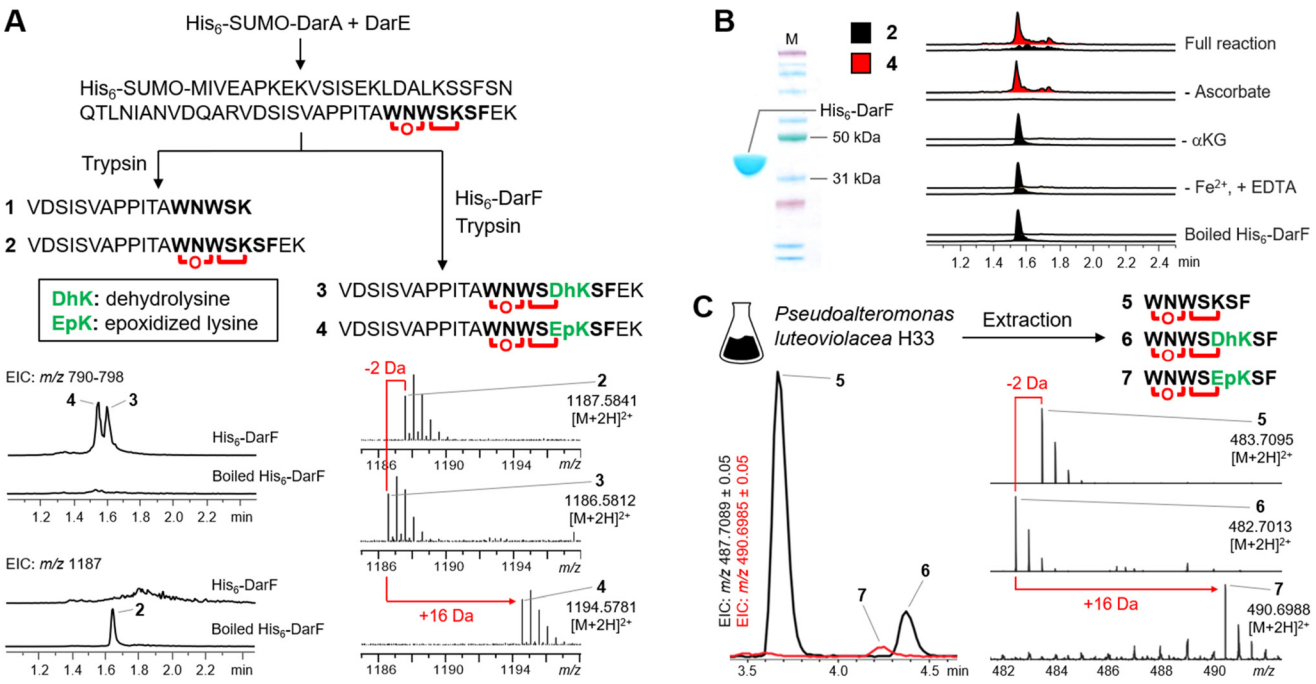


**Fig. 2** (A) Overview of DarF *in vitro* activity yielded dehydro- and epoxidized products. (B) Cofactor-dependent *in vitro* assay of DarF. (C) Detection of dehydrodarobactin A and epoxidized darobactin A from native strains. The EIC chromatogram and MS spectra of peaks **2**–**7**. DarF transformation on the peptide sequences is shown as green colored letters.

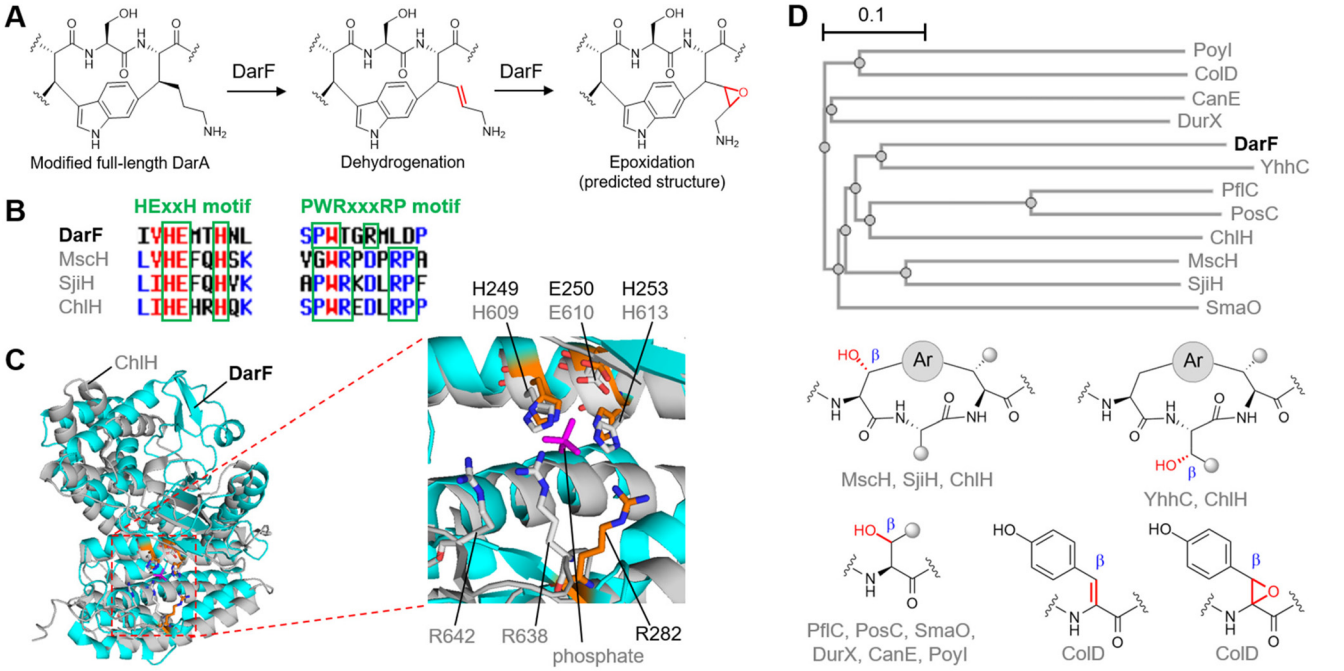


**Fig. 3** (A) Proposed DarF reaction on modified full-length DarA. (B) HExxH and PWRxxxRP motifs in DarF and HExxH enzymes. (C) Superimposition of the crystal structure of $His_6$-ChlH (PDB: 8S5F) with the predicted DarF structure. Protein structures of ChlH and DarF are shown as gray and cyan colored ribbons, respectively. Residues in ChlH and DarF are shown as gray and black colored letters, respectively. (D) Phylogenetic tree of DarF and known αKG-dependent enzymes in RiPP biosynthesis and their summarized reactions. Phe and His are shown as Ar labelled spheres.

sufficient material for NMR characterization. To verify our *in vitro* results, we then turned to *P. luteoviolacea* H33, a proven native producer of darobactin A and dehydrodarobactin A.[22] Analysis of the culture extract by UHPLC-MS confirmed the presence of darobactin A (**5**), dehydrodarobactin A (**6**), and epoxidized darobactin A (**7**) (Fig. 2C and S11–S13). Based on these findings, we proposed that DarF catalyzes a dehydrogenation–epoxidation reaction on the modified full-length DarA (Fig. 3A). The DarF-catalyzed dehydro-product is assumed to be identical to NMR verified dehydrodarobactin A (**6**),[22] while the structure of the DarF-catalyzed epoxidized product is predicted without NMR evidence.

To understand the structure of DarF, the AlphaFold3[28] predicted structure of DarF was input into the DALI server,[29] and the output showed that ChlH is the closest structurally similar protein (Fig. S14). All characterized HExxH enzymes ChlH, MscH and SjiH possess conserved HExxH and PWRxxxRP motifs, which correspond to the HxD/E···H motif and positively charged residues for Fe and αKG binding in αKG-dependent enzymes.[27] Sequence alignment of DarF with known HExxH enzymes showed the presence of a conserved HExxH motif for Fe coordination, but the absence of a PWRxxxRP motif in DarF (Fig. 3B and S15). Superimposition of the predicted DarF structure with $His_6$-ChlH (PDB: 8S5F) revealed that the positively charged residue Arg282 in DarF substituted the PWRxxxRP motif, suggesting that Arg282 may be involved in αKG binding (Fig. 3C and S16).

In RiPP biosynthesis, all characterized αKG-dependent enzymes,[30–34] including HExxH enzymes,[27,35] are known to catalyze β-hydroxylation, but the αKG-dependent dioxygenase ColD has recently been reported to catalyze a sequential dehydrogenation–epoxidation reaction on a Tyr residue.[36] Although DarF has a HExxH motif, the phylogenetic tree of these enzymes showed that DarF does not belong to the same clade as the HExxH enzymes ChlH, MscH and SjiH (Fig. 3D and S17). To the best of our knowledge, there is currently only one example of a sequential dehydrogenation–epoxidation reaction catalyzed by an αKG-dependent enzyme in RiPP biosynthesis,[36] and DarF is the second example. Notably, (1) the dehydroamino acids found in the cyclophane rings are only reported in dehydrodarobactin A,[22] (2) most of the dehydroamino acids found in bacterial RiPP biosynthesis are present in lanthipeptides (Fig. S18),[37] and (3) only three αKG-dependent enzymes ColD, AsqJ and BcmB have been reported to catalyze sequential dehydrogenation–epoxidation reactions in ribosomal and non-ribosomal peptide natural products biosynthesis (Fig. S19).[36,38–40]

## Conclusion

We re-investigated the HExxH enzyme DarF and found that it catalyzes a dehydrogenation–epoxidation reaction, yielding dehydrodarobactin A and epoxidized darobactin A. This study does not rule out previous *in vitro* reports of DarF acting as a protease to degrade the leaderless substrate, darobactin A.[21] A recent study has revealed that the HExxH enzyme PflC cata-

lyzes (1) oxidative chemistry on the precursor peptides or (2) proteolytic cleavage on leaderless substrates.[35] To our knowledge, DarF is the second enzyme, after ColD,[36] that catalyzes dehydrogenation–epoxidation in RiPP biosynthesis. This study connects a missing gene to the corresponding encoded functionality in dehydrodarobactin A and unlocks a new toolkit for daropeptide modification.

## Conflicts of interest

There are no conflicts to declare.

## Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information is available. See DOI: https://doi.org/10.1039/d6ob00496b.

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