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Biosynthesis of Cyclochlorotine: Identification of the Genes Involved in Oxidative Transformations and Intramolecular *O,N*-Transacylation

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Supporting Information Placeholder

hydroxycyclochlorotine
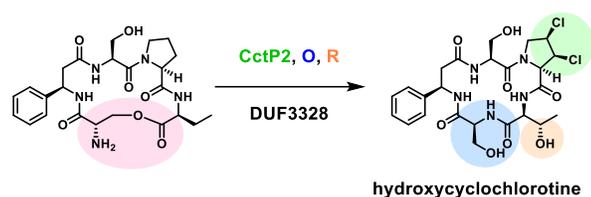
ABSTRACT: Mycotoxin cyclochlorotine (**1**) and structurally related astins are cyclic pentapeptides containing unique nonproteinogenic amino acids, such as β-phenylalanine, L-*allo*-threonine, and 3,4-dichloroproline. Herein, we report the biosynthetic pathway for **1**, which involves intriguing tailoring processes mediated by DUF3328 proteins, including stereo- and regiospecific chlorination and hydroxylation, and intramolecular *O,N*-transacylation. Our findings demonstrate that DUF3328 proteins, which are known to be involved in oxidative cyclization of fungal ribosomal peptides, have much higher functional diversity than previously expected.

Cyclochlorotine (**1**, Figure 1) is a hepatotoxic mycotoxin produced by *Talaromyces islandicus* (*Penicillium islandicum*), a fungal pathogen of rice and wheat and is known as a contaminant that causes *yellowed rice*.[1–3] Both **1** and its derivatives are cyclic pentapeptides that are closely related to antitumor astins produced by the fungal endophyte *Cyanodermella asteris*.[4] These compounds consist of nonproteinogenic amino acids, including 2-aminobutyric acid (Abu), β-phenylalanine (βPhe), L-*allo*-threonine, and 3,4-dichloroproline. Although the biosynthetic gene clusters for **1**[5] and astins[4] have been elucidated, the enzymes required for the chlorination of the proline (Pro) residue have not yet been identified. The absence of a typical halogenase, such as α-ketoglutarate (αKG) and iron-dependent enzyme, that can catalyze the regio- and stereospecific chlorination at unactivated carbon[6] suggests the involvement of a distinct type of enzymes. Additionally, the biosynthetic origin of L-*allo*-threonine has also not yet been elucidated. Intriguingly, three proteins containing DUF3328 domain (Pfam ID: PF11807, also known as UstYa family in Pfam) are highly conserved in both gene clusters, allowing us to hypothesize that these proteins are involved in the chlorination and/or other transformations in the biosynthesis of **1**.

DUF3328 proteins are widely and specifically distributed in the fungal kingdom, and their function has long been unknown. Since the discovery of the ustiloxin biosynthetic gene cluster,[7,8]
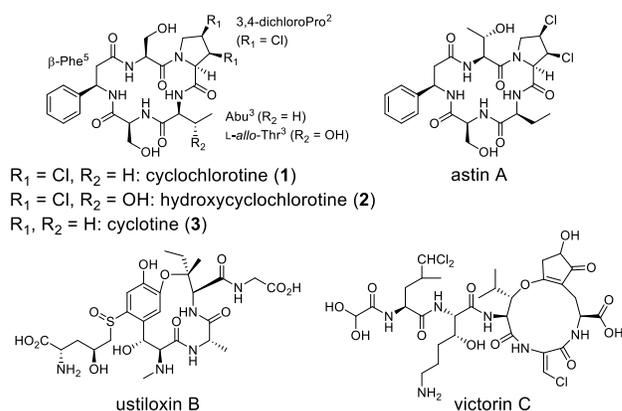

Figure 1. Cyclochlorotine (1) and its related fungal peptides.

DUF3328 proteins have been known to be common proteins conserved in the biosynthesis of fungal cyclic peptides of ribosomal origin[9–11] Although several heterologous expression experiments have indicated that DUF3328 proteins are responsible for the oxidative cyclization and hydroxylation,[12,13] further details regarding their function remain to be elucidated. Herein, we report on the biosynthetic pathway of **1**, which involves intriguing post-nonribosomal peptide synthetase (post-NRPS)

transformations, including the dichlorination of Pro, hydroxylation of Abu, and cryptic intramolecular O,N-transacylation. We show that three DUF3328 proteins (i.e., CctO, CctP2, and CctR) are essential for the above transformations, demonstrating that DUF3328 domain is a functionally diverse group of proteins involved in the modification of fungal natural products, such as ribosomally synthesized and post-translationally modified peptides (RiPPs) and non-ribosomal peptides (NRPs).
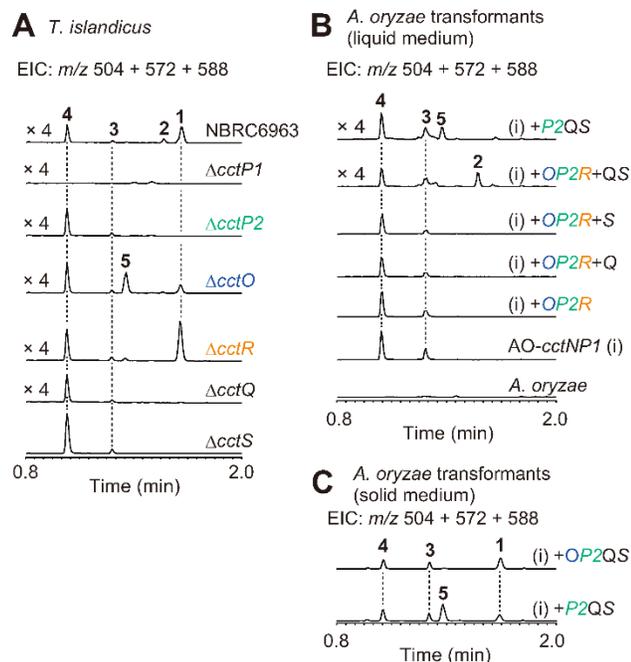

Figure 2. Liquid chromatography–mass spectrometry (LC-MS) traces for the metabolites. (A) *T. islandicus* NBRC 6963 and gene-knockout mutants. (B) Heterologous reconstitution of the biosynthetic pathway. (C) Metabolites of the selected transformants cultured in a solid (rice) medium

First, we analyzed the metabolites of *T. islandicus* NBRC 6963 and identified **1**, hydroxycyclochlorotine (**2**), and cyclotine (**3**) (Figures 1 and 2A). In addition, we also identified **4** as an isomeric metabolite of **3**; this compound was detected using high-performance liquid chromatography (HPLC), although its structure has not been determined.[5] During the isolation process, we observed that **4** readily decomposes when exposed to a high temperature (>45˚C), MeOH, base, and repeated concentration and dissolution. After several attempts, we successfully isolated **4**. Nuclear magnetic resonance (NMR) analyses revealed that **4** has an ester bond between Ser4 and Abu3 instead of a normal amide bond, establishing the structure of **4** as shown in Scheme 1 (Figure S1, Table S1). Additionally, ester **4** was found to readily convert into amide **3** under mild alkaline conditions (aq. NaHCO$_3$ or pyridine, room temperature; Scheme 1, Figure S2A). Time course analysis of the metabolites in fermentation of *T. islandicus* revealed that **4** was gradually converted into **3** (Figure S3). This conversion occurred even in a simple incubation of **4** in a fermentation medium (Figure S4). Such rapid O,N-transacylation suggests that compound **3** produced by *T. islandicus* may be derived from a nonenzymatic transformation of **4**. When the methanol solution of **4** was heated at 45˚C overnight, the ester **4** was converted into methyl ester **4a** ($C_{25}H_{38}O_8N_5$) and

a small amount of **4b** ($C_{24}H_{33}O_8N_4$), whose structures were proposed by MS/MS fragmentation analyses (Scheme 1, Figure S5ABC). Similar elimination also occurred in the dimethylsulfoxide (DMSO) solution of **4** (Figure S6).

Scheme 1. Chemical transformation of 4 and 5.
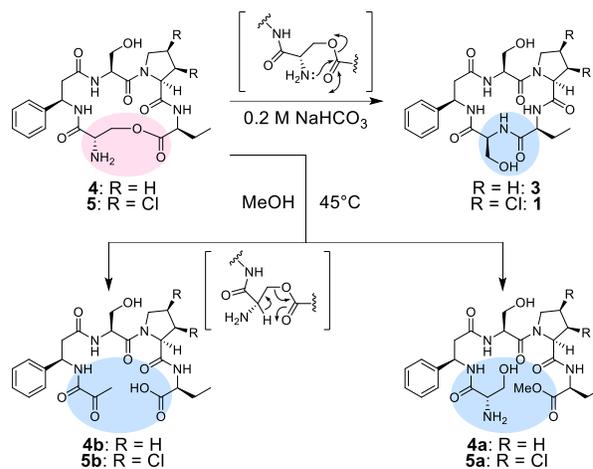
To gain insights into the biosynthesis of **1**, we analyzed the nucleotide sequence of the *cct* gene cluster to check the annotation of each gene, as to our knowledge no previous studies have functionally analyzed the DUF3328 genes in the *cct* cluster.[5] According to manual inspection, we divided *cctP*, a gene encoding unusual fusion of phenylalanine aminomutase (PAM) and DUF3328,[5] into two separate ORFs: *cctP1* for PAM and *cctP2* for DUF3328 (Figure 3A, Figure S7).

According to the sequence analysis, we constructed $\Delta cctO$, $\Delta cctP1$, $\Delta cctP2$, and $\Delta cctR$ mutants (Figure S8, Table S2). Deletion of *cctP1* stopped the production of **1** and other related metabolites (Figure 2A). Addition of DL-βPhe to the culture medium of this mutant restored the production of **1** and its derivatives (Figure S9), showing that βPhe is synthesized by CctP1. Deletion of *cctP2* abolished the production of chlorinated derivatives **1** and **2** and caused the accumulation of **4**. Addition of synthetic 3,4-dichloroproline[14] to the culture medium of the $\Delta cctP2$ mutant did not change the metabolite profile, suggesting that the dichlorination is a post-NRPS process (Figure S10). We also examined the deletion of *cctR*, which abolished the production of **2** (Figure 2A).

Deletion of *cctO* resulted in the distinct accumulation of a new compound **5** (Figure 2A), which is isomeric to **1**. As observed for **4**, compound **5** was converted into **1** under mild alkaline conditions, indicating that this compound is a dichlorinated derivative of **4** (Scheme 1, Figure S1, S2B, Table S1). Similar to **4**, the methanolysis product **5a** and pyruvylamide **5b** were obtained by heating the methanol solution of **5** (Scheme 1, Figure S5ADE). The lactone **5** was found to be more susceptible to elimination than that of **4** and, thus, slowly converted into **5b** during the NMR measurement (DMSO-$d6$, ca. 10 days) in quantitative yield (Figure S1, Table S3). The predominant accumulation of **5** in the $\Delta cctO$ mutant suggests that this intramolecular transacylation is an enzymatic process catalyzed by CctO (Figure 2A).
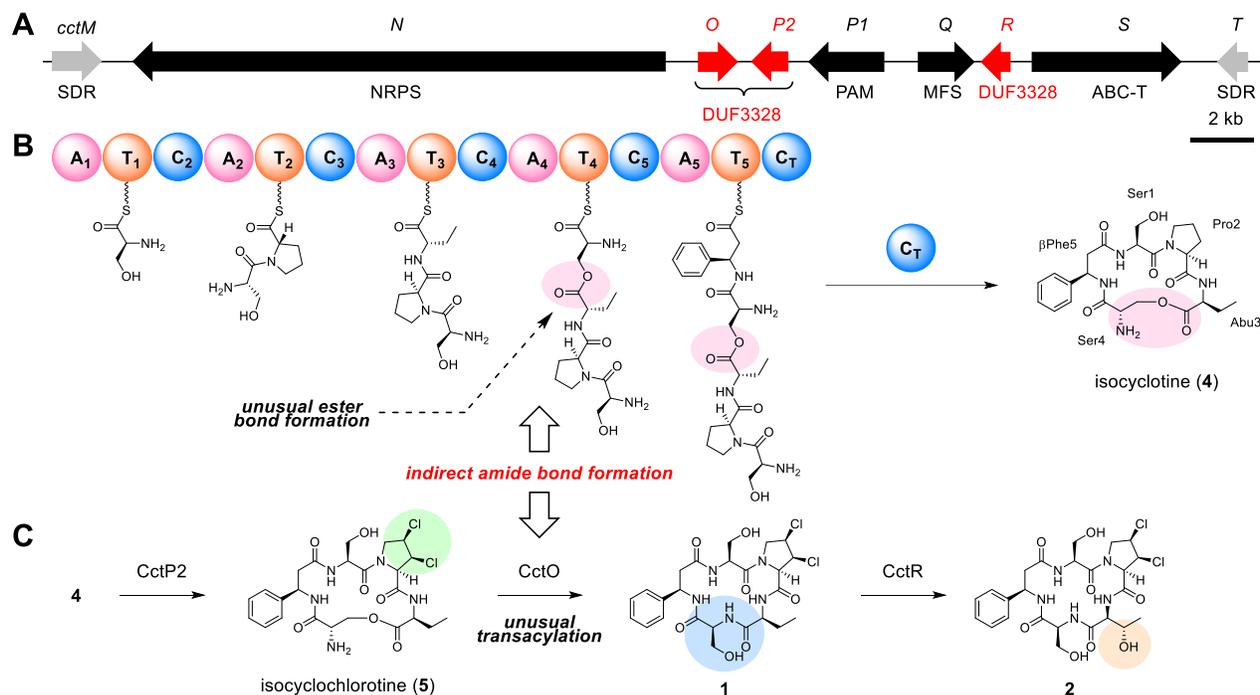
Figure 3. Biosynthesis of cyclochlorotine by *T. islandicus* NBRC 6963. (A) Revised *cct* gene cluster. The three genes encoding DUF3328 proteins (Pfam ID: PF11807), are colored in red. Abbreviations: SDR, short-chain dehydrogenase/reductase; ABC-T, ATP-binding cassette transporter. (B) The proposed pathway leading to **1** and **2**.

Given the above results, we hypothesize that the NRPS CctN initially catalyzes the condensation of L-serine (Ser), Pro, Abu, Ser, and βPhe in this order, as previously predicted.[4,5] During the chain elongation, side-chain hydroxy group of Ser4 would be used as a nucleophile, giving **4** rather than **3** as a product of terminal condensation-like (C_T) domain[15]-catalyzed cyclization (Figure 3B). After the dichlorination of Pro2 likely catalyzed by CctP2, the CctO-mediated transacylation of **5** can furnish **1**. The subsequent hydroxylation of **1** by CctR yields **2** as the final product (Figure 3B). To investigate this hypothesis, we examined the bioconversion of **4** and **1** by the *ΔcctP1* mutant, which contains CctO, CctP2, and CctR in an active form. However, neither chlorination nor hydroxylation activities were observed, likely because of the poor incorporation of hydrophilic peptides (Figure S11). Therefore, we planned heterologous expression as described below.

For the reconstitution of the biosynthetic machinery, we used the recently established CRISPR/Cas9-based genome editing technique for the host strain *Aspergillus oryzae* to achieve screening-free transformation.[16] First, we constructed the transformant AO-*cctNP1*, which harbors the NRPS gene *cctN* and the PAM gene *cctP1* (Figure S12, Table S4). As expected, AO-*cctNP1* was found to produce **4** with a small amount of **3** (Figure 2B). Next, we introduced *cctO*, *cctP2*, and *cctR* into AO-*cctNP1* to construct AO-*cctNP1+OP2R*. However, further modifications did not proceed (Figure 2B), indicating that additional factors are necessary.

While examining the fermentation conditions, we found that adding KCl to the production medium effectively increased the product ratio of chlorinated derivatives to nonchlorinated ones (Figure S13). With this observation in mind, we found out that two transporter genes, *cctQ* and *cctS*, remain to be characterized. We hypothesized that CctS, an ATP-binding cassette

transporter (ABC-C subfamily), functions as a chloride channel for the biosynthesis of **1** because ABC-C transporters include ATP-gated chloride channels.[17] For the remaining transporter CctQ belonging to a major facilitator superfamily (MFS), we speculate that CctQ acts as a self-resistance mechanism for product toxicity.

As expected from above hypothesis, **1** and **2** were successfully produced when *cctQ* and *cctS* were introduced into the transformant AO-*cctNP1+OP2R* (Figure 2B). Moreover, AO-*cctNP1+OP2R+Q* and AO-*cctNP1+OP2R+S* showed a similar metabolite profile to that of AO-*cctNP1+OP2R*, indicating that both proteins are indispensable for the biosynthetic process (Figure 2B). Consistently, knockout of *cctQ* and *cctS* in the producing strain abolished or significantly reduced the production of **1** and **2**, further supporting their importance in the biosynthesis (Figure 2A). The fact that **1** and **3** were not toxic to *A. oryzae* (Figure S14) would deny a role of CctQ in self-resistance.

Recently, many studies on cellular compartmentalization involved in the biosynthesis of fungal secondary metabolites have been reported.[18,19] Considering these examples on the intracellular trafficking of intermediates, we speculate that DUF3328 proteins are located in specific cellular compartment in which two transporters are colocalized to transport peptide precursors and chloride ions. The importance of MFS transporters in the DUF3328-related pathway was also noted for ustiloxin B and asperipin-2a.[8,13] According to the results of this study, the DUF3328 proteins for fungal RiPP biosynthesis may also be located in similar organelles in which CctO, CctP2, and CctR are localized.

We then expressed *cctO*, *cctP2*, and *cctR* in various combinations. Omitting either *cctO* or *cctR* produced essentially the same results as those obtained with the *ΔcctO* or *ΔcctR* mutant, respectively (Figure S15). The expression of a putative

halogenase gene *cctP2* with *cctN*, *cctP1*, *cctQ*, and *cctS* resulted in the chlorinated product **5** with **1** (Figure 2B), indicating that **4** rather than **3** is the preferred substrate of CctP2. The predominant formation of **5** was more obvious in a solid medium than in a liquid medium (Figure 2C). Interestingly, CctP2 does not exhibit any homology to known halogenases that installs chlorines into the unactivated carbon center,[6,20] suggesting that CctP2 is a new type of halogenase that activates inert C–H bonds like αKG-dependent enzymes. Notably, the presence of a similar gene was reported in the biosynthetic gene cluster for the fungal RiPP victorin[11] and polyketide atpenin,[21] suggesting that DUF3328-mediated chlorination is involved in the biosynthesis of various fungal metabolites. This hypothesis was further supported by the sequence similarity network (SSN) analysis of DUF3328 family proteins, which showed that the DUF3328 protiens AstS and ApnIII, encoded in the biosynthetic gene clusters for astins and atpenin, are classified to the same cluster to CctP2 (Figure S16).

Coexpression of *cctO* with *cctQ* and *cctS* in AO-*cctNP1* was found to have a little effect on the ratio of **3** and **4** (Figure S15), whereas substantial accumulation of **5** was observed only when *cctO* was omitted (Figure 2BC). On the basis of this result, we propose that CctO catalyzes the intramolecular *O,N*-transacylation from **5** to **1**. The close similarity between the biosynthetic gene clusters for **1** and astins suggests that the NRPS-catalyzed formation of serinyl ester and the subsequent *O,N*-transacylation, which resembles native chemical ligation,[22] are a common strategy for both compounds. Currently, the exact role of this highly unusual bypass is not clear, but it is possibly related to DUF3328 protein–catalyzed chlorination.

In the present study, we unveiled the biosynthetic pathway of **1** and **2**. Intriguingly, three DUF3328 proteins, which are known to be involved in the fungal RiPP pathway, were found to play a role in the post-NRPS transformations. These proteins are involved in halogenation, transacylation, and hydroxylation, indicating that the DUF3328 comprises much higher functional diversity than previously known. As we deciphered the function of each protein and identified possible substrates, further biochemical studies will unveil the mechanism underlying these enigmatic reactions. In addition, we also demonstrated that two transporters, CctQ and CctS, are indispensable for the biosynthetic process, indicating that expression of transporters should be considered during the genome mining and functional reconstitution of the related pathways.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental procedures, Supplementary Figures and Tables, and NMR spectra (PDF)

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