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Author manuscript
Org Lett. Author manuscript; available in PMC 2017 March 18.

Published in final edited form as:
Org Lett. 2016 March 18; 18(6): 1446–1449. doi:10.1021/acs.orglett.6b00380.

Combinatorial Generation of Chemical Diversity by Redox Enzymes in Chaetoviridin Biosynthesis

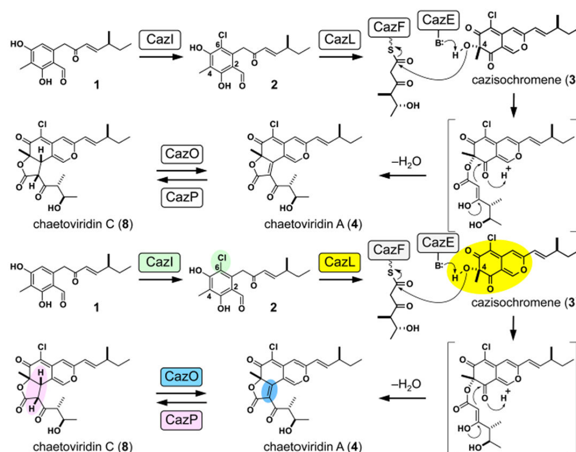
Michio Sato[†], **Jaclyn M. Winter**[‡,§], **Hiroshi Noguchi**[†], **Yi Tang**[‡,‖], and **Kenji Watanabe**[*,†]

[†]Department of Pharmaceutical Sciences, University of Shizuoka, Shizuoka 422-8526, Japan

[‡]Department of Chemical and Biomolecular Engineering, University of California, Los Angeles, California 90095, United States

[‖]Department of Chemical and Biomolecular Engineering, and Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90095, USA

Abstract



Chaetoviridins constitute a large family of structurally related secondary metabolites isolated from *Chaetomium* fungi. To elucidate the biosynthesis pathway and understand how the chemical diversity of chaetoviridins is generated, gene deletion and in vitro characterization of the four post-PKS modifications enzymes were undertaken. CazL and CazP were identified to have substrate promiscuity that facilitates the formation of non-chlorinated analogs. In addition, enzymatic oxidation and reduction combined with spontaneous dehydration and lactonization of the intermediates further expand the chemical diversity.

Chaetoviridins are a group of fungal secondary metabolites produced by various *Chaetomium* spp. that exhibit antifungal and antibiotic activities.[1] Initially isolated from *C.*

[*]**Corresponding Author** kenji55@u-shizuoka-ken.ac.jp.
[§]**Present Addresses** (For JW) Department of Medicinal Chemistry, University of Utah, Utah 84112, USA.

Supporting Information Data from the NMR and MS determination of the compounds, and additional experimental information. This material is available free of charge via Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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globosum var. *flavo-viride* by Takahashi, *et al.*,[2] chaetoviridins and closely related chaetomugilins are categorized as azaphilones with a chlorine atom in their chemical composition. Those compounds are assembled by iterative polyketide synthases, which are megasynthases containing a single set of catalytic domains necessary to carry out a polyketide chain extension and modifications that are programmed to function repetitively in different combinations. We previously identified the *caz* biosynthetic gene cluster in *C. globosum* that is responsible for the production of chaetoviridins and chaetomugilins.[3] The characterization of CazF, a highly-reducing polyketide synthase (HR-PKS), established that it acts in both a sequential and a convergent manner with CazM, a nonreducing polyketide synthase (NR-PKS), to form the polyketide framework of chaetoviridins and chaetomugilins (Figure 1). Through in vitro reconstitution, we demonstrated the production of chaetoviridin A **4** from the pyranoquinone intermediate cazisochromene **3** using CazF and an acyltransferase CazE,[3] as well as the formation of the intermediate cazaldehyde A **1** through modular interaction of CazF and CazM.[4] However, the post-PKS modifications that lead to the generation of chemical diversity among chaetoviridins and chaetomugilins remain poorly understood. Here we present the identification and in vitro characterizations of four redox enzymes, CazI, CazL, CazP and CazO, that are responsible for chlorination, pyranoquinone formation, reduction and oxidation of the polyketide core that give rise to the vast structural diversity found in chaetoviridins and chaetomugilins.

First, to fully understand the entire chaetoviridin biosynthetic pathway, we sought to determine the function of non-PKS enzymes encoded in the gene cluster. In particular, we focused on four redox enzymes, CazI, CazL, CazP and CazO. BLASTP[5] search indicated CazI as an FADH2-dependent halogenase, CazL as an flavin-containing monooxygenase (FMO), CazP as a dehydrogenase and CazO as a flavin-dependent oxidoreductase (Table S2 and Figure S9 in the Supporting Information).

We initially performed targeted gene deletion by double homologous recombination gene replacement using an *hph* (hygromycin B phosphotransferase gene) cassette on the *C. globosum* *CgpyrG*/ *CgligD* strain, CGKW14 (Figures S1–S2).[6] Four transformants having *hph* integrated individually into *cazI*, *cazL*, *cazP* or *cazO* were successfully obtained after confirming the deletion of the target genes by diagnostic PCR (Figure S3). Five-day culture of *cazI*/CGKW14 grown on MYG liquid medium was extracted with ethyl acetate, and the dried extract was subjected to metabolite analysis by liquid chromatography–mass spectrometry (LCMS). As expected, deletion of *cazI* resulted in an accumulation of **1**, the predicted substrate for halogenation by CazI (Figure S10a). Similarly, deletion of *cazL* led to an accumulation of **2**, the proposed substrate of CazL (Figure S10b). The chemical structures of all the compounds isolated from the knockout mutants were characterized by high-resolution (HR) electrospray ionization (ESI) LCMS, ^{1}H NMR and ^{13}C NMR (Table S3 and S4, Figures S14–S21).

As to the remaining two genes, the UV trace from HPLC analysis of the extract from *cazP*/ CGKW14 (Figure S10c) showed a nearly complete loss of the formation of chaetoviridin B **6** (Table S7 and Figures S29–S30) and an accumulation of 4'-epi-*N*-2-hydroxyethyl-azachaetovoridin A **7**[6] (Table S8 and Figures S31, S32). Since **6** could form spontaneously from chaetoviridin C **8**, the depletion of **6** suggests that the production of **8** is lost in this

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mutant. Similarly, the accumulation of **7** suggests **4** is what is collecting in the absence of CazP. Similarly, the UV trace from HPLC analysis of the extract from *cazO*/CGKW14 (Figure S10d) showed a significant loss of **4** (Table S5 and Figures S22– S23) and an accumulation of **8** (Table S9 and Figures S33– S34). Combined, these results suggest that the predicted dehydrogenase CazP is responsible for the reduction of **4** to **8**, and the predicted oxidase CazO catalyzes the oxidation of **8** to form **4**.

Preparation of the deletion strains gave us insight into the biosynthetic mechanisms involved in the formation of chaetoviridins and chaetomugilins, but they also provided us with intermediates in the biosynthetic pathway that can be used as substrates in more detailed in vitro assays. All of such intermediates were characterized by NMR analyses as described in the Supporting information. Among the intermediates isolated in this study, **2** and **5** were new compounds.

To characterize in detail the biochemical function of CazI, we cloned *cazI* from mRNA extracted from *C. globosum*, and expressed it heterologously in *Escherichia coli* (Figures S4 and S6). CazI showed a high amino acid sequence similarity to the previously characterized flavin-dependent halogenase RadH (70% positives, 54% identities) from the radicicol biosynthetic pathway in *C. chiversii*.[7] Therefore, the predicted halogenase activity of CazI was examined in vitro using **1** that was isolated from the culture of *cazI*/CGKW14 as a substrate. As predicted, the purified recombinant CazI was able to catalyze the halogenation of **1** to form **2** (Figure 2a i vs. ii).

Next, we attempted to characterize the activity of the predicted FMO, CazL. We speculated that CazL would be responsible for the formation of the pyranoquinone based on the amino acid sequence similarity to AfoD[8] (73% positives, 55% identities) and AzaH[9] (57% positives, 41% identities) from the asperfuranone and the azanigerone biosynthetic pathway, respectively, that are responsible for the pyran ring formation on substrates that are highly similar to **1**. We cloned *cazL* from *C. globosum* mRNA, and expressed it heterologously in *Saccharomyces cerevisiae* (Figures S5 and S7). The recombinant CazL was subjected to an in vitro assay using **2** as a substrate, and we were able to confirm that CazL was responsible for the pyranoquinone formation (Figure 2b i vs. ii). Interestingly, however, CazL was also able to accept the non-chlorinated compound **1** as a substrate (Figure 2b iii vs. vi) to form a pyranoquinone-containing product **5** (Table S6 and Figures S24–S28). The chemical structures of all of the products from the in vitro assays that were previously uncharacterized were elucidated by HRESIMS, ^{1}H NMR and ^{13}C NMR. These results indicate that CazL tolerates lack of chlorination at the C6 position of the aromatic ring in its substrate. To understand the substrate preference of CazL, kinetic constants for the two substrates **2** and **1** were determined from initial velocity measurements. CazL displayed a k_{cat} of 51.58 ± 7.80 min^{-1} for the formation of **3** with a K_m of 68.12 ± 22.10 μM for **2**, whereas a similar k_{cat} of 30.88 ± 2.30 min^{-1} for the formation of **5** and a K_m of 73.04 ± 9.93 μM were obtained when **1** was used as the substrate (Figure S11). These results suggest that CazL catalyzes the transformation of the halogenated substrate only about two-fold more efficiently than the non-halogenated precursor. Considering that CazL would also have to compete with CazI for **1**, the predominant product formed by CazL would be **3**. However, the observed sizable activity of CazL against **1** can explain the low-level but actual formation of non-halogenated

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chaetoviridin H **10**[10] and chaetomugilin U **11**.[11] This type of relaxed substrate specificity is rather common among secondary metabolite biosynthetic enzymes and serves as the basis for the chemical diversity generation of natural products.[12] Our study demonstrated that this is also the case for the biosynthesis of chaetoviridins and chaetomugilins.

For the in vitro characterization of CazP, we heterologously expressed the cloned *cazP* in *E. coli* (Figure S4 and S6). When the recombinant CazP was provided with **4** and NADPH, we observed the formation of **8** (Figure 3a i vs. ii), indicating that CazP is responsible for the reduction of the C8–C2' olefin of **4**. However, we also found the formation of **6**. As stated earlier, we hypothesized that **8** could be converted into **6** through a spontaneous rearrangement, which can be initiated by a hydrolytic opening of the δ-lactone ring and a rotation of the molecule around the C8–C2' bond. Subsequent tetrahydrofuran ring formation followed by a dehydrative cyclization of the terminal polyketide chain into a δ-lactone can yield **6** (Figure 3b). To confirm the spontaneous transformation of **8** into **6**, we followed a HEPES solution of **8** at a neutral pH kept at room temperature. From this experiment, we were able to establish that **8** indeed converted to **6** spontaneously (Figure S12). Furthermore, we suspect that the lactonized product **6** could be oxidized by an unidentified enzyme to form the hydroxylated compound, chaetomugilin A **12**.[3] At this point, based on the previously observed lack of discrimination between halogenated and non-halogenated substrates by CazL, we speculated that CazP may also accept **10**.[13] Like other substrates used in this study, **10** was isolated from the wild-type *C. globosum* (Table S10 and Figures S13a, S35, S36) as a substrate and forms a reduced product. Our in vitro assay proved this to be the case (Figure 3c); CazP accepted **10** as a substrate to form **13** (Figure S13c). We also observed the formation of **11** (Figure S13b), a presumed product of the spontaneous lactonization of **13**.

To characterize CazO in detail, we produced the protein recombinantly in *S. cerevisiae* using the *cazO* gene cloned from *C. globosum* mRNA (Figure S5 and S8). Then, the activity of the recombinant CazO was examined in an in vitro assay using **8** isolated from the *cazO*/ CGKW14 strain (Figure S10d) as a substrate. In the absence of CazO, the substrate completely transformed into **6** spontaneously. However, when the active sample of CazO was provided in the reaction mixture, we observed the formation of the oxidized product **4** in addition to the spontaneously forming **6** (Figure 3d). We suspect that chaetoviridin E **9**[13] can be formed from **4** via dehydration catalyzed by an unknown enzyme. Similarly, **7** can be formed readily by ethanolamine attacking **4**.[14]

In this study, we combined targeted gene deletion studies and in vitro assays to identify the main pathway leading to the formation of chaetoviridins through the formation of cazisochromene **3** and chaetoviridin A **4** (Figure 1). Through the study, we identified seven of the intermediates formed and the four enzymes, CazI, CazL, CazP and CazO, responsible for the chlorination and oxidation steps involved in the transformation of **1** to **4**. We also discovered the unanticipated relaxed substrate tolerance of the FMO CazL that allowed formation of non-chlorinated products. This finding led us to discover the same chlorination-insensitive substrate specificity of the dehydrogenase CazP. Our current study further adds to the notion that relaxed substrate specificity in the post-PKS modification redox enzymes is

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key to the generation of chemical diversity in natural product biosynthesis.[15,16] In addition, combination of enzymatic oxidation and reduction with spontaneous conversion of the core chemical framework via dehydration and lactonization further expands the variety of chaetoviridin and chaetomugilin analogs being formed. These mechanisms underlie the occurrence of a large number of variants, 43 to date,[1] of this class of compounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We wish to thank the financial support from Japan Society for the Promotion of Science (JSPS) Program for Advancing Strategic International Networks to Accelerate the Circulation of Talented Researchers (No. G2604) (K.W.). This work was also supported in part by Takeda Science Foundation (K.W.), Nagase Science and Technology Foundation Japan (K.W.), Grant-in-Aid for Scientific Research from JSPS (K.W.), Uehara Memorial Foundation (K.W.), Institution for Fermentation, Osaka (K.W.) and Tokyo Biochemical Research Foundation (K.W.). Y.T. is supported by NIH (1DP1GM106413).

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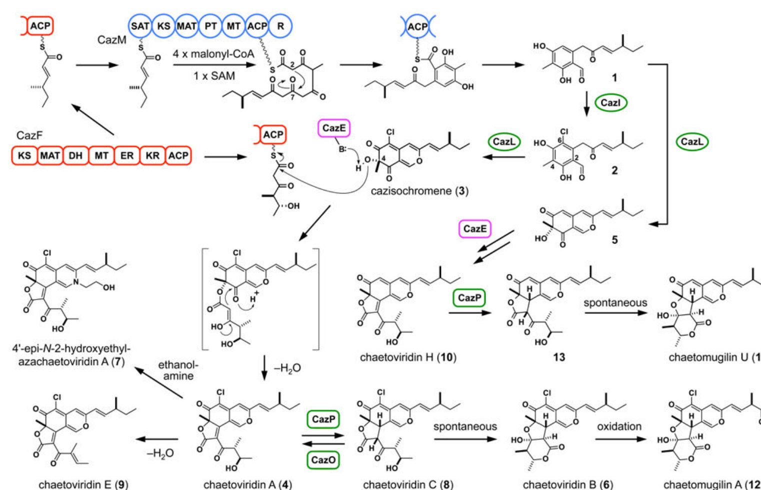

Figure 1.

Chemical diversity of post-PKS assembly modifications in the chaetoviridin biosynthetic pathway. Proposed biosynthesis of chaetoviridin A (**4**), 4'-epi-*N*-2-hydroxyethyl-azachaetoviridin A (**7**), chaetomugilin U (**11**) and chaetomugilin A (**12**). Domain abbreviations: ketosynthase (KS), malonyl-CoA:acyl carrier protein acyltransferase (MAT), dehydratase (DH), methyltransferase (MT), enoyl reductase (ER), ketoreductase (KR), acyl carrier protein (ACP), starter-unit:ACP-transacylase (SAT), product template (PT) and reductive (R) domain.

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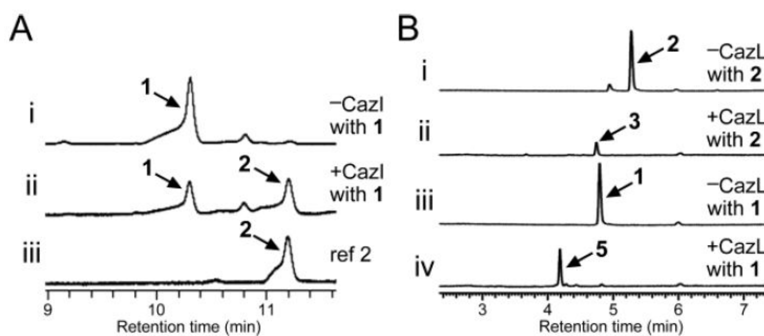

Figure 2.

In vitro characterization of CazI and CazL. (a) HPLC analysis of CazI using **1** as a substrate. HPLC traces were monitored at 254 nm. (i) Negative control with **1** lacking CazI. (ii) Reaction containing CazI and **1** incubated for 16 h at room temperature. (iii) Authentic reference of **2**. (b) HPLC analysis of CazL using **2** and **1** as substrates. HPLC traces were monitored at 280 nm. (i) Negative control with **2** lacking CazL. (ii) Reaction containing CazL and **2** after 1 h at 30 °C. (iii) Negative control with **1** lacking CazL. (iv) Reaction containing CazL and **1** incubated for 1 h at 30 °C.

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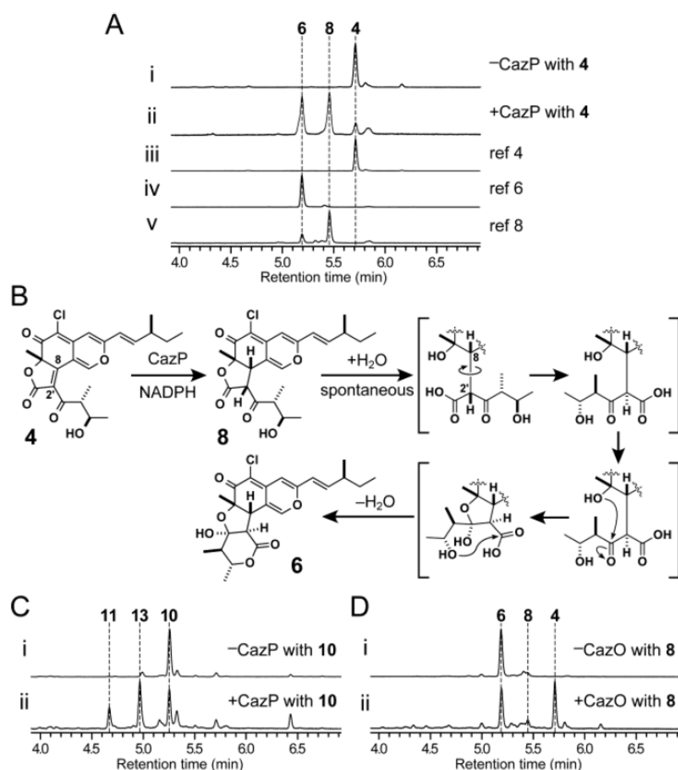

Figure 3.

HPLC analyses of in vitro assays on CazP and CazO. All HPLC traces were monitored at 280 nm. (a) In vitro characterization of CazP using **4** as a substrate. (i) Negative control with **4** lacking CazP. (ii) Reaction containing CazP and **4** incubated for 15 min at 30 °C. Authentic references of (iii) **4**, (iv) **6** and (v) **8** are also shown. (b) Proposed reaction mechanism of lactonization from **4** in **6** via **8** by CazP. (c) In vitro characterization of CazP using **10** as a substrate. (i) Negative control with **10** lacking CazP. (ii) Reaction containing CazP and **10** incubated for 3 h at 30 °C. (d) In vitro characterization of CazO using **8** as a substrate. (i) Negative control with **8** lacking CazO. Most of **8** has converted into **6** spontaneously in the reaction mixture. (ii) Reaction containing CazO and **8** incubated for 1 h at 30 °C.