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COMMUNICATION

Analysis of the cercosporin polyketide synthase CTB1 reveals a new fungal thioesterase function†

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The polyketide synthase CTB1 is demonstrated to catalyze pyrone formation thereby expanding the known biosynthetic repertoire of thioesterase domains in iterative, non-reducing polyketide synthases.

Thioesterase (TE) domains catalyze product release from fatty acid synthases, non-ribosomal peptide synthetases and polyketide synthases. In the main, these reactions occur by simple hydrolysis but, in the latter two protein families, can take place by macrolactamization or macrolactonization, respectively.[1] For the subset of iterative, non-reducing polyketide synthases (NR-PKSs) found in fungi, TE domains have recently been shown to play a critical "editing" role during polyketide extension[2] in addition to terminal synthetic roles in carbon–carbon bond formation (Claisen/Dieckmann ring formation[1,3,4]) and cleavage (deacylation[5]). We report here discovery of the further function of a fungal TE domain to accelerate pyrone formation in the release of *nor*-toralactone (**1**) from the NR-PKS CTB1.

Cercosporin (**2**, Scheme 1) is a photoactivated phytotoxin produced by several *Cercospora* species.[6] The perylenequinone core of cercosporin is essential to its toxicity and acts as a photosensitizing agent upon absorption of visible light to produce singlet oxygen ($^1O_2$) and superoxide radical (O_2^-).[7] Cercosporin does not have a direct cellular target, but causes indiscriminate oxidative damage to cell membranes, proteins, lipids, and nucleic acids.[8]

The substituted perylenequinone nucleus of cercosporin confers the interesting property of atropisomerism. How chirality is established is a biosynthetic question of fundamental interest. The cercosporin biosynthetic gene cluster has been identified in *C. nicotianae*.[9] The first assembly step takes place on CTB1 (GenBank accession no. AAT69682), which contains the basis set of six catalytic domains characteristic of the NR-PKS family and a dual-tandem acyl-carrier protein (ACP_2), a common variant. The starter unit acyltransferase (SAT) domain of CTB1 has been shown to initiate polyketide extension by the selective utilization of acetyl-CoA,[10] which is elongated to the heptaketide **3** in the β-ketoacyl synthase (KS) domain by successive condensations with six malonyl units introduced by the malonyl acyltransferase (MAT) domain. The product template (PT) domain catalyzes C4–C9 and C2–C11 aldol cyclizations and dehydrations to a trihydroxynaphthalene, which is thought to be delivered to the thioesterase (TE) domain as **4** for product release.[6] We anticipated that the product of CTB1 would be the carboxylic acid **5** by conventional TE-mediated hydrolysis, but, in fact, pyrone **1** was the cleanly observed major product. The two simplest mechanisms (Scheme 1) that can be proposed for its formation are (**A**) canonical TE-mediated hydrolysis to **5**, which can be visualized to form the pseudoacid **6** and dehydrate to **1**, or (**B**) the TE-bound bicyclic intermediate **4** could be directly cyclized to **1*** by either rapid, spontaneous enol lactonization or TE-catalyzed pyrone formation—an unprecedented TE function.

Protein deconstruction has proved to be a powerful tool for determining PKS activity *in vitro*.[3] This method requires the identification of interdomain regions in multidomain proteins, which, in this instance, was achieved using bioinformatics methods.[11] In this way, constructs of mono- and multidomain protein fragments were prepared for heterologous expression. Recombination of these protein fragments to generate complete and partial systems *in vitro* led to reconstituted enzymatic activity and deduction of individual domain function.

The complete set of CTB1 domains (SAT-KS-MAT + PT + ACP_2 + TE) was combined in equimolar concentrations with acetyl-CoA and malonyl-CoA. The major product of this reaction (Fig. 1A) was **1** as determined by comparison to a standard prepared by total synthesis (See ESI†, Fig. S2). The appearance of **1** was unexpected. However, spontaneous intramolecular cyclization and dehydration of the bicyclic intermediate **4** could produce **1** without the intervention of the TE domain. We sought to characterize the TE domain to resolve this ambiguity.

In vitro reactions of CTB1 were carried out in which the TE domain was excluded (SAT-KS-MAT + PT + ACP_2).

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† Electronic supplementary information (ESI) available: Experimental details including cloning, protein expression and purification, [^{18}O]acetyl-CoA synthesis and purification, ACP activation, reconstitution reactions, analytical methods and *nor*-toralactone total synthesis. See DOI: 10.1039/c2cc36010a

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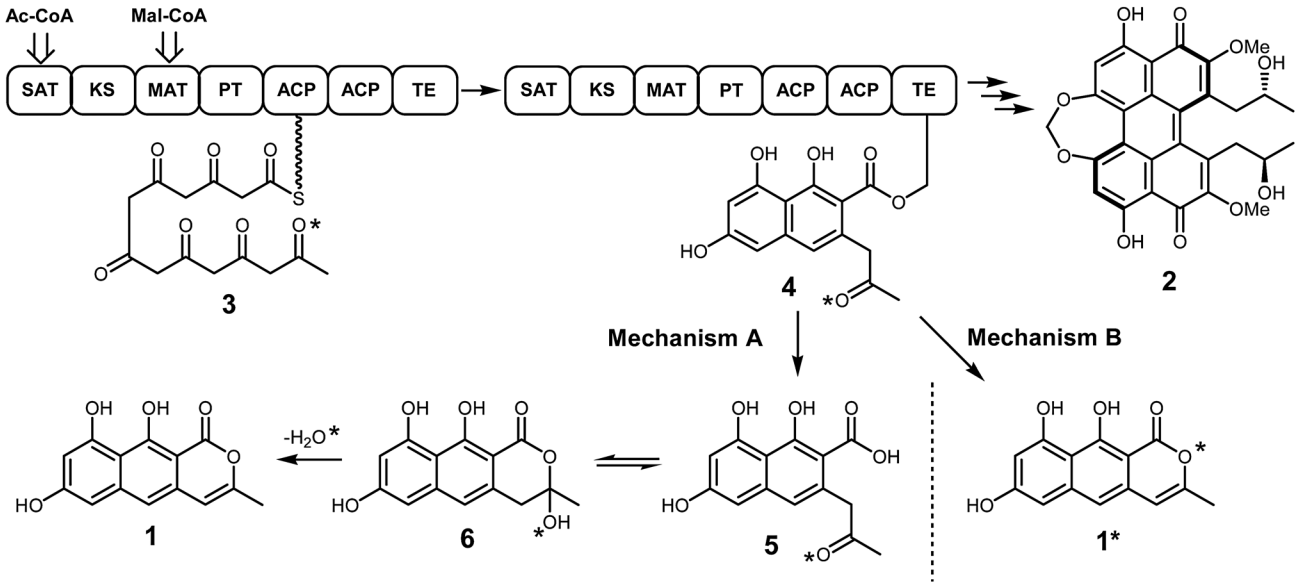

Scheme 1 Proposed mechanisms for *nor*-toralactone (**1**) biosynthesis by CTB1. Star indicates position of ^{18}O label.

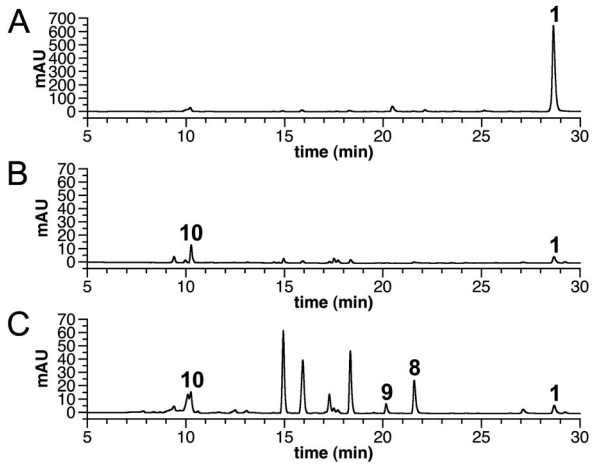

Fig. 1 HPLC chromatograms at 280 nm of product extracts for reactions of (A) a fully reconstituted CTB1, (B) a reconstituted CTB1 lacking the TE domain and (C) a minimally reconstituted CTB1 (SAT-KS-MAT + ACP_2).

The overall synthetic activity of CTB1 was dramatically reduced (*ca.* 50-fold) in these reactions as measured by total product output (Fig. 1B). Pyrone **1** was produced in small amounts while no other major product was observed. Without the TE domain, spontaneous pyrone formation was the likely release mechanism.[3,12] The low production of **1** indicated a slow rate of spontaneous release strongly suggesting a central role for the TE domain in accelerating pyrone formation.

In keeping with this observation, a mutant TE domain in which the catalytic Ser2008 was replaced with Ala was similarly unproductive (Fig. S3C, ESI†). However, when the catalytic His2171 was mutated to Gln, the TE domain demonstrated two thirds of wild type production (Fig. S3D, ESI†). The catalytic His is believed to serve as a general base during the formation of the acyl-enzyme intermediate and then again to facilitate product-releasing nucleophilic attack of the acyl-enzyme intermediate.[1,4] A different residue may serve this role in the mutant TE thereby allowing for the increased residual activity. Alternatively, functionally-related enzymes within the α/β-hydrolase family have been shown to be relatively tolerant of the His to Gln mutation.[13] It is proposed that these enzymes do not utilize the classical serine protease mechanism. Rather, the His Ser dyad promotes the formation of a reactive intermediate that directly activates the catalytic Ser for nucleophilic attack. It is unclear if such a mechanism is at play in CTB1.

A minimal CTB1 system in which both the PT and TE domains were excluded (SAT-KS-MAT + ACP_2) was proficient in acetate homologation, leading to a variety of "derailment" products (Fig. 1C). These derailment products were consistent with full-length heptaketide intermediates as determined by LCMS (Fig. S4, ESI†). Two such products were structurally characterized to be YWA1 (**8**)[12] and pannorin (**9**)[14] by comparison to known chemical properties and an authentic standard, respectively. These products were likely generated through spontaneous aldol cyclizations and intramolecular transesterification of the linear heptaketide intermediate **11** (Scheme 2A). Additionally, triacetic acid lactone (**10**)[15] was observed, a product of self-condensation from a truncated triketide intermediate (Scheme 2B). All of these side products were produced in significantly lower quantities when the PT or TE was present underscoring the central roles of these domains in both directed cyclization and overall catalytic efficiency.

Despite solidifying its overall role in catalysis, these observations did not address the mechanism by which the TE domain catalyzes the synthesis of **1**. As noted above, two possible courses of reaction were considered (Scheme 1). The fate of the polyketide oxygen at C13 is different for each mechanism. In pathway **A**, the oxygen would be lost as water, while in pathway **B** it would be retained as the endocyclic oxygen of the pyrone. The oxygen at this position is uniquely derived from the starter unit acetyl-CoA. This behavior allowed us to selectively label it through the use of [^{18}O]acetyl-CoA. Fortunately, CTB1 showed a strong "starter unit effect" in which the progenitor of the first acetate unit in homologation was predominantly transferred from the SAT domain.

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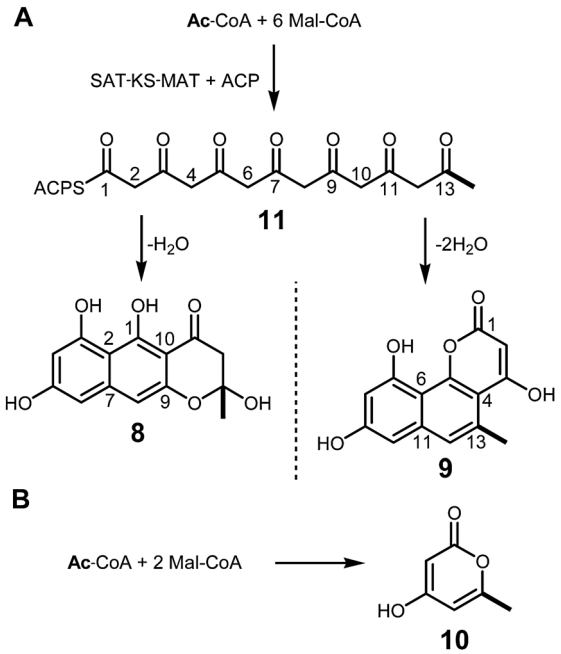

Scheme 2 (A) Spontaneous cyclization of liner heptaketide intermediate **11** led to production of YWA1 (**8**) and pannorin (**9**). (B) Truncated triketide product triacetic acid lactone (**10**). Highlighted acetate unit is derived from acetyl-CoA.

Reconstitution reactions in which acetyl-CoA was withheld showed less than a quarter of the production of **1** by HPLC relative to reactions in which both acetyl-CoA and malonyl-CoA were included (Fig. S5, ESI†). The residual activity was believed to arise from acetyl-CoA contamination of malonyl-CoA stocks through spontaneous decarboxylation, but may also arise from PKS-mediated decarboxylation following malonyl loading.

Reconstitution reactions were carried out as before with either unlabeled acetyl-CoA or [^{18}O]acetyl-CoA. The products were analyzed by LCMS to measure heavy isotope content. Pyrone **1*** produced by the fully reconstituted CTB1 showed a distinct mass shift consistent with the incorporation of a single ^{18}O atom (*m*/*z* 261.0651 [MH$^+$]). However, a minor portion, estimated at about 23% by relative signal intensities, did not contain the heavy isotope (*m*/*z* 259.0610 [MH$^+$], Fig. S6, ESI†). This fraction was attributed to unlabeled acetyl incorporation, as the proportion was similar to the residual production in reactions described above where acetyl-CoA was withheld. In sum, these incorporation data were fully consistent with mechanism **B**.

In most derailment products, ^{18}O incorporation was seen in the minor component relative to the unlabeled portion. The loss of heavy isotope is likely due to facile exchange of the labeled oxygen with the aqueous medium. Compound **8** showed very little ^{18}O incorporation (*m*/*z* 277.0691 [MH$^+$, 100%], 279.0725 [3]). The label in this product would be incorporated into a hemiketal and expected to readily exchange. No ^{18}O incorporation was observed in the derailment product **9** (*m*/*z* 259.0596 [MH$^+$, 100%]), as the labeled oxygen is lost as water during aromatization of the central ring. On the other hand, compound **10** was substantially enriched in ^{18}O (*m*/*z* 129.0430 [MH$^+$, 100%], 127.0392 [37]). The labeled atom in this α-pyrone is the endocyclic oxygen, a position that is expected to be stable in aqueous solvents.

The isotope incorporation data are unambiguous that **1** is the direct product of CTB1. The mechanism by which the TE domain of CTB1 favors pyrone formation over hydrolysis is not precisely known. It is likely that the thioesterase promotes keto/enol tautomerization in the active site thereby favoring enol lactonization. In contrast, TE substrates undergoing Claisen cyclization for product release contain a β-diketone side chain,[3,4] not a methyl ketone as in **1**, whose lower pK_a (*ca.* 9 *vs.* 19) doubtless plays a determining role in the catalysis of carbon–carbon bond formation. This observation marks a further expansion of the chemistry catalyzed by NR-PKS thioesterase domains.[1]

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