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Resorcylic Acid Lactone Biosynthesis Relies on a Stereotolerant Macrocyclizing Thioesterase

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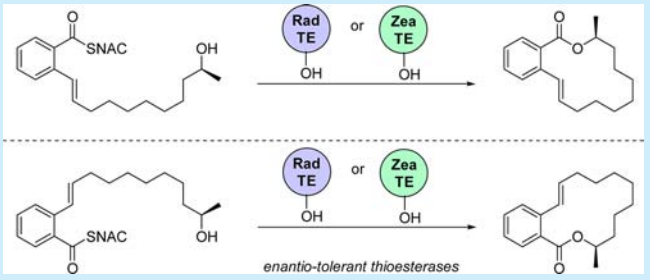
Supporting Information

ABSTRACT: Zearalenone and radicicol are highly related resorcylic acid lactones with the rare property of having opposite stereochemical configurations of the secondary alcohol involved in lactone formation. The ability of the thioesterases from the zearalenone and radicicol biosynthetic pathways to macrocyclize both D and L configured synthetic substrate analogs was biochemically characterized and showed that both enzymes were highly stereotolerant, macrocyclizing both substrates with similar kinetic parameters. This observed stereotolerance is consistent with a proposed evolution of both natural products from a common ancestral resorcylic acid lactone.



Resorcylic acid lactones (RALs) are a class of macrocyclic fungal polyketides all containing a resorcylate (2,4-dihydroxybenzoate) typically embedded into a 14-member ring lactone.[1,2] Zearalenone **1**, an estrogen receptor agonist,[3] and radicicol **2**, a HSP90 inhibitor,[4] are archetypical examples of the class (Figure 1). Unusual among closely related

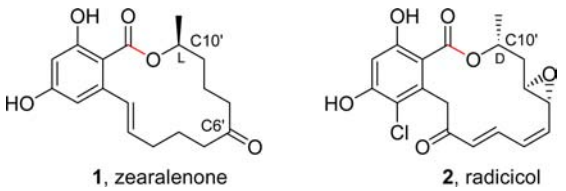


Figure 1. Fungal polyketides zearalenone **1** and radicicol **2** have the opposite configurations at C10′. Lactone bond formed by the TE is highlighted in red.

macrocyclic polyketides are the opposing configurations of the lactone alcohol group seen in zearalenone with the L (*S*) configuration and in radicicol, with the D (*R*) configuration. Apart from the RALs, this phenomenon is only observed in the mixed nonribosomal peptide-polyketide depsipeptides, the turnagainolides.[5] From a biosynthetic perspective this unusual feature provides a unique glimpse into the evolutionary process of accessing new structural features from an ancestral compound. Herein we show that the thioesterases (TEs) responsible for macrocyclizing these RALs are stereotolerant making them ideal as potential biocatalysts and showing the unique plasticity of fungal polyketide biosynthetic pathways.

RAL biosynthesis (Figure 2) is catalyzed by two iterative polyketide synthase (PKS) proteins, a highly reducing PKS (hrPKS) and a nonreducing PKS (nrPKS).[6−17] The hrPKS has a full complement of reductive domains and generates the alkyl portion of the RALs. The alcohol required for macrocyclization

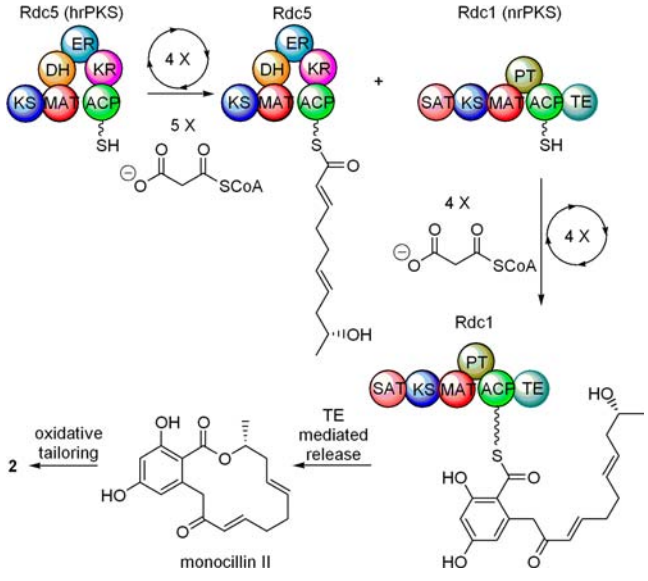


Figure 2. Biosynthesis of the polyketide precursor of radicicol, monocillin II by Rdc5 and Rdc1 is typical of RAL biosynthesis.

as well as other functional groups such as the oxygenation in **1** or the olefins in **2** are introduced by cryptic programming that enables the hrPKS to skip various reductive domains based on the length of the growing chain. The nrPKS, which lacks all reductive domains, takes the hrPKS product and adds additional malonate units generating a poly β-keto intermediate that is cyclized by a product template domain[18,19] into the resorcylate group. The completed polyketide chain is then

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released via macrocylization from the nrPKS by a thioesterase (TE) domain.[20]

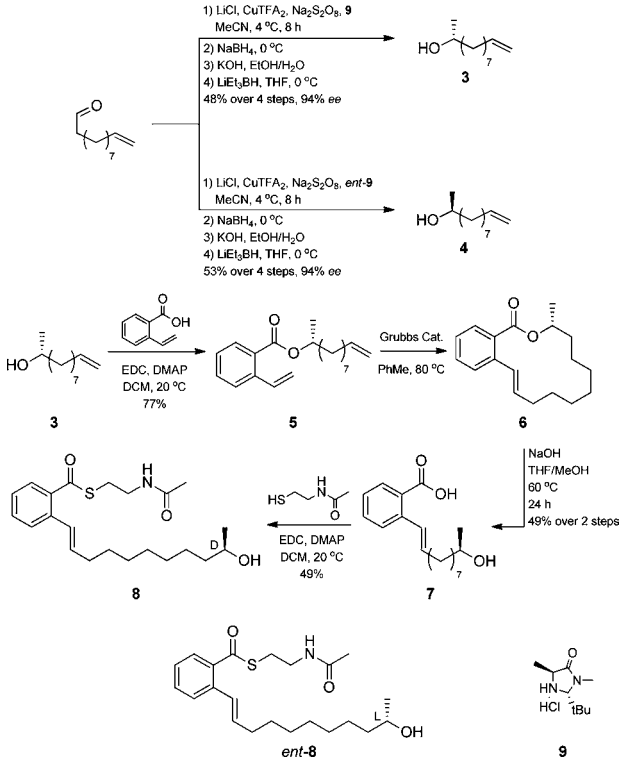
The iterative nature of the hrPKS coupled with its cryptic programming enables the alkyl chains of the highly related RALs to differ substantially. For example, recent work by Vederas and Tang on the RAL pathway for hypothemycin biosynthesis shows that the ketoreductase (KR) facial selectivity for hydride delivery changes based on overall chain length.[17] This observation explains why a single KR from the hrPKS of the hypothemycin and by analogy zearalenone biosynthetic pathways can generate alcohols at C10′ and C6′ (which is ultimately oxidized to a ketone) with L and D stereochemistry, respectively. The first reduction by the hrPKS sets the chemistry at C10′, and this configuration can be inverted to that seen in radicicol by replacement of the $\beta_5\alpha_5\alpha_6$ motif in the KR active site with the sequence from the radicicol hrPKS KR. This is of particular relevance to macrocyclization as this alcohol is involved in formation of the macrolactone in these natural products.

Characterization of full length nrPKS proteins from RAL pathways suggests that the TEs embedded into these proteins are capable of macrocyclizing both L and D configured substrates.[12,14,17] *In vivo* and *in vitro* work with Hpm3, the nrPKS from hypothemycin biosynthesis, shows that both the native D and epimeric L configured macrocycles can be obtained.[14,17] Similarly *in vivo* characterization of Rdc1, the nrPKS from radicicol biosynthesis, showed that both the native D and enantiomeric L macrocycle could be generated.[12] This stereotolerant activity would be in stark contrast to their bacterial analogues[21,22] and represent unique activity for TE domains. However, as the TE has only been characterized in the context of the full length nrPKS, it is unclear if the TE is stereotolerant or stereoselective but much faster than the other steps in nrPKS substrate processing. To resolve this issue, *in vitro* biochemical characterization of isolated RAL TEs is required.

While the structural similarities between **1** and **2** indicate that the biosynthetic pathways are highly related, analysis of the entire PKS protein sequence shows that they share less than 29% identity and thus must have diverged from a common ancestor long ago. This ancient divergence is supported by the differences in gene cluster synteny.[23,24] The orientation of the PKS genes is maintained in the zearalenone and radicicol clusters; however, additional tailoring genes are inserted between the two PKS genes in the radicicol cluster. With substantial time for divergent evolution, it is reasonable to hypothesize that the TEs (47% identity) from the pathways for **1** and **2** could have specialized to macrocyclize their substrates stereoselectively. We thus expected to observe substantial kinetic stereoselectivity for the TE domains from zearalenone biosynthesis (Zea TE) and radicicol biosynthesis (Rad TE).

To examine the stereoselectivity of the Zea TE and Rad TE, we synthesized enantioenriched substrates mimicking the native linear completed polyketide intermediates. The substrates were designed to be synthetically tractable and differ only in the absolute stereochemistry of the lactone oxygen. The synthesis of these substrates is shown in Scheme 1. The key step was the use of a MacMillan diastereoselective α-chlorination[25] followed by reduction and epoxidation with inversion of configuration to generate the terminal epoxide, which could be reductively opened to deliver alcohols **3** and **4** in reasonable yield and excellent *ee* (94% *ee*, Scheme 1). This methodology represents a significant advantage over other methodologies used in the

Scheme 1. Synthesis of Eneantioenriched Substrate 8[a]



[a]*ent*-**8** was synthesized in an analogous manner from **4**, see the Supporting Information.

synthesis of RAL natural products[1,2] such as the Jacobsen hydrolytic kinetic resolution (HKR)[26] or the use of expensive asymmetric epoxides, such as enantioenriched propylene oxides.[27]

The enantioenriched alcohols were coupled to vinylbenzoic acid, which can be readily accessed from ethyl 2-bromobenzoate through a Hiyama coupling[28] and subsequent hydrolysis (see the Supporting Information). The resulting ester, **5**, was macrocyclized by treatment with Grubbs second generation metathesis catalyst. The resulting alkene containing macrocycle was found to be entirely *E*-configured (>95:5) as the *Z*-configured macrocyle is strongly disfavored due to transannular ring strain in the 14-member macrocycle. Hydrolysis followed by coupling with *N*-acetylcysteamine generates the two enantioenriched thioester substrates, **8** and *ent*-**8**. The *N*-acetylcysteamine thioester is employed to activate the carboxylate for reaction with the active site Ser of the TE and to mimic the phosphopantetheine arm of the ACP domain which delivers the linear polyketide to the TE *in vivo*.[11,21,22,29−39] While the *N*-acetyl cysteamine group does not provide the hydrogen bond interaction seen between the native phosphopantetheine arm and the TE,[40] it has been demonstrated to be effective *in vitro*.[11,21,22,29−39]

The excised Zea TE was recombinantly expressed and purified as previously described.[11] The Rad TE was generated by PCR amplification of the TE domain of *rdc1* from *Pochonia chlamydosporia*.[9] The excised Rad TE gene was cloned into an *Escherichia coli* expression vector under the control of the T7 promoter. Rad TE was overexpressed and purified by metal-affinity chromatography to high purity (>90%, see the Supporting Information).

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Treatment of **8** and *ent*-**8** with recombinant, purified Zea TE and Rad TE showed that both TEs are stereotolerant, effectively macrocyclizing the L and D configured substrates (Figure 3). Kinetic analysis of macrocycle formation was performed by a discontinuous HPLC-based assay. TEs (5 μM except 2 μM for Rad TE with **8**) were incubated at room temperature with **8** or *ent*-**8** at concentrations between 0.1−5.0 mM in 50 mM phosphate buffer (pH 7.4) with 10% *v*/*v* DMSO. Aliquots were taken during the first 15 min of the reaction and analyzed by HPLC for macrocycle production. A standard curve based on authentic macrocycle was used to quantify production. All time points were within the linear range of initial velocities for product formation. Rad TE macrocyclized both substrates (**8**, k_{cat} = 0.180 ± 0.003 s^{-1}, K_M = 0.19 ± 0.02 mM, k_{cat}/K_M = 970 ± 180 M^{-1} s^{-1}; *ent*-**8**, k_{cat} = 0.15 ± 0.01 s^{-1}, K_M = 0.14 ± 0.03 mM, k_{cat}/K_M = 1100 ± 400 M^{-1} s^{-1}, K_i = 4.5 ± 0.9 mM). Zea TE macrocyclized both substrates at slower rates (**8**, k_{cat} = 0.088 ± 0.001 s^{-1}, K_M = 0.39 ± 0.02 mM, k_{cat}/K_M = 230 ± 70 M^{-1} s^{-1}; *ent*-**8**, k_{cat} = 0.06 ± 0.01 s^{-1}, K_M = 1.4 ± 0.6 mM, k_{cat}/K_M = 40 ± 20 M^{-1} s^{-1}). Macrocycle formation was confirmed in all cases by MS analysis. Little hydrolysis of either substrate to the seco acid was observed (Figure 3 and Figure S3), which is in agreement with our previous study of macrocyclization of a primary alcohol substrate by Zea TE.[11] In contrast *in vitro* studies of macrocyclization by bacterial PKS TEs from the picromycin[35] and epothilone C[32] pathways showed significant hydrolysis even when presented the SNAC thioester of their native substrates. Intriguingly, Rad TE generated substantial glycerol ester when incubated for prolonged periods with *ent*-**8** (Figure 3D).

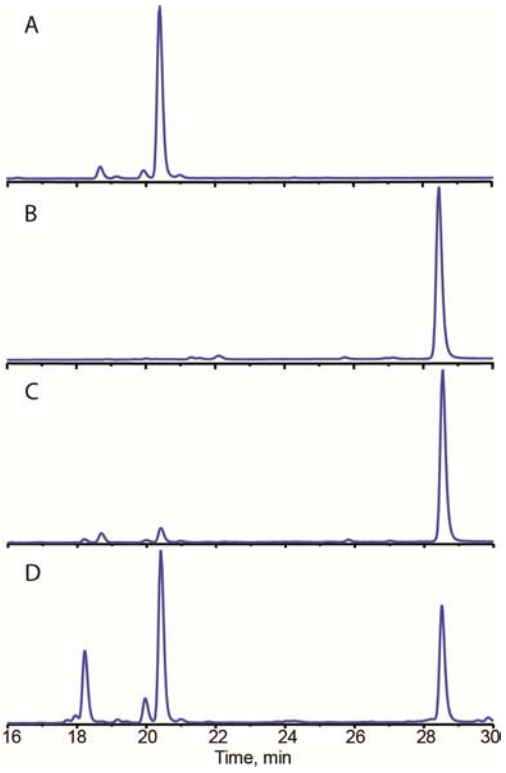

Figure 3. HPLC traces for incubations of **8** and *ent*-**8** with Rad TE. Incubations of Zea TE with substrates are in the Supporting Information. (A) No enzyme blank (5 mM **8**, 50 mM phosphate buffer pH 7.4, 24 h, rt); boiled enzyme blank showed a comparable result. (B) Racemic macrocycle, **6** (see the Supporting Information for synthesis). (C) **8** with Rad TE (5 mM **8**, 5 μM Rad TE, 50 mM phosphate buffer pH 7.4, 24 h, rt). (D) *ent*-**8** with Rad TE (5 mM *ent*-**8**, 5 μM Rad TE, 50 mM phosphate buffer, pH 7.4, 24 h, rt). The peak at 18 min is the glycerol ester of *ent*-**8**. *ent*-**8** concentration is at K_i leading to lower conversion than seen for **8**.

We also noted that with increasing concentration of the L substrate, *ent*-**8**, the rate of macrocyclization with the Rad TE decreased. Our data was modeled exceptionally well (R^2 = 0.9807) by Copeland's model for substrate inhibition (Equation S1).[41] This model assumes a second molecule of substrate binds, allosterically, to the substrate-enzyme complex. One other allosteric interaction with a polyketide TE was previously reported by Scaglione et al. in the tautomycetin pathway.[39] Unlike the Rad TE's inhibitory interaction, this study found a cooperative allosteric interaction. These findings should be interpreted with care as they are the result of *in vitro* assays with the TE removed from the context of its native pathway and treated with non-native levels of substrates.[42]

A working hypothesis in the field has been that nucleophile stereochemistry plays an important role in controlling TE-mediated macrocyclization. While this hypothesis is supported for bacterial PKS TEs,[21,22] it does not appear to hold true for fungal RAL TEs. This study provides clear *in vitro* kinetic characterization of stereotolerant PKS TEs. The relaxed substrate selectivity of these RAL TEs makes them appealing candidates for use in engineered combinatorial PKS pathways.[43] In addition, this activity warrants further study of the substrate scope of these TEs, as they may function as general macrocyclization catalysts for chemoenzymatic syntheses.

The screening hypothesis, an evolutionary model for describing the chemical diversity of natural products, predicts that there is an evolutionary cost to having high selectivity in the late stage of natural product biosynthesis.[44,45] The low stereoselectivity of RAL TEs is thus consistent with minimizing this evolutionary cost. Furthermore, by viewing the biosynthetic pathway as a series of logic gates where each enzymatic step asks a different "question" about the structure of the substrate, a stereoselective TE would be asking a redundant "question" since the stereochemistry of the nucleophilic alcohol is tightly controlled by an upstream process, the KR domain of the hrPKS. We propose that this substrate flexibility increases fitness and adaptability of the pathway as it enables the overall pathway to accommodate changes to the linear polyketide product brought about by mutations that impact the cryptic programming of the hrPKS and thus the configuration as well as steric and electronic environment of the nucleophilic alcohol. This TE stereotolerance may have facilitated the divergent evolution of the oppositely configured macrolactones from a common RAL ancestor.

In summary, we have kinetically characterized *in vitro* two fungal RAL PKS TEs for their ability to macrocyclize substrates with D and L nucleophile stereochemistry. We show that these RAL TEs are stereotolerant and macrocyclize either enantio-configured substrate with very little competing hydrolysis. This stereotolerance is in accordance with the prediction of the screening hypothesis and enables the RAL pathways to adapt to changes in the polyketide substrate due to the cryptic programming of the hrPKS. In comparison bacterial PKS TEs are highly stereoselective even though the screening hypothesis prediction should be equally true for them. Resolving the discrepancy between these two types of TEs will give much

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needed insight into the different evolutionary pressures shaping iterative and modular PKS pathway evolution.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic protocols, experimental procedure, and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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