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Article

Functional Characterization of the Biosynthesis of Radicicol, an Hsp90 Inhibitor Resorcylic Acid Lactone from *Chaetomium chiversii*

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SUMMARY

Fungal polyketides with the resorcylic acid lactone (RAL) scaffold are of interest for growth stimulation, the treatment of cancer, and neurodegenerative diseases. The RAL radicicol is a nanomolar inhibitor of the chaperone Hsp90, whose repression leads to a combinatorial blockade of cancer-causing pathways. Clustered genes for radicicol biosynthesis were identified and functionally characterized from the endophytic fungus *Chaetomium chiversii*, and compared to recently described RAL biosynthetic gene clusters. Radicicol production is abolished upon targeted inactivation of a putative cluster-specific regulator, or either of the two polyketide synthases that are predicted to collectively synthesize the radicicol polyketide core. Genomic evidence supports the existence of flavin-dependent halogenases in fungi: inactivation of such a putative halogenase from the *C. chiversii* radicicol locus yields dechloro-radicicol (monocillin I). Inactivation of a cytochrome P450 epoxidase furnishes pochonin D, a deepoxy-dihydro radicicol analog.

INTRODUCTION

Fungal polyketides sharing the resorcylic acid lactone (RAL) scaffold display an impressive array of biological activities (Winssinger and Barluenga, 2007). Zearalenone (**4**) (Figure 1), isolated from different *Fusaria* (teleomorph: *Gibberella*) species, is an estrogen agonist used for growth promotion in cattle (Winssinger and Barluenga, 2007). Hypothemycin (**5**) (Figure 1) and similar RALs with a *cis*-enone moiety are highly specific inhibitors of a subset of the mitogen-activated protein (MAP) kinases that coordinate the responses of the cell to its environment (Winssinger and Barluenga, 2007). Radicicol (**1**) and its natural analogs, monocillin I (**2**) and pochonin D (**3**) (Figure 1), bind to the Bergerat-fold ATP-binding pocket of the GHKL superfamily proteins, including heat shock protein 90 (Hsp90) (Roe et al., 1999). The chaperone Hsp90 is essential for the folding and activation of a wide range of oncogenic proteins but processes very few housekeeping enzymes. Inhibition of Hsp90 promotes the degradation of the oncogenic clients and leads to the combinatorial blockade of multiple cancer-causing pathways (McDonald et al., 2006). Hsp90 inhibitors also reduce protein aggregation in models of several neurodegenerative diseases (Luo et al., 2007) and increase thermotolerance in plants (McLellan et al., 2007). Radicicol and geldanamycin (Figure 1), a bacterial ansamycin whose derivatives are undergoing clinical trials, are competitive inhibitors binding to the same target (Roe et al., 1999). Radicicol is the most potent inhibitor of Hsp90 in vitro, but the reactivity of its epoxide group, and the sensitivity of its conjugated double bonds to Michael additions, renders it inactive in vivo (Winssinger and Barluenga, 2007). Intensive synthetic efforts are directed at radicicol-like RALs that might overcome this metabolic instability (Dakas et al., 2007; Winssinger and Barluenga, 2007).

Fungal polyketides are biosynthesized from malonyl-CoA precursors by polyketide synthases (PKS) that resemble a single module of a bacterial type I PKS (Cox, 2007). Fungal PKSs incorporate a single ketoacyl synthase (KS) domain for the recursive decarboxylative condensations of the precursors, covalently loaded as acyl thioesters onto the acyl carrier protein (ACP) domain(s) by a single acyltransferase (AT) domain. Fungal nonreducing PKSs (nrPKSs) producing unreduced polyketide chains additionally feature an N-terminal starter unit acyltransferase (SAT) domain (Crawford et al., 2006), a centrally located product templating (PT) domain (Crawford et al., 2008a), and a C-terminal thioesterase (TE) domain. Fungal highly reducing PKSs (hrPKSs) producing reduced polyketide chains do not contain SAT, PT, or TE domains but house single catalytic domains with ketoacyl reductase (KR), dehydratase (DH), and enoyl reductase (ER) activities. Fungal nrPKSs and hrPKSs orchestrate recursive biosynthetic processes involving the iterative use of their catalytic domains, where the programming rules that determine polyketide chain length, reduction (if any) of the β-ketoacyls, and the regiospecific cyclization of the final polyketide products remain obscure (Cox, 2007).

Fungal RALs contain a resorcylate moiety (derived from an unreduced polyketide chain) bridged by a 12- or 14-member macrolactone ring (derived from a reduced polyketide chain).

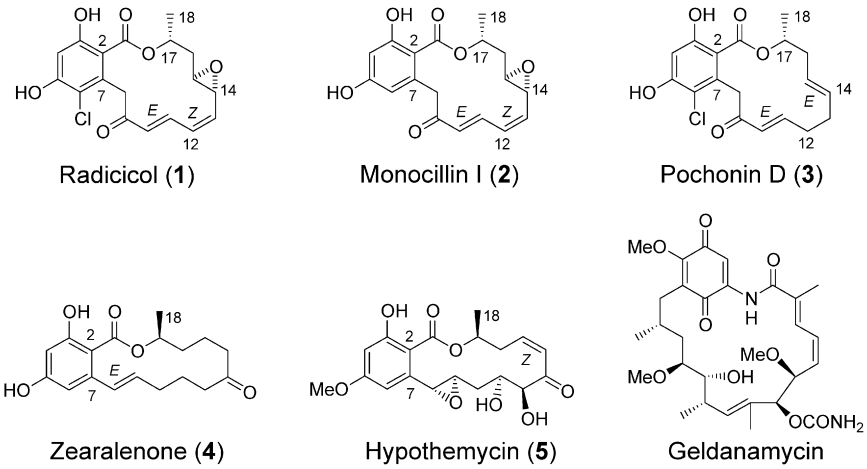

Figure 1. Structures of RALs and Geldanamycin

Whereas most fungal polyketides are biosynthesized by a single PKS, the assembly of RALs requires both an hrPKS and an nrPKS, encoded by clustered genes (Gaffoor and Trail, 2006; Kim et al., 2005; Reeves et al., 2008). Thus, the zearalenone hrPKS (PKS4) was proposed to biosynthesize a reduced hexaketide, corresponding to C18–C7. This advanced starter unit is condensed with three additional malonyl-CoA extender units on the cognate nrPKS (PKS13). The formation of the acyl resorcylate by aldol condensation, and the release of the macrolactone by intramolecular ester bond formation, are also carried out by the nrPKS. The results of the recent enzymatic characterization of PKS13 are congruent with this model, and show that PKS13 is a versatile resorcylic acid macrolactone synthase (Zhou et al., 2008). While this manuscript was in review, a report on the characterization of the hypothemycin biosynthetic gene cluster from *Hypomyces subiculosus* and the sequencing of a putative radicicol biosynthetic locus from *Pochonia chlamydosporia* has been published, proposing similar biosynthetic models (Reeves et al., 2008).

The presence of a chlorine atom at C6 contributes to the cytotoxicity of radicicol (**1**) but has only a small influence on Hsp90 binding (Turbyville et al., 2006). In contrast, the dechloro analog of pochonin D (**3**) is attenuated in its binding to Hsp90 (Moulin et al., 2005). Although halogenated small-molecule natural products are abundant in nature, substrate- and regiospecific halogenase enzymes have only recently been characterized from various prokaryotic secondary metabolite producers (Neumann et al., 2008; van Pee et al., 2006). Flavin-dependent halogenases that generate hypohalite equivalents are prevalent among these enzymes (Hornung et al., 2007). Although sequence similarities indicated that the putative radicicol biosynthetic locus of *P. chlamydosporia* encodes a deduced halogenase (Reeves et al., 2008), no flavin-dependent or any other substrate- and regiospecific halogenase enzymes have been functionally characterized from fungi.

In a screening program for Hsp90 inhibitors from fungal isolates of the Sonoran desert, we have reisolated radicicol from *Chaetomium chiversii*, an endophyte of the Mormon tea (*Ephedra fasciculata*), and monocillin I from *Paraphaeosphaeria quadriseptata*, occurring in the rhizosphere of the Christmas cactus (*Opuntia leptocaulis*) (Turbyville et al., 2006). Both strains were also shown to produce the alkyl isocoumarin congeners of their respective RALs (Wijeratne et al., 2006). Here we present a comparative sequence analysis of the radicicol biosynthetic gene cluster of *C. chiversii*, including genomic evidence for a conserved family of putative flavin-dependent halogenases in fungi. Targeted gene disruptions and isolation of the corresponding metabolites, if any, provided functional proofs for the roles of an hrPKS, an nrPKS, a flavin-dependent halogenase, a cytochrome P450, and a positive regulator in radicicol biosynthesis, and support a unifying model for the biosynthesis of radicicol/monocillin/pochonin-type RALs and their isocoumarin congeners.

RESULTS

Isolation of the Radicicol Biosynthetic Locus

We have used several pairs of PCR primers for the amplification of PrnC-type flavin-dependent halogenase and separately hrPKS gene segments from *C. chiversii* CS-36-62 genomic DNA as described in Supplemental Data (available online). One of the resulting amplicons encoded an hrPKS segment with high similarity (58% identity/69% similarity) to the zearalenone hrPKS (PKS4; GenBank accession numbers throughout; ABB90283), whereas another amplicon obtained with the halogenase-specific primers encoded a protein segment with high similarity to bacterial halogenases (e.g., ABM21576, 43% identity/65% similarity). Both PCR products were used as probes to screen a genomic DNA library of *C. chiversii*. Two nonoverlapping sets of fosmids were identified that hybridized with one or the other probe only, proving that the two amplicons originated from distinct genomic loci. Sequence sampling of the fosmid set hybridizing with the halogenase-like probe revealed genes for a putative pfam04820 halogenase-*O*-methyltransferase bifunctional protein (*ccHmt*) and an hrPKS (*ccPks01*). The deduced CcPKS01 showed only low similarity to the zearalenone hrPKS (39% identity/56% similarity). In contrast, sequencing three representative fosmids covering the zearalenone hrPKS-like amplicon revealed a 45,229 bp contig (Table 1) with clustered genes encoding an hrPKS (CcRADS1) and an nrPKS (CcRADS2), both highly similar to the zearalenone PKS pair. Further genes in the cluster (Figure 2A) encoded a putative pfam04820 halogenase (RadH), a cytochrome P450 (CYP, RadP), a major facilitator superfamily (MFS) transporter (RadE), and a bZIP leucine zipper transcription factor (RadR), all with plausible functions in radicicol biosynthesis (see below). The cluster is bordered by open reading frames (ORFs) encoding housekeeping enzymes (Orf1, a putative trehalose-6-phosphate synthetase/trehalose phosphatase and Orf3, a cystathionine γ-synthase/β-lyase). Two conserved hypothetical proteins

Table 1. Similarities of the Deduced Proteins from the *C. chiversii* Radicicol Biosynthetic Locus

Gene model	Protein length (aa)	Homolog[a], *Organism*[b], Function of the homolog	Identity / Similarity (%)	Conserved domain	E-value[c]
orf1	1008	**CAP65155**, *P. anserina*, unnamed protein	79 / 88	COG0380, trehalose-6-phosphate synthase	$4e^{-118}$
				pfam02358, trehalose phosphatase	$9e^{-60}$
orf2	154	**EAQ92919**, *C. globosum*, hypothetical protein	64 / 67	COG3791, uncharacterized conserved protein	$1e^{-06}$
radH	520	**ACD39771**, *P. chlamydosporia*, Rdc2 halogenase	74 / 84	pfam01494, FAD-binding domain	0.002
		EAQ86388, *C. globosum*, hypothetical protein	54 / 70	pfam04820, Tryptophan halogenase	$3e^{-09}$
ccRads2	2138	**ACD39770**, *P. chlamydosporia*, Rdc1 nrPKS	61 / 75	SAT-KS-AT-PT-ACP-TE	
		ACD39753, *H. subiculosus*, Hpm3 nrPKS	57 / 73		
		ABB90282, *G. zeae*, PKS13 nrPKS	57 / 71		
radR	453	**EAA32450**, *N. crassa*, hypothetical protein	45 / 62[d]	pfam00170, bZIP leucine zipper transcription factor	0.004
radE	538	**ACD39772**, *P. chlamydosporia*, Rdc3 MFS transporter	64 / 76	pfam07690, major facilitator superfamily	$2e^{-16}$
		ACD39756, *H. subiculosus*, Hpm6 MFS transporter;	27 / 45		
		EAS30292, *Co. immitis*, MFS transporter	49 / 69		
ccRads1	2431	**ACD39774**, *P. chlamydosporia*, Rdc5 hrPKS	68 / 81	KS-AT-DH-ER-KR-ACP	
		ACD39758, *H. subiculosus*, Hpm8 hrPKS	60 / 75		
		ABB90283, *G. zeae*, PKS4 hrPKS	61 / 76		
radP	501	**ACD39773**, *P. chlamydosporia*, Rdc4 CYP	69 / 80	pfam00067, cytochrome P450	$4e^{-55}$
		ACD39751, *H. subiculosus*, Hpm1 CYP	33 / 52		
		EDU51262, *Py. tritici-repentis*, CYP	43 / 62		
orf3	588	**EAQ91923**, *C. globosum*, cystathionine γ-synthase	59 / 72	COG0626, cystathionine β-lyases / cystathionine γ-synthases	$3e^{-44}$
orf4	1391	**EAQ91930**, *C. globosum*, predicted protein	37 / 52[d]	ND[e]	NA[f]
orf5	301	**EAQ91943**, *C. globosum*, tricarboxylate transporter	93 / 96	pfam00153, mitochondrial carrier protein	$1e^{-15}$ [g]
					$1e^{-11}$ [g]
					$1e^{-07}$ [g]

[a] Closest homolog in the *P. chlamydosporia* radicicol and *H. subiculosus* hypothemycin biosynthetic loci (if applicable), or in the rest of GenBank.
[b] Genus abbreviations: *C*, *Chaetomium*; *Co*, *Coccidioides*; *G*, *Gibberella*; *H*, *Hypomyces*; *N*, *Neurospora*; *P*, *Podospora*; *Py*, *Pyrenophora*.
[c] E value for the match against the conserved domain.
[d] Similarity on a shorter protein segment only.
[e] ND, not detected.
[f] NA, not applicable.
[g] Three tandem copies of this conserved domain.

(Orf2 and Orf4) and a tricarboxylate transporter (Orf5) are also encoded in the sequenced locus (Table 1).

CcRADS1, CcRADS2, and RadR Are Essential for Radicicol Biosynthesis

To ascertain the function of the putative radicicol biosynthetic cluster, internal fragments of the putative PKSs *ccRads1* and *ccRads2*, as well as the disconnected *ccPks01*, were replaced on the *C. chiversii* genome with the *hph* hygromycin resistance marker gene using *Agrobacterium*-mediated transformation (Zhang et al., 2003). The *radR* gene encoding a putative cluster-specific regulator was similarly targeted for gene replacement. Only 1%–5% of the hygromycin-resistant progeny originated from double-homologous recombination in the targeted

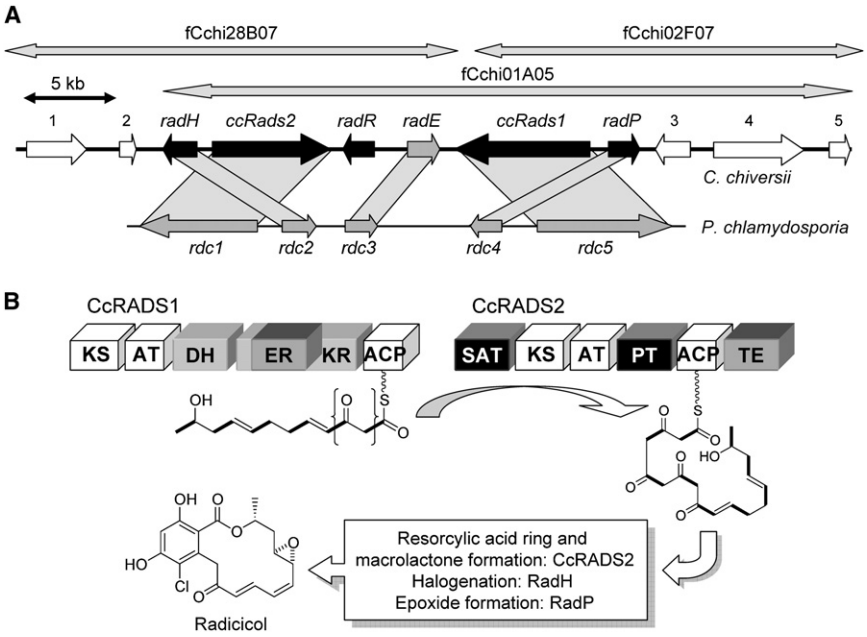

Figure 2. The Radicicol Biosynthetic Locus of *C. chiversii* and Proposed Biosynthesis of Radicicol

(A) White arrows: open reading frames encoding hypothetical proteins; black arrows: genes shown to be involved in radicicol biosynthesis by gene disruption; gray arrows: genes proposed to be involved in radicicol biosynthesis; two-way gray arrows: the sequenced fosmids. The putative radicicol biosynthetic locus of *P. chlamydosporia* (Reeves et al., 2008) is shown for comparison.

(B) Model for the biosynthesis of radicicol. The timing of macrolactone release versus polyketide tailoring following resorcylic acid ring closure is not determined.

genes (Figures 3A and 3B). All transformants with ectopic or homologous integration of the disruption cassettes showed unaltered morphology. Radicicol production was unaffected by ectopic integration of any of the disruption cassettes. Similarly, radicicol production was undisturbed by the targeted disruption of the *ccPks01* gene (Figure 3D). However, radicicol production was completely and specifically abolished in all the isolates with the *ccRads1*, *ccRads2* (Figures 3E and 3F), or the *radR* knockouts (data not shown). Thus, the CcRADS1 and CcRADS2 PKSs and the putative RadR regulator, but not the CcPKS01 PKS, are necessary for radicicol biosynthesis in *C. chiversii*. Whereas the recombinant zearalenone nrPKS PKS13 was reported to accept short-chain fatty-acid starters from primary metabolism to produce acyl resorcylates in vivo (Zhou et al., 2008), no significant amounts of shunt products were seen to be produced by either *C. chiversii* RADS knockout strains.

The deduced CcRADS1 (Figure 2B) is a 2431 amino acid, 263.5 kDa enzyme belonging to the fungal clade I hrPKSs (Baker et al., 2006), with 61% identity and 76% similarity to PKS4, the zearalenone biosynthetic hrPKS (ABB90283). CcRADS1 also shows 60% identity and 75% similarity to Hpm8, the hypothemycin hrPKS from *H. subicolosus* (ACD39758), and 68% identity and 81% similarity to Rdc5, the putative radicicol biosynthetic hrPKS from *P. chlamydosporia* (ACD39774), both described concurrently with our work (Reeves et al., 2008). These RAL hrPKSs all share identical domain compositions: KS-AT-DH-ER-KR-ACP. Their reductive domains are predicted to be fully active based on their conserved amino acid sequence motifs and the structures of the reduced polyketide portions of their RAL products. Thus, the DH domains feature the HxxxGxxxxP active site motif that encompasses part of the catalytic His/Glu dyad (Khosla et al., 2007), albeit with the central Gly shifted one amino acid to the N terminus (CcRADS1: amino acids 973–985). The ER and KR domains house well-conserved NADPH-binding motifs (CcRADS1: amino acids 1848–1857 and 2055–2064, respectively), with the KR domains carrying the full Lys-Ser-Tyr catalytic triad (CcRADS1: K2159, S2184, Y2197) (Khosla et al., 2007). These hrPKSs are predicted to initiate RAL biosynthesis by assembling the reduced polyketide chains that serve as starter units for the corresponding nrPKSs (Figure 2B, see below).

The deduced CcRADS2 is a 2138 amino acid, 230.5 kDa enzyme belonging to the fungal clade I nrPKSs (Baker et al., 2006), with 57% identity and 71% similarity to PKS13, the zearalenone biosynthetic nrPKS (ABB90282). CcRADS1 also shows 57% identity and 73% similarity to Hpm3, the hypothemycin nrPKS (ACD39753), and 61% identity and 75% similarity to Rdc1 (ACD39770), the putative radicicol biosynthetic nrPKS from *P. chlamydosporia* (Reeves et al., 2008). These RAL nrPKSs all feature identical domain compositions: SAT-KS-AT-PT-ACP-TE (Figure 2B). The SAT domains of the RAL nrPKSs are proposed to act as chain initiation selectivity filters and to load the reduced polyketide chains supplied by the hrPKS partners as starter acyl units onto the ACP domains (Crawford et al., 2006, 2008c; Gaffoor and Trail, 2006). The conserved Cys in the proposed SAT active site motif Gx**C**xG (Crawford et al., 2008b) is replaced in all RAL nrPKSs by a Ser (CcRADS2: S124). By analogy to AT domains, this Ser could form an active site dyad with a conserved His (CcRADS2: H249), while a conserved Gln (CcPKS2: Q16) stabilizes the oxyanion hole during acyl transfer (Crawford et al., 2006; Khosla et al., 2007). However, replacement of the active site Ser with Ala has been shown not to affect starter unit recognition in the heterologously expressed PKS13 (Zhou et al., 2008). In zearalenone, hypothemycin, and radicicol, the nonaketide-ACP thioester intermediates undergo regioselective C2-C7 aldol cyclization to yield the resorcylic acid moiety, possibly guided by the PT domain (Crawford et al., 2008a). Whereas most fungal nrPKS TE domains act as Claisen cyclases creating C-C bonds (Cox, 2007; Crawford et al., 2008a), the TE domains of the RAL nrPKSs release their macrolactone products by ester formation, with C17-OH serving as the nucleophile (Zhou et al., 2008). The catalytic His of the TE of PKS13, the zearalenone nrPKS, was suggested to be replaced by an Arg that might facilitate acyl transfer instead of hydrolysis (Zhou et al., 2008). However, the PGD**H**FTM motif with the catalytic His is well

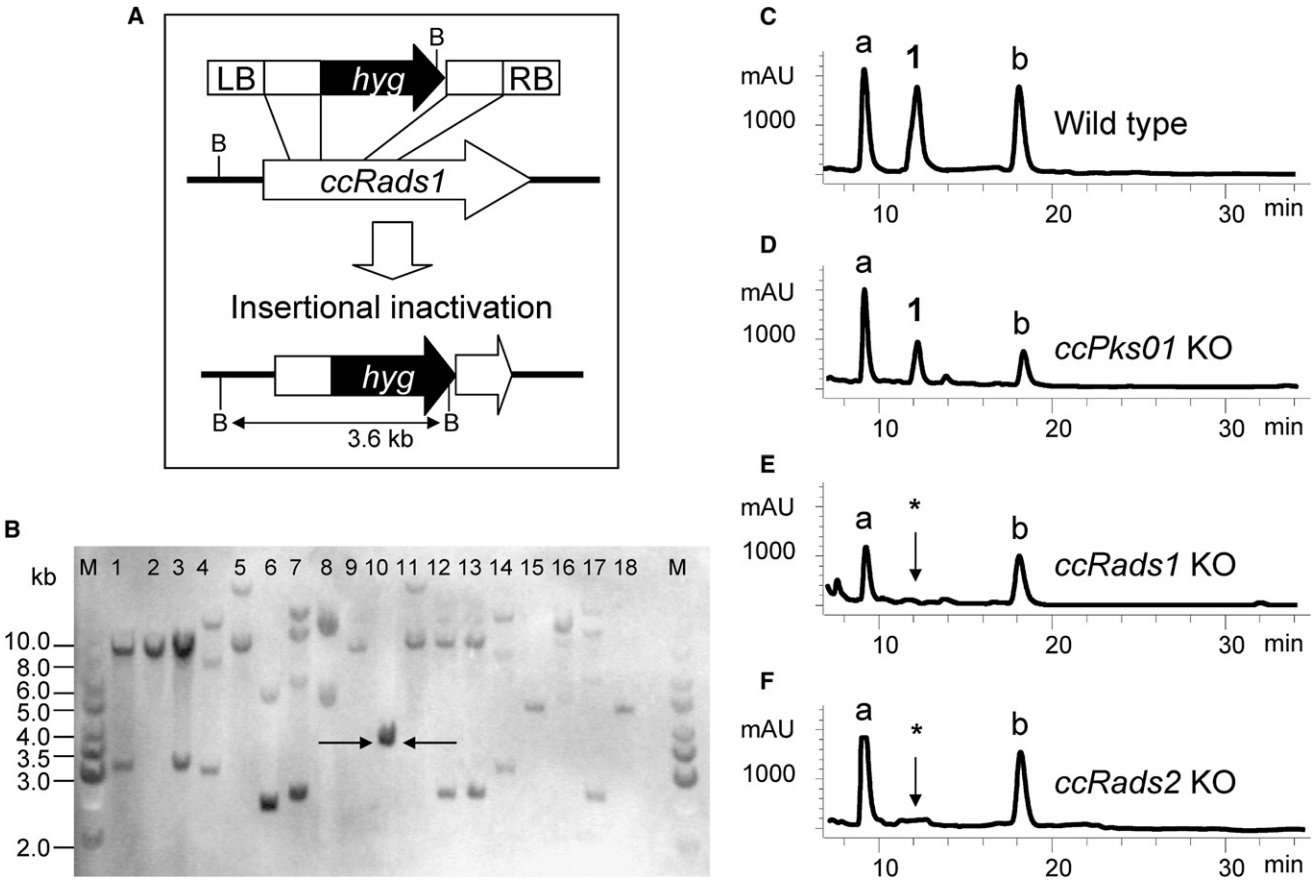

Figure 3. Insertional Inactivation of *ccRads1* and *ccRads2*

(A) Scheme for the double-homologous recombination between the *C. chiversii* genome and the T-DNA with the *ccRads1* disruption cassette. LB and RB, left and right borders of the T-DNA; B, BamHI restriction site.

(B) Southern hybridization against BamHI-digested total DNA of several transformants. The arrows point to the expected hybridizing fragment. M, marker.

(C–F) HPLC traces of extracts from fermentations of *C. chiversii* strains.

(C) Strain CS-36-62 (wild-type).

(D) The strain disrupted in *ccPks01* (*ccPks01* KO).

(E) The strain disrupted in *ccRads1* (*ccRads1* KO).

(F) The strain disrupted in *ccRads2* (*ccRads2* KO).

*, the expected position of the peak due to radicicol (**1**) (absent). Peaks a and b are due to 6-hydroxymethyleugenin and 6-methoxymethyleugenin, respectively.

conserved in all RAL nrPKSs (CcRADS2: eaD**H**leM, amino acids 2106–2112; PKS13: PaD**H**leM, amino acids 2006–2012) as part of a Ser-Asp-His catalytic triad (CcRADS2: S1930, D1957, H2109) (Fujii et al., 2001; Khosla et al., 2007).

The deduced RadR is a 453 amino acid protein showing similarities to various hypothetical fungal transcription factors with the pfam00170 basic region leucine zipper domain near their N terminus (RadR: amino acids 28–78). RadR is also similar to ZEB2 (ABB90285, 24% identity/39% similarity) that was shown to act as a transcriptional regulator for zearalenone biosynthesis in *Gibberella zeae* (Kim et al., 2005). Interestingly, neither the *H. subicolosus* hypothemycin nor the putative *P. chlamydosporia* radicicol biosynthetic gene cluster (Figure 2A) encodes similar regulators (Reeves et al., 2008). The amino acid sequence of RadR and the abolishment of radicicol biosynthesis in the knockout strain are consistent with RadR being a cluster-specific positive regulator for radicicol biosynthesis in *C. chiversii*.

radH Encodes a Fungal Flavin-Dependent Halogenase

The *C. chiversii* radicicol biosynthetic gene cluster also encodes enzymes with potential roles in post-PKS tailoring. Thus, *radH* encodes a 520 amino acid, 56.4 kDa protein that is highly similar to unidentified fungal proteins with the pfam04820 conserved domain like EAQ86388 of *C. globosum* (54% identity/70% similarity), and to the putative halogenase Rdc2 from *P. chlamydosporia* (ACD39771, 74% identity/84% similarity). RadH also displays clear similarities to bacterial halogenases like CrpH from *Nostoc* sp. (ABM21576, 38% identity/58% similarity). RadH shares with bacterial flavin-dependent halogenases a FAD-binding domain (pfam01494) with the nucleotide-binding motif GxGxxG (amino acids 13–18), and the partially overlapping pfam04820 (tryptophan) halogenase conserved domain with the WxWxIP active site cavity motif (amino acids 234–239). The bulky Trp side chains have been proposed to distance the enzyme-bound substrate from the flavin hydroperoxide to

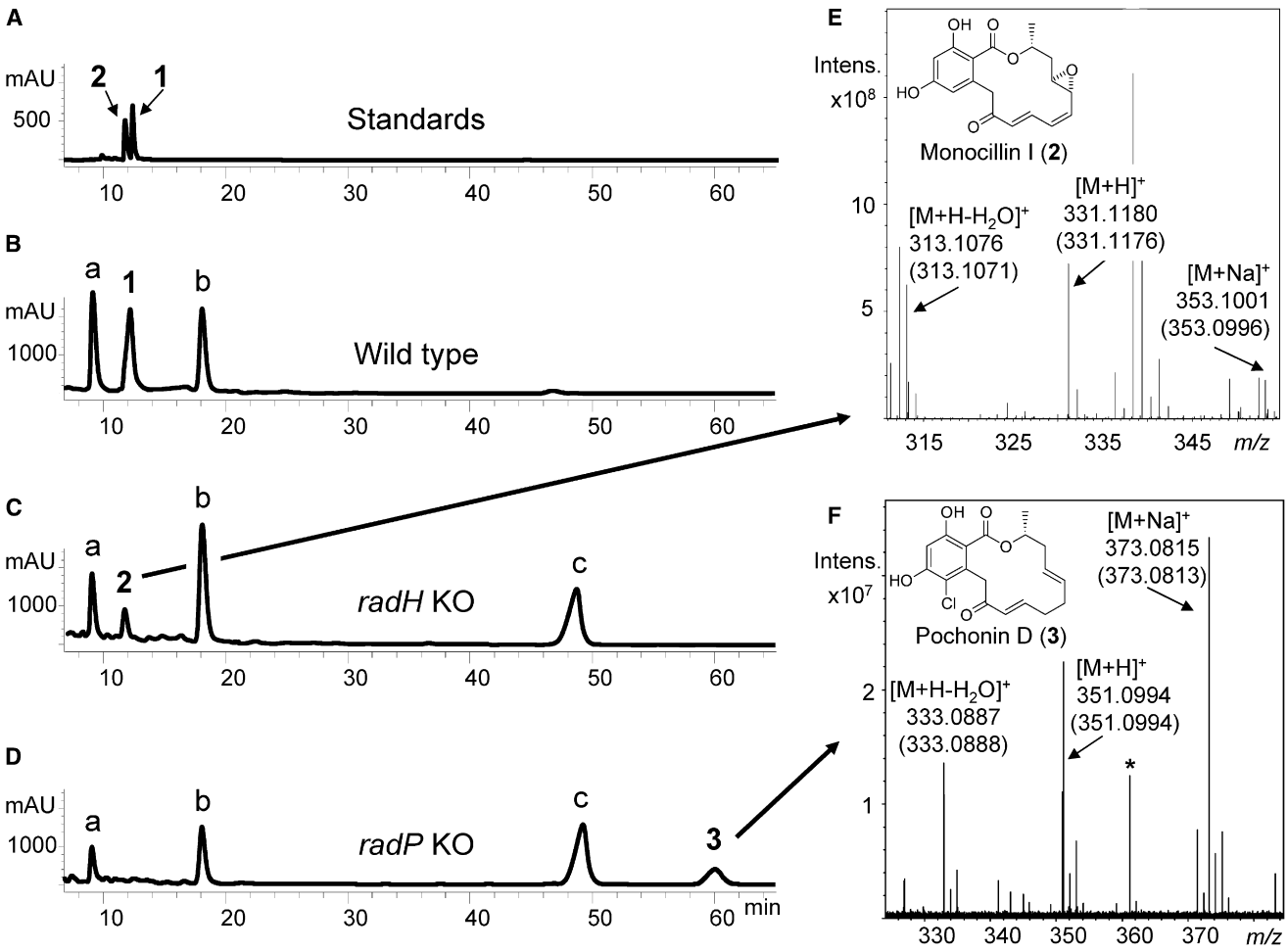

Figure 4. Insertional Inactivation of *radH* and *radP*

(A) HPLC trace of a mixture of authentic monocillin I (**2**) and radicicol (**1**).

(B–D) HPLC traces of extracts from fermentations of *C. chiversii* strains.

(B) Strain CS-36-62 fermentation (wild-type).

(C) The strain disrupted in *radH* (*radH* KO).

(D) The strain disrupted in *radP* (*radP* KO).

(E) FTICR-MS spectrum of monocillin I (**2**) isolated from the fermentation of *C. chiversii* disrupted in *radH*.

(F) FTICR-MS spectrum of pochonin D (**3**) isolated from the fermentation of *C. chiversii* disrupted in *radP*. *, internal standard.

Peaks a, b, and c are due to 6-hydroxymethyleugenin, 6-methoxymethyleugenin, and eugenitin, respectively.

prevent a direct monooxygenase reaction (Neumann et al., 2008; van Pee et al., 2006). Instead, a chloride ion bound near the cofactor attacks the oxidized flavin to produce hypochlorous acid, captured by the strictly conserved Lys (RadH: K74) as a lysine chloramine that reacts with high regiospecificity with the substrate (Neumann et al., 2008).

Because no fungal halogenases have been experimentally characterized to date to the best of our knowledge, we sought experimental proof for the involvement of RadH in the chlorination of the polyketide scaffold of radicicol by performing a directed gene knockout of *radH*. The production of radicicol was found undisturbed in isolates with ectopic integration of the disruption cassette. However, radicicol production was completely abolished, and a new HPLC peak with similar, but not identical mobility to radicicol was detected in all fermentation extracts with the *radH* knockout isolates (Figure 4C). Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS; Figure 4E) and ^{1}H NMR spectroscopy (Supplemental Data) of the isolated compound proved that this product is 6-dechloro-radicicol, identical with monocillin I (**2**) previously isolated from various fungi (Winssinger and Barluenga, 2007). No other RAL product was detected in these fermentations. The production of the biogenetically unrelated eugenitin (peak *c*, Figure 4B) was also increased in many transformants, irrespective of their origin from ectopic or homologous integration. Monocillin I could not be converted to radicicol by feeding the isolated compound to the *ccRads1* or *ccRads2* knockout strains during fermentations, or during attempts to reconstitute the halogenation reaction using crude extracts prepared from these strains (Supplemental Data). However, the amino acid sequence of RadH and the production of the dechloro-radicicol analog in the corresponding knockout strain are consistent with RadH being a flavin-dependent

substrate- and regiospecific halogenase that installs the chlorine atom at C6 of radicicol.

radP Encodes a Putative CYP Epoxidase

Another deduced tailoring enzyme found encoded in the radicicol biosynthetic cluster is RadP, a 501 amino acid, 57.2 kDa putative protein with the pfam00067 cytochrome P450 (CYP) domain. The deduced RadP shows high similarity to a variety of CYP monooxygenases including EDU51262 from *Pyrenophora tritici-repentis* (43% identity/62% similarity), and the putative radicicol hydroxylase Rdc4 from *P. chlamydosporia* (ACD39773, 69% identity/80% similarity). RadP features the conserved Thr (T307) implicated in O_2 binding (Buchatskii et al., 2001), as well as the conserved E/DxxR motif (amino acids 359–362) and the conserved FxxGxH**C**xGxxxA motif with the invariable Cys (amino acids 437–450, C444), both involved in heme binding. Radicicol features an epoxide moiety at C14(15) that might be expected, in analogy with epothilone biosynthesis (Molnár et al., 2000), to be installed by a CYP epoxidase acting on a PKS-generated double bond. To establish the role of RadP as radicicol epoxidase, the *radP* gene was inactivated by directed gene knockout. Ectopic integrants, representing more than 99% of the transformants in this case, produced radicicol undisturbed. However, radicicol production was completely abolished in all *radP* knockout isolates, with the concomitant appearance of a single new RAL with decreased chromatographic mobility (Figure 4D) and a significant red shift compared to radicicol (from 276 to 314 nm). This new RAL was isolated and subjected to FTICR-MS (Figure 4F), ^{1}H NMR, and DQF-COSY spectroscopy (Supplemental Data). Although the limited amount of isolated material precluded us from obtaining ^{13}C NMR spectra, the above data revealed this product to be 14(15)-deepoxy-12(13)-dihydro-radicicol, identical to pochonin D (**3**) previously isolated from *P. chlamydosporia* (Hellwig et al., 2003). Pochonin D could not be converted to radicicol by feeding the isolated compound to the *ccRads1* or *ccRads2* knockout strains during fermentations, or during attempts to reconstitute the epoxidation reaction using crude extracts prepared from these strains (Supplemental Data). However, the amino acid sequence of RadP and the production of a deepoxy-radicicol analog in the corresponding knockout strain are consistent with the proposed role of RadP as the radicicol C14(15) CYP epoxidase.

DISCUSSION

The current work describes the functional characterization of the genes for the biosynthesis of radicicol, an Hsp90 inhibitor RAL polyketide, from the endophytic fungus *C. chiversii*. Clustered genes encoding two PKSs, a halogenase, and a CYP were identified by exploiting the expected similarity of the radicicol biosynthetic genes to bacterial flavin-dependent halogenases and the nrPKS and hrPKS genes for the biosynthesis of the RAL zearalenone. Targeted disruptions of the nrPKS, the hrPKS, and a putative transcriptional regulator specifically abolished radicicol biosynthesis, whereas knockout of the putative halogenase led to the production of the dechloro analog of radicicol (monocillin I). Targeted disruption of the CYP furnished a deepoxy analog that was also devoid of the C12-C13 *cis* double bond. Disruption of *orf3* encoding a putative cystathionine γ-synthase/β-lyase led to no change in radicicol production, establishing the right border of the *C. chiversii* cluster (data not shown). Attempts to disrupt *orf1* encoding a putative trehalose-6-phosphate synthetase at the left flank of the cluster were unsuccessful and not pursued further.

The putative *P. chlamydosporia* radicicol biosynthetic cluster whose sequencing coincided with our work (Reeves et al., 2008) contains orthologous genes to the *C. chiversii* cluster (Table 1) but appears to be scrambled by two inversions (Figure 2A). The *C. chiversii* cluster-specific regulator, encoded by *radR*, is missing from the sequenced *P. chlamydosporia* locus. The radicicol biosynthetic machineries in both fungi must recruit an NAD(P)H-dependent flavin reductase to provide reduced cofactor for the halogenase, and a NADPH-dependent CYP reductase to supply electrons to the CYP. Because neither halogenases nor CYPs require exclusive reductase partners (Molnár et al., 2005; van Pee et al., 2006), these two functions must be provided by housekeeping enzymes. No significant synteny of the *C. chiversii* radicicol locus was detected with the completely sequenced *C. globosum* genome (http://www.broad.mit.edu/annotation/fungi/fgi/).

The overwhelming majority of fungal polyketides are produced by single PKS enzymes with one set of active site domains that are used iteratively in a programmed recursive biosynthetic process. Remarkably, the biosynthesis of fungal RALs, as shown for zearalenone (Gaffoor and Trail, 2006; Kim et al., 2005), hypothemycin (Reeves et al., 2008), and now for radicicol, exploits both an hrPKS and an nrPKS for the biosynthesis of a single continuous polyketide chain. With the nrPKS utilizing the product of the hrPKS as a starter unit, the collaborating RAL PKSs represent a conceptual step toward modularity. A close parallel to RAL biosynthesis is the assembly of norsolorinic acid, where a specialized fatty-acid synthase (a PKS equivalent) produces a hexanoate starter extended by an nrPKS (Crawford et al., 2008b). The biosynthesis of T toxin requires two hrPKSs to build a single linear polyketide chain (Baker et al., 2006). In contrast, the two PKS systems for lovastatin, compactin and squalestatin, produce two separate polyketide chains that are conjoined via ester bonds by post-PKS tailoring enzymes (Cox, 2007).

The nonaketide backbone of zearalenone and hypothemycin were proposed to be assembled, on the basis of their reduction patterns, through three rounds of nonreductive chain extensions on nrPKSs that use a reduced hexaketide starter from their hrPKS partners (Gaffoor and Trail, 2006; Reeves et al., 2008; Zhou et al., 2008). The structure of radicicol does not allow for a similarly clear prediction. Thus, it is currently unknown whether CcRADS2 (and the putative Rdc1 from *P. chlamydosporia*) extends a β-keto hexaketide starter unit using three malonyl-CoA building blocks, or elaborates a pentaketide starter unit with four malonyl-CoA extenders (Figure 2B). A correlation was previously proposed between the primary sequences of the SAT domains and their starter unit substrates (Cox, 2007; Crawford et al., 2008b), and separately between the sequences of PT domains and the number of chain extensions performed by the nrPKSs (Cox, 2007). The sequences of the SAT domains of the zearalenone, hypothemycin, and the two radicicol nrPKSs are remarkably similar to each other while distinct from known C2 and C6 acceptors (Crawford et al., 2008b). Similarly, the PT domains of these nrPKSs

show high mutual identities, but are removed from known hexaketide synthases like those that produce 1,3,6,8-tetrahydroxynaphthalene (BAA18956), heptaketide synthases like those that yield YWA1 (AAC39471), or octaketide synthases like those that produce norsolorinic acid (AAS66004). However, the limited number of nrPKSs with characterized starter units and product chain length specificities limits the scope of these comparisons and denies insight into the division of labor between the radicicol PKS partners.

Although the first halogenating enzyme (a nonspecific chloroperoxidase) was isolated from a filamentous fungus, no substrate- and regiospecific halogenases were functionally characterized previously from any fungal sources (van Pee et al., 2006). The radicicol biosynthetic gene clusters from *C. chiversii* and *P. chlamydosporia* each encode a putative enzyme (RadH and Rdc2, respectively) with high similarity to characterized flavin-dependent bacterial halogenases with the pfam04820 (tryptophan) halogenase conserved domain. The targeted disruption of RadH from *C. chiversii* leads to the production of the 6-dechloro analog of radicicol, monocillin I, providing to our knowledge the first functional evidence for a flavin-dependent halogenase in a fungal strain. Bacterial flavin-dependent halogenases form two subfamilies: PrnC-type halogenases acting on phenol and pyrrole moieties, and PrnA-type halogenases processing tryptophan residues (Neumann et al., 2008; van Pee et al., 2006). A further correlation between the phylogenetic divergence of PrnC-type halogenases and their substrate chemotypes has also been noted (Hornung et al., 2007). A survey of the available 34 fungal genome sequences (http://www.broad.mit.edu/annotation/fungi/fgi/) revealed 29 uncharacterized conserved proteins with the pfam04820 conserved domain. A phylogenomic analysis (Figure S1) indicates an intimate clustering of the deduced fungal pfam04820 proteins with selected bacterial PrnC- and PrnA-type halogenases (Hornung et al., 2007) with multiple clades supported by significant bootstrap values. PrnA-type bacterial halogenases acting on free Trp (Yeh et al., 2005) form the first clade basal to all other sequences, reflecting the low sequence similarity of PrnA- and PrnC-type enzymes (van Pee et al., 2006). The next clade contains selected bacterial and fungal oxidoreductases with the PRK10015 conserved domain: PRK10015 and pfam04820 proteins belong to the same protein fold superfamily. The small fungal clade I contains pfam04820 enzymes with no functionally characterized members. Present in the *Aspergillus* cluster (Eurotiomycetes), *Magnaporthe grisea* (Sordariomycetes), *Botrytis cinerea* and *Sclerotinia sclerotiorum* (both Leotiomycetes), these putative enzymes share conserved motifs with, but branch basal to, the bacterial PrnC-type halogenases. The first clade of bacterial PrnC-type halogenases includes proteins similar to the anabaenopeptolide tyrosine halogenase (CAC01605) acting on a carrier protein-bound substrate (Rouhiainen et al., 2000). The bacterial PrnC clade II includes enzymes acting on free pyrrole substrates, as well as chlorinases from the tetracycline and neocarzilin clusters modifying polyketides. The RadH radicicol halogenase (as well as the putative Rdc2 from *P. chlamydosporia*) belongs to the fungal clade II enzymes: these proteins might modify polyketides or other secondary metabolites. The phylogenetic distribution of these putative fungal halogenases is sporadic: they are present in *Coprinus cinerea* (Homobasidiomycetes), the *Aspergillus* cluster, and in *Chaetomium* and *Pochonia* spp. (Sordariomycetes), but are apparently absent in other Sordariomycetes like the *Fusarium* cluster. Finally, the bacterial clade III PrnC halogenases tailor carrier protein-bound polyketides and nonribosomal peptides or their CoA-bound precursors (Dorrestein et al., 2005).

The only biosynthetic step not accounted for by the radicicol clusters is the synthesis of the *cis* double bond at C12-C13. Although PKSs almost always produce *E* double bonds, different mechanisms for *Z* double-bond formation by prokaryotic modular PKS systems have been proposed (Alhamadsheh et al., 2007; Tang et al., 2004). Conceptually, the biosynthesis of *cis* double bonds by the *iterative* fungal hrPKSs is even more problematic, because a single set of active sites should yield both geometries. With only a single KR domain, the formation of *cis* double bonds cannot be dictated by the configuration of the hydroxyl moieties as proposed for separate A- or B-type KR domains in bacteria (Caffrey, 2003). Indeed, the KR sequences from the two radicicol hrPKSs (CcRADS1 and Rdc5) broadly correspond to B-type bacterial KRs present in modules producing *E* double bonds. However, a polyketide carbon chain rotation during an "out of register" dehydration, as proposed for epothilone biosynthesis (Tang et al., 2004), cannot be excluded. In other prokaryotic systems, *Z* double-bond formation was proposed to be the result of accessory proteins that modulate the DH activity (Ikeda et al., 1999). Further, a "tailoring" glutathione S-transferase was shown to equilibrate *E* and *Z* double bonds in hypothemycin analogs (Reeves et al., 2008). However, no such accessory or tailoring activities are encoded in the radicicol biosynthetic gene clusters.

A comparative metabolic analysis leads us to propose a unifying scheme for the biosynthesis of RALs and their isocoumarin congeners in *C. chiversii*, *P. chlamydosporia*, and *Pa. quadriseptata* (Hellwig et al., 2003; Wijeratne et al., 2006). The hrPKS and the nrPKS collectively produce an ACP-bound 12(13)-dihydro-14(15)-deepoxy nonaketide (Figure 2B). After the resorcylic acid moiety is templated on the nrPKS, TE-catalyzed intramolecular ester bond formation yields RALs, or spontaneous or TE-catalyzed hydrolysis releases isocoumarins (Figure 5). The absence of conversion of purified pochonin D and monocillin I by PKS knockout mutants of *C. chiversii* or their crude protein extracts leaves open the question of whether the ACP-bound resorcylate thioesters or the free RALs and isocoumarins serve as substrates for the subsequent tailoring reactions (Dorrestein et al., 2005; Ma et al., 2008). Notably, in vitro reconstitution of the CYP-mediated hydroxylation of hypothemycin was similarly unsuccessful with free substrates, but proceeded in vivo using PKS-generated substrates (Reeves et al., 2008). Either way, the first tailoring reaction is an optional C6 chlorination to yield pochonin D, to our knowledge the first detected natural product from the pochonin-radicicol/chaetochiversin series. Skipping the halogenation step shunts the pathway toward the production of monocillins and paraphaeosphaerins. C14(15) epoxide formation provides the known natural products monocillin III and pochonin A, still without the C12(13) *cis* double bond. The production of C12 α-hydroxylated natural products by *Pa. quadriseptata* (hydroxymonocillin III and paraphaeosphaerin C), and the biosynthesis of pochonin B by *P. chlamydosporia* suggest a regio- and stereospecific hydroxylation reaction. The absence of natural RALs (or their isocoumarin analogs) with C12-OH but

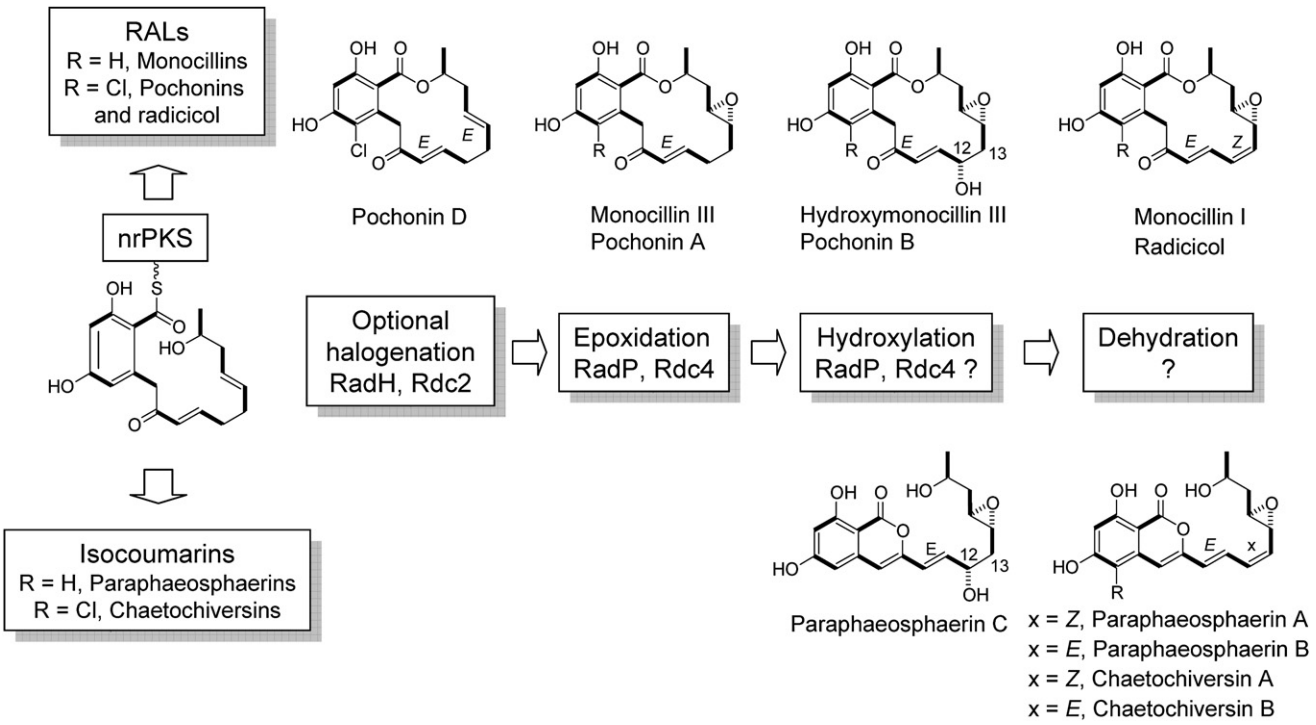

Figure 5. Model for the Biosynthetic "Tailoring" of Radicicol-type RALs and Biogenetically Related Isocoumarins
See text for details.

without the C14(15) epoxide moiety suggests the precedence of epoxidation over C12 hydroxylation. Because C12 corresponds to an α carbon of the relevant polyketide chain extension unit, the C12-OH cannot be derived by the PKS. It is possible, however, that RadP and its equivalents catalyze C12 hydroxylation in addition to C14(15) epoxide formation: a single CYP performing two distinct but specific tailoring steps is known in pikromycin/methymycin biosynthesis (Xue et al., 1998). Finally, elimination of water yields the C12(13) *Z* double bond in the *cis* RALs monocillin I and radicicol, and in the *cis* isocoumarins paraphaeosphaerin A and chaetochiversin A. The lack of acidity of the proton at C13 in the C12-OH RALs excludes spontaneous dehydration and suggests that this dehydration is catalyzed by an unidentified enzyme. The natural occurrence of the *E* isocoumarins paraphaeosphaerin B and chaetochiversin B suggests that these *trans* isocoumarins derive from a spontaneous *cis/trans* isomerization during fermentations and/or the workup of the extracts.

SIGNIFICANCE

The resorcylic acid lactone (RAL) scaffold constitutes a rich pharmacophore whereby different RALs act as specific inhibitors of the stress chaperone Hsp90 or mitogen-activated protein kinases, and ligands for hormone receptors. Importantly, the inhibition of the evolutionarily conserved chaperone Hsp90 by the RAL radicicol leads to a combinatorial blockade of multiple cancer-causing pathways in vitro. The metabolic instability of radicicol, however, prevents its deployment for broad-spectrum cancer chemotherapy, and led to extensive combinatorial synthetic efforts to produce more stable radicicol analogs. The cloning and functional analysis of clustered genes for the biosynthesis of radicicol from the endophytic fungus *Chaetomium chiversii* will facilitate combinatorial biosynthetic strategies to derive unnatural radicicol analogs and other RALs. The functional identification of a pair of highly reducing and nonreducing PKSs that are predicted to collaborate in the production of a single polyketide chain for the radicicol core raises interesting questions about the evolution of modularity in PKSs, and on the programming rules that determine β-keto group reduction, starter unit choice, product chain length, and the folding and cyclization of the nascent polyketide in fungal iterative PKSs. The functional identification of a regiospecific chlorinase encoded in the radicicol biosynthetic cluster highlights a group of conserved proteins in sequenced fungal genomes that might similarly be involved in substrate- and regiospecific halogenation reactions. These fungal proteins share conserved active site architecture and a conserved cofactor-binding domain with bacterial flavin-dependent halogenases. Hitherto described only from prokaryotes, the existence of flavin-dependent halogenases is now documented, to our knowledge, for the first time in the kingdom of fungi.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions

Chaetomium chiversii CS-36-62 (Turbyville et al., 2006) was maintained on potato dextrose agar (PDA; Difco). Agar surface culture fermentations were done

by growing *C. chiversii* or its derivatives on PDA plates at 28°C for 7 days. *Escherichia coli* Epi300 and fosmid pCCFOS1 (Epicenter) were used for *C. chiversii* genomic library construction, and *E. coli* DH10B and plasmid pJET1 (Fermentas) served for routine cloning and sequencing. *Agrobacterium tumefaciens* LBA4404 (Invitrogen) with the plasmid pAg1-H3 (Zhang et al., 2003) was used for *C. chiversii* transformation.

Cloning, Sequencing, and Sequence Analysis

Putative PKS- and halogenase-encoding sequences were amplified by PCR from *C. chiversii* total DNA as the template, and used to isolate overlapping fosmids from a genomic DNA library as described in Supplemental Data. Overlapping fragments of ~2 kb in size were generated from the selected fosmids by partial HaeIII digests, and cloned into pJET1 to produce double-stranded templates for DNA sequencing. Sequencher 4.7 (Gene Codes) and the VectorNTI suite 9.0 (Invitrogen) were used for sequence assembly and analysis. HMM-based gene models were built with FGENESH (Softberry). Additional exon/intron boundary predictions from SPLICEVIEW (http://l25.itba.mi.cnr.it/~webgene/wwwspliceview.html/) and multiple sequence alignments were also considered for the refinement of the initial gene models. The UMA algorithm was used to predict domain boundaries in PKSs (Udwary et al., 2002). The sequences of the radicicol biosynthetic locus (EU980390) and the *ccPks01* (EU980392) and *ccHmt* genes (EU980391) appear in GenBank.

Phylogenomic Analysis of Trp Halogenase Superfamily Proteins

Twenty-nine deduced proteins annotated as pfam04820 Trp halogenase and five deduced proteins annotated as PRK10015 oxidoreductase were collected from the currently (May 2008) available 34 fungal genome sequences at the Fungal Genome Initiative (FGI) website (http://www.broad.mit.edu/annotation/fungi/fgi/). Selected bacterial PrnC-type (Hornung et al., 2007) and PrnA-type halogenases (all with the pfam04820 conserved domain) and bacterial PRK10015 oxidoreductases were also included. A multiple sequence alignment of these sequences with the *C. chiversii* RadH, the putative halogenase-methyltransferase bifunctional protein ccHmt, and the putative *P. chlamydosporia* Rdc2 radicicol halogenase were generated in VectorNTI. Bootstrapped trees were calculated in CLUSTALX with the neighbor-joining method using 1000 repeats, and the phylogram was plotted with NJPlot (http://pbil.univ-lyon1.fr/software/njplot.html/).

Gene Disruptions in *C. chiversii*

The constructions of the gene disruption vectors for *Agrobacterium*-mediated transformation (Zhang et al., 2003) are described in Supplemental Data. Putative transformants were selected on PDA plates supplemented with 350 μg/ml hygromycin and 500 μg/ml carbenicillin (final concentrations) after 5–7 days of incubation at 28°C. Correct integration of the disruption cassettes at both the 5′ and the 3′ flanking DNA regions were validated by Southern hybridizations using the digoxigenin-labeled (Roche Applied Science) *hph* gene as the probe. Three ectopic integrant and three homologous replacement isolates were evaluated independently for RAL production for each gene knockout experiment.

Extraction and Analysis of RALs

Radicicol, monocillin I, and pochonin D were isolated from *C. chiversii* agar surface fermentations (Turbyville et al., 2006). HPLC analyses and reverse-phase preparative HPLC were done on an HP-1050 equipped with a Kromasil C18 column (5 μm, 250 mm × 4.6 mm); MeOH:H_2O = 60:40 at a flow rate of 0.8 ml/min; detection at 220 nm. Accurate mass measurements, ^{1}H NMR, and DQF-COSY spectra for isolated RALs are reported in Supplemental Data.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, one figure, and Supplemental References and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(08)00408-0/.

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