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ORIGINAL RESEARCH PAPER

Specific inhibition of the halogenase for radicicol biosynthesis by bromide at the transcriptional level in *Pochonia chlamydosporia*

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Abstract *Pochonia chlamydosporia* produces radicicol (**1**), a potent antifungal and anticancer product. NaBr, but not NaF, NaCl or NaI, inhibited the biosynthesis of **1** in *P. chlamydosporia* in a dose-dependent manner, accompanied by the formation chlorine-lacking monocillins II–V (**2–5**), indicating that the dedicated halogenase, Rdc2 had been inhibited. RT-PCR analysis confirmed that transcription of *rdc2* was selectively inhibited by Br^-, whereas the putative P450 epoxidase gene, *rdc4*, was not affected.

Keywords Bromide · Halogenase · Inhibition · Radicicol biosynthesis · Transcription

Introduction

Filamentous fungi are a rich source of pharmaceutically important products. Whole-genome sequencing projects indicate that the biosynthetic potential of fungi has been underestimated as there are many silent or cryptic genes that could be investigated for the production of new molecules (Scherlach and Hertweck 2009). In addition, media composition, such as halogen salts and growth conditions, also have a significant impact on secondary metabolite profiles thereby providing new opportunities to access untapped chemical diversity of fungi (Paranagama et al. 2007; Stander et al. 2000; Wijeratne et al. 2010). However, the mechanisms by which fungal biosynthetic pathways are influenced by medium components and culture conditions are poorly understood, which has hampered our understanding of fungal secondary metabolism. Thus, studies on regulation of biosynthetic enzymes are required to further explore fungal systems for diverse natural products.

Radicicol (**1**, Fig. 1a) is a chlorine-containing polyketide produced by *Pochonia chlamydosporia* (Shinonaga et al. 2009) and several other filamentous fungi (Ayer et al. 1980; Evans and White 1966; McCapra et al. 1964). It has significant antifungal (Fujita et al. 1999) and anticancer activities (Shimada et al. 1995), and is a potent heat shock protein 90 (Hsp90) inhibitor (Turbyville et al. 2006). It belongs to a family of fungal resorcylic acid lactones (RALS) such as hypothemycin and zearalenone. The biosynthetic gene clusters of **1** from two producing strains, *P. chlamydosporia* (Reeves et al. 2008) and *Chaetomium chiversii* (Wang et al. 2008), have been recently sequenced. Both gene clusters contain four key biosynthetic genes encoding reducing polyketide

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synthase (R-PKS), non-reducing PKS (NR-PKS), halogenase and P450 oxygenase. A recent research on hypothemycin biosynthesis (Zhou et al. 2010) has demonstrated that assembly of the RAL structure is the result of cooperative actions of the dedicated R-PKS and NR-PKS. Thus, a similar mechanism can be proposed for the formation of the core structure of **1** by Rdc5 and Rdc1 in *P. chlamydosporia*, with further modifications by two post-PKS tailoring enzymes Rdc2 (chlorination) and Rdc4 (epoxidation) (Fig. 1b). Although the gene sequence information of the radicicol biosynthetic gene cluster is available, how the radicicol biosynthetic enzymes are regulated has not been well understood yet. In the present paper, we report the effect of halides on radicicol biosynthesis and for the first time demonstrate specific inhibition of the dedicated halogenase by bromide at the transcriptional level.

Materials and methods

Strains, media and culture conditions

Pochonia chlamydosporia ATCC 16683 was used. *Escherichia coli* BL21-CodonPlus (DE3)-RIL (Stratagene, CA, USA) was routinely grown in LB medium at 37°C and used for expression of Rdc2.

Effect of halides on radicicol biosynthesis

Pochonia chlamydosporia was grown on potato/dextrose/agar (PDA) medium alone or supplemented with NaF, NaCl, NaBr or NaI at 5 and 50 mM at 30°C for 7 days, followed by extraction of the cells with methanol. The extracts were dried under reduced pressure, and analyzed and quantified on an Agilent Single Quad HPLC–MS using a Zorbax SB-C18 reversed-phase analytical column (5 μm, 150 × 4.6 mm^2) at 280 nm. A gradient of acetonitrile/water (10–90%, v/v) containing 0.1% (v/v) trifluoroacetic acid was programmed over 25 min at 1 ml min^{-1}.

Extraction and isolation of monocillin IV (**4**)

Pochonia chlamydosporia was grown on 600 ml PDA medium supplemented with 50 mM NaBr in 20 Petri dishes. The cultures were incubated at 30°C for 7 days and then extracted 3 times with 1 l of methanol. After evaporation of the solvent, 2.8 g residue was obtained. The extract was then separated on a 25 g silica gel column (63–200 μm, EMD Chemical Inc., Germany), sequentially eluted with hexane/acetone (8:1, 4:1, 2:1, 1:1, v/v) and then pure acetone. **4** was detected in the hexane–acetone (4:1 and 2:1, v/v) eluents. A total of 10.5 mg of **4** was isolated in pure form.

Monocillin IV (**4**): Colorless crystal. ESIMS (+) *m/z* 319 $[M+H]^+$. ^1H and ^{13}C NMR data are listed in Supplementary Table 1.

Reverse transcription-PCR (RT-PCR) analysis of transcription of *rdc2* and *rdc4*

To identify whether the inhibition of biosynthesis of **1** by NaBr is due to the inhibitory effect on transcription

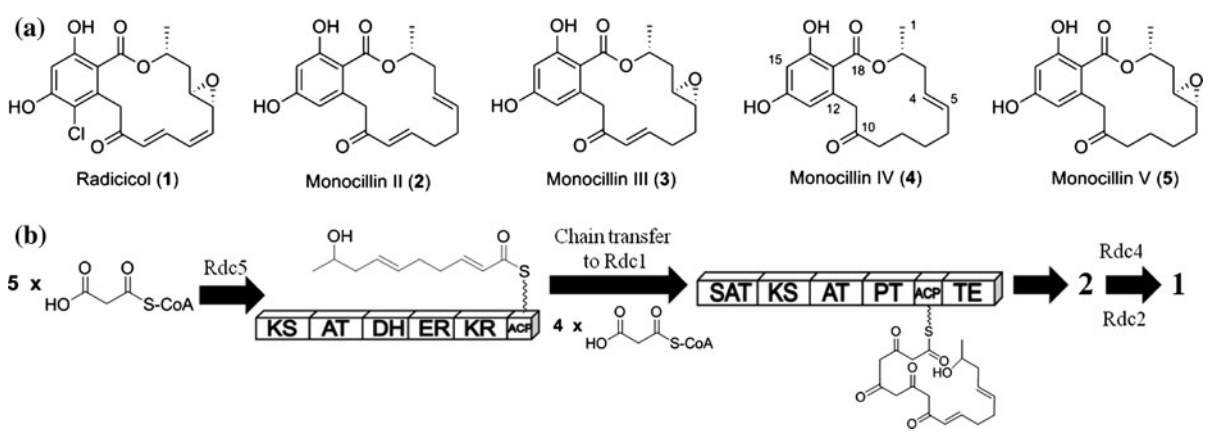

Fig. 1 **a** Structure of radicicol (**1**) and the chlorine-lacking metabolites **2–5**; **b** proposed biosynthetic pathway of **1**

of the tailoring enzymes, RT-PCR analysis was performed. *P. chlamydosporia* was grown in 50 ml of potato/dextrose broth at 30°C and 250 rpm, with and without 50 mM NaBr. After 7 days, the cultures were filtered. The supernatants were extracted with ethyl acetate. After evaporation of the solvent, the extracts were analyzed by HPLC under the same conditions as described above.

The mycelia were ground in liquid nitrogen. Total RNA was extracted from the ground mycelia using a RNeasy Plant Mini Kit (Qiagen, CA, USA), which was subsequently used as the template to synthesize the cDNA using a SuperScript III First-Strand cDNA Kit (Invitrogen, CA, USA). Specific primers including rdc2-5′-*Nde*I (AA*CATATG*TCGGTACCCAAGT CTTG) and rdc2-3′-*Hin*dIII (AA*AAGCTT*TCAAACT TTGTTGAGGCCAA) were used to clone the *rdc2* gene. Similarly, rdc4-5′-*Nde*I (AA*CATATG*AATG TATCGCCTCAGTT) and rdc4-3′-*Eco*RI (AA*GAA TTC*TCAATCTGCTCTCTCATGCT) were used for amplification of *rdc4*. Pure water and the genomic DNA were used in the PCR analysis as negative and positive controls, respectively. Phusion High-Fidelity DNA Polymerase (New England Biolabs, MA, USA) was used for cloning. The PCR program for amplification of both genes included an initial denaturation step of 5 min at 98°C, a 20-cycle touchdown program (30 s denaturation at 98°C, 30 s annealing at temperatures reduced by −0.5°C/cycle from 75°C to 65°C, 70 s extension at 72°C), an additional 20-cycle regular program (30 s denaturation at 98°C, 30 s annealing at 65°C, 70 s extension at 72°C), and a 10-min final extension at 72°C. The PCR products were checked by 0.8% agarose gel electrophoresis and visualized by ethidium bromide staining.

Effect of NaBr on expression of *rdc2* in *E. coli*

The intron-free *rdc2* gene cloned from the cDNA was ligated into pET28a vector between *Nde*I and *Hin*dIII to yield an expression plasmid pJZ54. This plasmid was then transformed into *E. coli* BL21-CodonPlus (DE3)-RIL and the enzyme was expressed at 28°C for 16 h after induction by 200 μM IPTG. To test whether the expression of *rdc2* in *E. coli* is influenced by bromide, 50 mM NaBr was added into the LB medium with IPTG when the OD_{600} reached 0.4–0.6. The cells were harvested by centrifugation and lysed by sonication. The lysates were then centrifuged at 15,000 × *g* for 10 min. Both the resulting supernatants (soluble proteins) and pellets (insoluble proteins that were dissolved in 8 M urea) were checked by SDS-PAGE.

Results and discussion

Effect of four different halides on radicicol biosynthesis

Halides have significant effects on the production of ochratoxins in *Aspergillus ochraceus* (Stander et al. 2000). To test whether halides can influence radicicol biosynthesis, NaF, NaCl, NaBr or NaI were added into PDA cultures of *P. chlamydosporia*, at 5 and 50 mM. None of the halides inhibited growth but the yield of **1** was significantly inhibited by NaBr (Table 1). HPLC of the products is shown in Fig. 2 showing the production of four new metabolites, **2–5**. The same inhibition was observed with KBr, indicating that it is Br^- that plays a key role in the inhibition of radicicol biosynthesis.

We next identified what new metabolites were synthesized when radicicol biosynthesis was inhibited. ESI–MS data revealed that molecular weights of **2–5** were 316, 332, 318, and 334, respectively. None of these compounds showed the characteristic ion

Table 1 The yields of **1** in *P. chlamydosporia* on PDA medium with different sodium halides

Medium	Yield (mg l^{-1})
Control	291 ± 16
5 mM NaI	290 ± 12
50 mM NaI	228 ± 20
5 mM NaBr	30 ± 3
50 mM NaBr	3.6 ± 0.3
5 mM NaCl	286 ± 21
50 mM NaCl	257 ± 11
+ 5 mM NaF	285 ± 17
50 mM NaF	274 ± 13

The 7-day old PDA cultures of *P. chlamydosporia* were minced and extracted with methanol for three times. After evaporation of the solvent, the samples were re-dissolved in methanol and analyzed by HPLC at 280 nm. A standard curve of **1** was established for quantification of the radicicol yields in the control (blank PDA) and cultures supplemented with different halides. Results represent the mean of three independent experiments

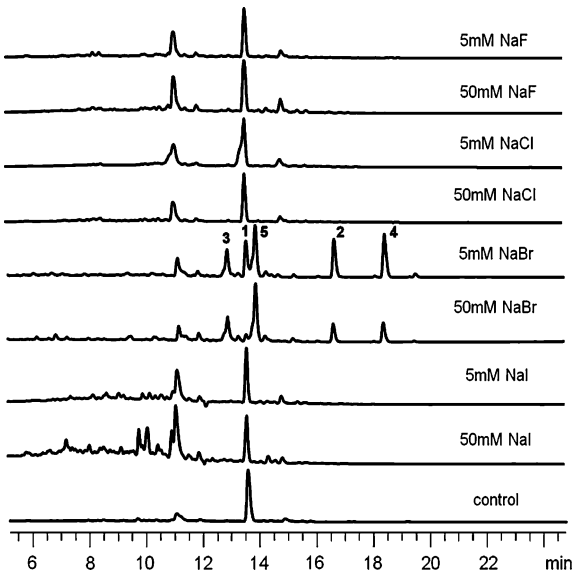

Fig. 2 HPLC profiles of methanol extracts of *P. chlamydosporia* ATCC 16683 grown on blank PDA medium and PDA supplemented with NaF, NaCl, NaBr, or NaI at two different 5 and 50 mM. The 7-day old cultures were minced and extracted with methanol. The extracts were analyzed and quantified by HPLC at 280 nm. Peak **1** is radicicol, and the new peaks 2–5 were identified based on spectral data as monocillins II–V, respectively. The HPLC traces shown are representative of three independent experiments

peaks (3:1) of mono-chlorinated molecules, indicating that they are chlorine-lacking metabolites. Based on the corresponding UV and MS spectra, 2–5 were predicted to be monocillins II–V (Fig. 1a) (Shinonaga et al. 2009; Wijeratne et al. 2004), which have been previously isolated from various fungi (Ayer et al. 1980; Shinonaga et al. 2009; Wijeratne et al. 2004).

We also scaled up the culture and isolated **4** for complete NMR analysis. The low field carbon signals including the C-10 keto group, C-18 lactone group, 6 aromatic carbons, and two olefinic carbons, were present in the ^{13}C NMR spectrum, while no epoxide signals were observed. In the ^{1}H NMR spectrum, two *meta*-coupled aromatic protons (H-13 and H-15, J = 2.4 Hz) were present at δ 6.26 and 6.15, respectively, confirming the absence of Cl in the molecule. Based on the ^{1}H-^{1}H COSY and HMBC spectra, the double bond can be located between C-4 and C-5 and thus **4** was characterized as monocillin IV. All the carbon and proton signals were assigned (Supplementary Table 1), which were in agreement with those previously reported (Shinonaga et al. 2009). Similarly, **2**, **3** and **5** were confirmed to be monocillins II, III and V (Fig. 1a), respectively, by comparison of the NMR data with the literature (Shinonaga et al. 2009; Wijeratne et al. 2004).

Because of the inhibition of radicicol biosynthesis and simultaneous production of four chlorine-lacking metabolites **2–5**, it can be concluded that the dedicated halogenase Rdc2 has been inhibited by NaBr. Similar to this discovery, a previous study also reported that large amounts of bromide (100 mM $CaBr_2$) can alter the secondary metabolism of another fungus, *Lachnum papyraceum*, to generate a different major metabolite papyracillic acid, although the mechanism still remains unknown (Shan et al. 1996).

Effect of bromide on Rdc2 expression in *P. chlamydosporia*

Inhibition of Rdc2 in *P. chlamydosporia* by bromide can be a result of regulation of enzyme activity or protein synthesis. In vitro enzymatic studies have shown that Br^- can be used as a halogen donor by Rdc2 and has no effect on the enzymatic activity (Zeng and Zhan 2010). Thus, the possibility of activity inhibition can be ruled out and protein synthesis of Rdc2 in *P. chlamydosporia* must be inhibited by bromide. Protein synthesis can be regulated at the transcriptional or translational level. To find out whether transcription of *rdc2* is affected by bromide, we used RT-PCR to analyze mRNA synthesis of *rdc2* by *P. chlamydosporia*. The results (Fig. 3a) showed that no mRNA transcript of *rdc2* was synthesized in the presence of 50 mM NaBr, thus confirming that expression of *rdc2* was inhibited by bromide at the transcriptional level. In contrast, bromide has no obvious effect on transcription of *rdc4*, as no difference was detected between the sample with 50 mM NaBr and the control, both giving rise to the corresponding *rdc4* PCR product (Fig. 3b). This result is consistent with the production of the epoxide-containing monocillins III (**3**) and V (**5**). Therefore, it is concluded that only the halogenase in the radicicol biosynthetic pathway is specifically inhibited by Br^-.

Effect of bromide on Rdc2 expression in *E. coli*

We also examined whether expression of intron-free *rdc2* in *E. coli* is influenced by bromide at 50 mM. Both soluble and insoluble proteins in *E. coli* were

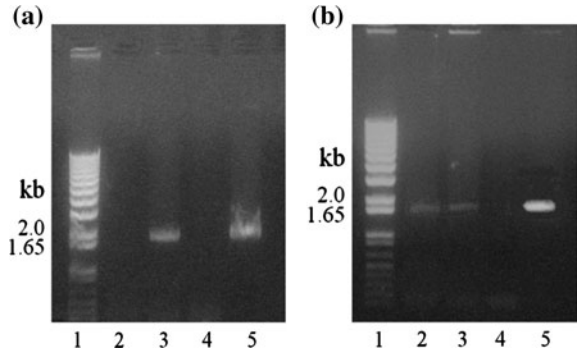

Fig. 3 RT-PCR analysis of transcription of *rdc2* (**a**) and *rdc4* (**b**). *Lane 1* 1 kb Plus DNA ladder, *Lanes 2–5* different templates. *Lane 2,* cDNA of *P. chlamydosporia* in PDB supplemented with 50 mM NaBr, *Lane 3* cDNA of *P. chlamydosporia* in blank PDB, *Lane 4* water (negative control), *Lane 5,* genomic DNA of *P. chlamydosporia* (positive control). Total RNA was extracted from 7-day old *P. chlamydosporia* cultures in PDB, and used for cDNA synthesis through RT-PCR. The cDNAs were subsequently used for amplification of *rdc2* and *rdc4* using the specific primers. Water was used as the negative control, and the fungal gnomic DNA as the positive control to ensure the PCR conditions worked. Results showed that in the presence of 50 mM NaBr, the intron-free *rdc2* gene could not be cloned (Lane 2) from the cDNA, whereas cloning of *rdc4* was not influenced (Lane 2)

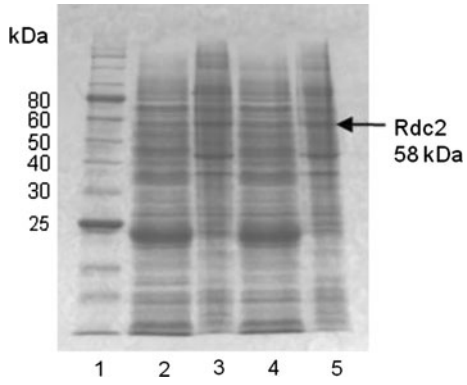

Fig. 4 SDS-PAGE analysis of Rdc2 expression in *E. coli*. *Lane 1* protein ladder, *Lane 2* soluble fraction of the lysate of cells from blank LB, *Lane 3* insoluble fraction of the lysate of cells from blank LB, *Lane 4* soluble fraction of the lysate of cells from LB supplemented with 50 mM NaBr, *Lane 5*: insoluble fraction of the lysate of cells from LB supplemented with 50 mM NaBr. Cells were harvested by centrifugation and lysed by sonication. Both soluble and insoluble (dissoved in 8 M urea) fractions were checked. The 58 kDa band of Rdc2 can be found in both soluble and insoluble fractions. No difference was observed between the samples with and without NaBr

analyzed and compared to the control by SDS-PAGE (Fig. 4). Although the expression level of Rdc2 in *E. coli* is relatively low, it was clear that NaBr has no effect on the expression of *rdc2* in pET28a under a T7 promoter in *E. coli* BL21(DE3)-CodonPlus-RIL strain. This indicated that, unlike in *P. chlamydosporia*, bromide does not interfere with transcription of *rdc2* in *E. coli*, likely due to the totally different systems in the fungus and bacterium. This also suggested a specific regulatory protein in the radicicol producing strain might be affected by Br^-, which subsequently leads to the inhibition of transcription of *rdc2*. Investigation of the specific target of Br^- is currently underway.

In summary, we have tested the effect of four halides on radicicol biosynthesis in *P. chlamydosporia* ATCC 16683. Bromide inhibits the formation of **1** by specifically targeting the dedicated halogenase Rdc2. RT–PCR analysis indicated that the expression of *rdc2* in *P. chlamydosporia* is inhibited by bromide at the transcriptional level. This work thus provides new insight into the effects of halides on secondary metabolism in filamentous fungi.

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