J|A|C|S
COMMUNICATIONS

Published on Web 12/14/2006

Production of Dihydroisocoumarins in *Fusarium verticillioides* by Swapping Ketosynthase Domain of the Fungal Iterative Polyketide Synthase Fum1p with That of Lovastatin Diketide Synthase

Xiangcheng Zhu,† Fengan Yu,† Xing-Cong Li,‡ and Liangcheng Du*,†

Department of Chemistry, University of Nebraska—Lincoln, Lincoln, Nebraska 68588, and National Center for Natural Products Research, School of Pharmacy, The University of Mississippi, University, Mississippi 38677

Received October 12, 2006; E-mail: ldu@unlserve.unl.edu

The recent sequencing of over 40 fungal genomes showed that fungi are rich in polyketide synthase (PKS) genes. However, the polyketide products synthesized by the PKS remain largely unexplored. The mechanism by which the fungal PKS controls the structure of the products is not well understood.[1] Here, we report the production of new polyketides in a filamentous fungus, *Fusarium verticillioides*, by genetically engineering a PKS gene and provide evidence for the role of the ketosynthase (KS) domain in determining fungal polyketide structure.

In bacterial modular PKS, such as 6-deoxyerythronolide B synthases for erythromycins, the order, number, and domain composition of the modules dictate the length and reduction level of the polyketide chain.[1,2] Mediated by the hydrolytic function of the thioesterase (TE) domain, the product is released with a length depending on the number of elongation cycles. Like bacterial modular PKS, fungal PKS's are modular enzymes but typically contain only a single module (Figure 1).[1] Fungal PKS with the same domain organization can synthesize a carbon chain with very different sizes in different fungi. Two distinct domain organizations are found in fungal PKS.[3] The aromatic-type PKS's typically have an organization of KS-AT-ACP-ACP-CYC, in which CYC is a TE-like domain that contributes to the chain length determination in fungal aromatic polyketide biosynthesis.[3] In contrast, fungal non-aromatic-type PKS's have additional domains, such as DH, ER, KR, and MT, but do not have a TE domain (Figure 1). It remained unclear what factors contribute to chain length determination in fungal nonaromatic polyketides.[1,2]

We have chosen to study a group of PKS's synthesizing several important fungal nonaromatic polyketides, including the cholesterol-lowering drug lovastatin, the life-threatening mycotoxin fumonisins, and pathogenesis factor T-toxins. The PKS's have an almost identical 7-domain organization but make very different polyketides. Fum1p encoded by *FUM1* is responsible for synthesizing the 18-carbon backbone of fumonisins in *Fusarium verticillioides*.[4] We have previously produced a *F. verticillioides* mutant by swapping the KS domain of *FUM1* with that of *PKS1*, which is required for synthesizing T-toxins in another filamentous fungus, *Cochliobolus heterotrophus* (Figure 1).[5] The mutant produced fumonisins with a fixed carbon chain length (Figure 2A). The heterologous KS domain is able to functionally interact with six other domains of Fum1p to iteratively make the 12,16-dimethyl C18 chain. The C18 intermediate is subsequently released from the PKS by the decarboxylative condensation with alanine, which is catalyzed by Fum8p, to produce the C20 chain and the 2-amino group of fumonisins.[6] This seems to suggest that the KS domain did not contribute to the product size control, and as long as a functional KS is embedded in Fum1p, fumonisins would be produced in this fungus. To gain more insights into the biosynthetic mechanism, we replaced the KS domain of *FUM1* with the KS domain of *LovF*, which encodes lovastatin diketide synthase (LDKS) responsible for the 4-carbon side chain of lovastatin in *Aspergillus terreus*.[7] Since T-toxin PKS1 and LDKS synthesize polyketides with very different chain lengths, we figured that the experiments may reveal new information on the KS domain's role in chain length determination.

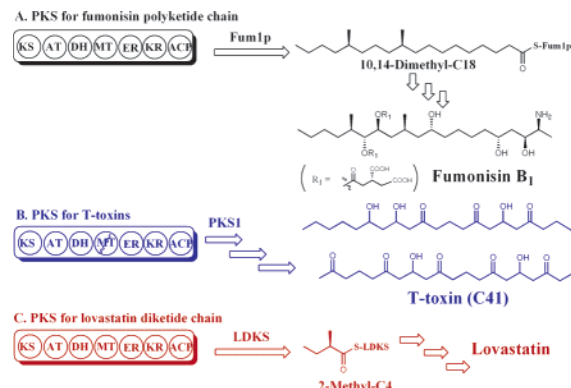

Figure 1. The domain organization of PKS for fumonisins (A), T-toxins (B), and lovastatin side chain (C) and the chemical structure of the corresponding nonaromatic polyketide products. The PKS modules consist of β-ketosynthase (KS), acyltransferase (AT), dehydratase (DH), methyltransferase (MT), enoylreductase (ER), β-ketoreductase (KR), and acyl carrier protein (ACP).

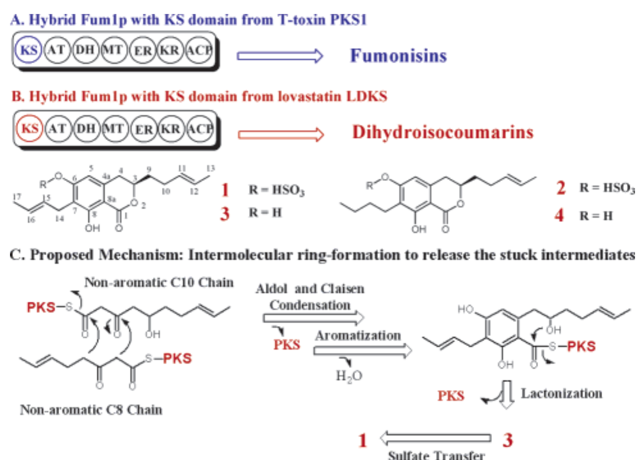

Figure 2. (A) *F. verticillioides* strain carrying *FUM1* with the KS domain of T-toxins *PKS1* produced fumonisins. (B) *F. verticillioides* strain carrying *FUM1* with the KS domain of lovastatin *LovF* produced new metabolites, dihydroisocoumarins. (C) A possible mechanism for the production of the aromatic dihydroisocoumarins by the domain-swapped PKS for fungal nonaromatic polyketides.

† University of Nebraska—Lincoln.
‡ The University of Mississippi.

10.1021/ja0672122 CCC: $37.00 © 2007 American Chemical Society

Using the two-stage screening approach for homologous recombinants in *F. verticillioides*,[8] we obtained mutants that contained a chimeric *FUM1* with a swapped KS domain from *LovF*. Identity of the mutants was confirmed by PCR, Southern blot (Figure S1), and DNA sequencing of the replaced KS domain region. The metabolites produced by the mutants were extracted from cultures and analyzed by HPLC-ELSD.[9] As controls, the wild-type *F. verticillioides* produced fumonisins, and the *FUM1*-disrupted mutant produced no fumonisins or new metabolites. The KS-replaced mutants produced four new compounds with a yield comparable to that of fumonisins in the wild-type (Figure S2). Subsequent isolation using preparative HPLC afforded four compounds (**1**−**4**). Compounds **3** and **4** were identified as 7-(*E*)-but-2-enyl-3,4-dihydro-6,8-dihydroxy-(3*R*)-(*E*)-pent-3-enylisochromen-1-one and 7-butyl-3,4-dihydro-6,8-dihydroxy-(3*R*)-(*E*)-pent-3-enylisochromen-1-one, respectively, by comparison of their spectral data (NMR and HRMS) and specific rotations with those reported in the literature.[10] Compounds **3** and **4** were recently isolated from *Geotrichum* sp., an endophytic fungus of the plant *Crassocephalum crepidioides,* and reported to have antimalarial, antituberculosis, and antifungal activities.[10] Compounds **1** and **2**, the predominant metabolites in the mutants, were determined as 6-sulfates of **3** and **4**, respectively, by NMR and HRTOFMS. Compounds **1** and **2** are new and have not been reported in the literature. The convincing evidence for such a sulfate substitution derived from the significant downfield shifting[11] of H-5 ($\Delta\delta$ −1.82 ppm), C-5, -7 and -8a ($\Delta\delta \sim -4.2$, −6.0, and −4.6 ppm, respectively), and upfield shifting[11] of C-6 ($\Delta\delta \sim +6.9$ ppm) in the NMR spectra of **1** and **2** when compared to those of **3** and **4** (Table S1). The absolute configuration of the single stereogenic carbon in **1** and **2** was also confirmed to be the same as that in **3** and **4** by their negative specific rotations.

The production of the above aromatic metabolites in *F. verticillioides* with an engineered fungal nonaromatic-type PKS is unprecedented. We proposed a possible mechanism for the production of the aromatic polyketides, which could result from an intermolecular ring formation between two (C8 and C10) linear nonaromatic polyketide intermediates (Figure 2C). The "short" intermediates that are covalently linked to the chimeric Fum1p could not be released by the "normal" mechanism via Fum8p and, thus, were "stuck" on the PKS. An aldol condensation and a Claisen condensation followed by an aromatization will release one of the stuck chains and form the aromatic ring. The subsequent lactonization will release the second chain and form the lactone functionality. The final sulfonation step, which is a common biosynthetic process in nature,[12] concludes the production of **1** and **2**. It has recently been proposed that aldol cyclization and aromatization of a linear, reduced polyketide chain lead to the formation of an aromatic polyene, aslaniol, which was heterologously produced in *A. oryzae* carrying a nonaromatic-type PKS gene (*pksF*) from *Alternaria solani*.[13]

Most fungal PKS's iteratively catalyze the carbon chain elongation to produce polyketides with distinct chain lengths. Our data suggest that the KS domain plays a key role in the control of the chain length. The studies on plant PKS[14] and bacterial aromatic-type PKS[15] have shown that the "volume" of the substrate binding pocket of plant PKS or that formed between the bacterial KS-CLF (chain length factor) is the determining factor for the polyketide chain length. Here, our results show that fungal chimeric PKS with a different KS domain makes different products. It is tempting to propose that the substrate binding pocket of the KS domain of PKS1 is large enough to allow the chimeric PKS to synthesize the C18 chain since PKS1 is involved in the synthesis of polyketides (T-toxins) larger than fumonisins. Thus, T-toxin KS within the context of Fum1p is able to produce a proper polyketide chain that can be released by the downstream enzyme Fum8p, which eventually leads to fumonisin production in the fungus. On the other hand, the volume of the KS domain of LDKS could be smaller than that of Fum1p or T-toxin PKS1 since LDKS synthesizes a short carbon chain (C4) (Figure 1). Thus, the chimeric Fum1p with the KS from LDKS could only make short chains that cannot be released by Fum8p. One possible way to release these stuck short chains is through intermolecular ring formation, leading to the title compounds, as illustrated in Figure 2C. Thus, the final outcome of the fungal nonaromatic polyketides also depends on the chain release mechanism. In the case of fumonisins, both the KS domain and the specific interaction between Fum1p and polyketide chain releasing enzyme Fum8p may be important for producing products with distinct structures.

Acknowledgment. This work was supported in part by NSF (MCB-0614916), NSF China (No. 30428023), and USDA/ARS Specific Cooperative Agreement No. 58-6408-2-0009. The research was performed in facilities renovated with support from NIH (RR015468-01). We thank Sara Basiaga and Joseph Dumais for technical assistance in NMR, and Ashraf Raza and Bharthi Avula for MS analysis.

Supporting Information Available: Details of mutant generation and identification, metabolite isolation, and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Staunton, J.; Weissman, K. J. *Nat. Prod. Rep.* **2001**, *18*, 380−416. (b) Axford, L. C.; Simpson, T. J.; Willis, C. L. *Angew. Chem., Int. Ed.* **2004**, *43*, 727−730.
(2) (a) Hopwood, D. A. *Chem. Rev.* **1997**, *97*, 2465−2498. (b) Khosla, C.; Gokhale, R. S.; Jacobsen, J. R.; Cane, D. E. *Annu. Rev. Biochem.* **1999**, *68*, 219−253. (c) Fischbach, M. A.; Walsh, C. T. *Chem. Rev.* **2006**, *106*, 3468−3496.
(3) (a) Fujii, I.; Watanabe, A.; Sankawa, U.; Ebizuka, Y. *Chem. Biol.* **2001**, *8*, 189−197. (b) Watanabe, A.; Ebizuka, Y. *Chem. Biol.* **2004**, *11*, 1101−1106.
(4) Proctor, R. H.; Desjardins, A. E.; Plattner, R. D.; Hohn, T. M. *Fungal Genet. Biol.* **1999**, *27*, 100−112.
(5) Zhu, X.; Yu, F.; Bojja, R. S.; Zaleta-Rivera, K.; Du, L. *J. Ind. Microbiol. Biotechnol.* **2006**, *33*, 859−868.
(6) Du, L.; Yu, F.; Zhu, X.; Zaleta-Rivera, K.; Bojja, R. S.; Ding, Y.; Yi, H.; Wang, Q. In *Polyketides: Biosynthesis, Biological Activities and Genetic Engineering*; Rimando, A. M., Baerson, S. R., Eds.; 2006; in press.
(7) Kennedy, J.; Auclair, K.; Kendrew, S. G.; Park, C.; Vederas, J. C.; Hutchinson, C. R. *Science* **1999**, *284*, 1368−1372.
(8) Yu, F.; Zhu, X.; Du, L. *FEMS Microbiol. Lett.* **2005**, *248*, 257−264.
(9) Xu, L.; Du, L. *J. Microbiol. Meth.* **2006**, *64*, 398−405.
(10) Kongsaeree, P.; Prabpai, S.; Sriubolmas, N.; Vongvein, C.; Wiyakrutta, S. *J. Nat. Prod.* **2003**, *66*, 709−711.
(11) Li, X.-C.; Joshi, A. S.; Tan, B.; ElSohly, H. N.; Walker, L. A.; Zjawiony, J.; Ferreira, D. *Tetrahedron* **2002**, *58*, 8709−8717.
(12) Mourão, P. A. S.; Assreuy, A. M. S. *J. Biol. Chem.* **1995**, *270*, 3132−3140.
(13) Kasahara, K.; Fujii, I.; Oikawa, H.; Ebizuka, Y. *Chembiochem* **2006**, *7*, 920−924.
(14) Jez, J. M.; Austin, M. B.; Ferrer, J.; Bowman, M. E.; Schroder, J.; Noel, J. P. *Chem. Biol.* **2000**, *7*, 919−930.
(15) (a) Tang, Y.; Tsai, S. C.; Khosla, C. *J. Am. Chem. Soc.* **2003**, *125*, 12708−12709. (b) Keatinge-Clay, A. T.; Maltby, D. A.; Medzihradszky, K. F.; Khosla, C.; Stroud, R. M. *Nat. Struct. Mol. Biol.* **2004**, *11*, 888−893.

JA0672122