Bioorganic & Medicinal Chemistry Letters 20 (2010) 4785–4788

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Aspergillus oryzae type III polyketide synthase CsyA is involved in the biosynthesis of 3,5-dihydroxybenzoic acid

Yasuyo Seshime [a,†], Praveen Rao Juvvadi [b,‡], Katsuhiko Kitamoto [b], Yutaka Ebizuka [c], Takamasa Nonaka [a], Isao Fujii [a,*]

[a] School of Pharmacy, Iwate Medical University, 2-1-1 Nishitokuta, Yahaba, Iwate 028-3694, Japan
[b] Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
[c] Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

ARTICLE INFO

Article history:
Received 10 February 2010
Revised 11 June 2010
Accepted 22 June 2010
Available online 25 June 2010

Keywords:
csyA
Type III polyketide synthase
Aspergillus oryzae
3,5-Dihydroxybenzoic acid
Alkyl-resorcinol/resorcylic acid synthase

ABSTRACT

As a novel superfamily of type III polyketide synthases in microbes, four genes *csyA*, *csyB*, *csyC*, and *csyD*, were found in the genome of *Aspergillus oryzae*, an industrially important filamentous fungus. In order to analyze their functions, we carried out the overexpression of *csyA* under the control of α-amylase promoter in *A. oryzae* and identified 3,5-dihydroxybenzoic acid (DHBA) as the major product. Feeding experiments using ^{13}C-labeled acetates confirmed that the acetate labeling pattern of DHBA coincided with that of orcinol derived from orsellinic acid, a polyketide formed by the condensation and cyclization of four acetate units. Further oxidation of methyl group of orcinol by the host fungus could lead to the production of DHBA. Comparative molecular modeling of CsyA with the crystal structure of *Neurospora crassa* 2′-oxoalkylresorcylic acid synthase indicated that CsyA cavity size can only accept short-chain acyl starter and tetraketide formation. Thus, CsyA is considered to be a tetraketide alkyl-resorcinol/resorcylic acid synthase.

Polyketides constitute a large family of natural compounds and some of them show clinically important properties as antibiotics, immunosuppressant, etc. Their carbon skeletons are constructed by polyketide synthases (PKSs) from acyl-CoA precursors.[1] Among the three types of PKSs known,[2] fungal polyketides are largely biosynthesized by iterative type I PKSs.[3] However, we recently reported the presence of genes for type III PKSs in filamentous fungi.[4] Type III PKSs are also known as chalcone synthase (CHS)-like PKSs with a simple ketosynthase architecture and independence of acyl carrier protein.[5] Recently, a single type III PKS present in *Neurospora crassa* was identified as an enzyme catalyzing the synthesis of pentaketide alkylresorcylic acid and named as 2′-oxoalkylresorcylic acid synthase (ORAS).[6]

Our previous studies revealed that the presence of four type III PKS genes (*csyA*, *B*, *C*, and *D*) in the genome of *Aspergillus oryzae* RIB40,[7] a genome project strain of industrially important fungus in Asian countries. Although orthologs of *csyA*, *csyC*, and *csyD* genes are present in a closely related species, *Aspergillus flavus*, *csyB* gene is unique to *A. oryzae*. Thus we first carried out overexpression of CsyB in *A. oryzae* M-2-3 and identified csypyrone B1 as the novel type III PKS product.[8] The *csyA* gene (Accession No. AB206758) encodes a protein comprising of 402 amino acids with a calculated molecular mass of 43.5 kDa. We observed that the expression of *csyA* gene in RIB40 was prominent and occurred throughout the 1–7 days of culture.[4] Although the similar expression of *csyD* gene was observed, CsyD lacks the conserved CHS catalytic triad (Cys-His-Asn), which is required for the priming and extension of the polyketide.[5] Consequently, we chose CsyA as a next target to analyze its function by overexpression and identification of its product compound(s).

The *csyA* cDNA amplified using the forward (5′-cac gtg ATG GCG CCC TTA ATT CAT GGT-3′) and reverse (5′-cac gtg TTA GGC CGG CCG AGC-3′) primers was cloned into the *Sma*I digested expression vector pTAex3[9] to place the gene under the control of *amyB* promoter. We have used *A. oryzae* M-2-3 as a host fungus to express heterologous fungal PKS genes.[10] Fortunately, no expression of *csyA* gene in this strain was confirmed by RT-PCR analysis under induction culture condition (data not shown). Thus the constructed expression plasmid pTA-csyA containing *argB* gene as selection marker was subsequently transformed into *A. oryzae* M-2-3 by protoplast-PEG method[11,12] for overexpression studies. The *A. oryzae* M-2-3 transformant with pTAex3 served as a control. Transformants selected on minimal medium were first cultured in DPY medium[12] and then transferred to the media containing maltose to induce the expression of *csyA*. After culturing in the induction

* Corresponding author. Tel.: +81 19 651 5111x5260; fax: +81 19 698 1923.
E-mail address: ifujii@iwate-med.ac.jp (I. Fujii).
† Present address: National Institute of Health Sciences, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan.
‡ Present address: Department of Pediatrics, Duke University Medical Center, Durham, NC 27710, USA.

0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.06.119

media for 3 days, the culture media was extracted with ethyl acetate under acidic condition, which was then subjected to HPLC analysis with an Inertsil ODS-3 reverse-phase column (4.6 × 150 mm, GL Science, Japan). In the pTA-csyA transformant extract, three distinct peaks designated as A1, A2, and A3 were detected at retention time of 5.5, 11.4, and 17.7 min, respectively, of which A1 was a major peak. None of these peaks were found in the extracts of control transformant nor the host fungus indicating the formation of these products specifically due to the overexpression of *csyA* (Fig. 1).

In order to further characterize the major product A1, we isolated it from the 5-L induction culture of pTA-csyA transformant. After removal of mycelia by filtration, combined induction culture media were applied onto a reverse phase silica gel column (3 × 50 cm, Cosmosil 75C_{18}-OPN, Nacalai Tesque, Japan). Elution was carried out with 10% aq acetonitrile containing 1% acetic acid

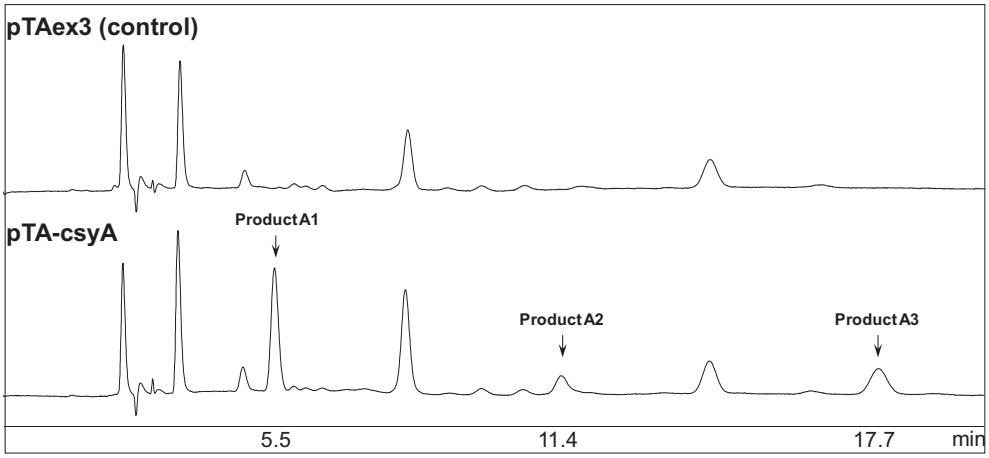

Figure 1. HPLC analysis of *Aspergillus oryzae* CsyA product. HPLC analysis with an Inertsil ODS-3 reverse-phase column (4.6 × 150 mm, GL Science, Japan) was performed with a solvent system of acetonitrile containing 1% acetic acid (solvent B) and H_2O containing 1% acetic acid (solvent A) at flow rate 0.8 ml/min (10% solvent B for 20 min) at 40 °C. Elution was monitored at 254 nm. pTA-csyA, extract prepared from the *A. oryza*e pTA-csyA transformant; pTAex3, control extract prepared from the *A. oryzae* pTAex3 transformant.

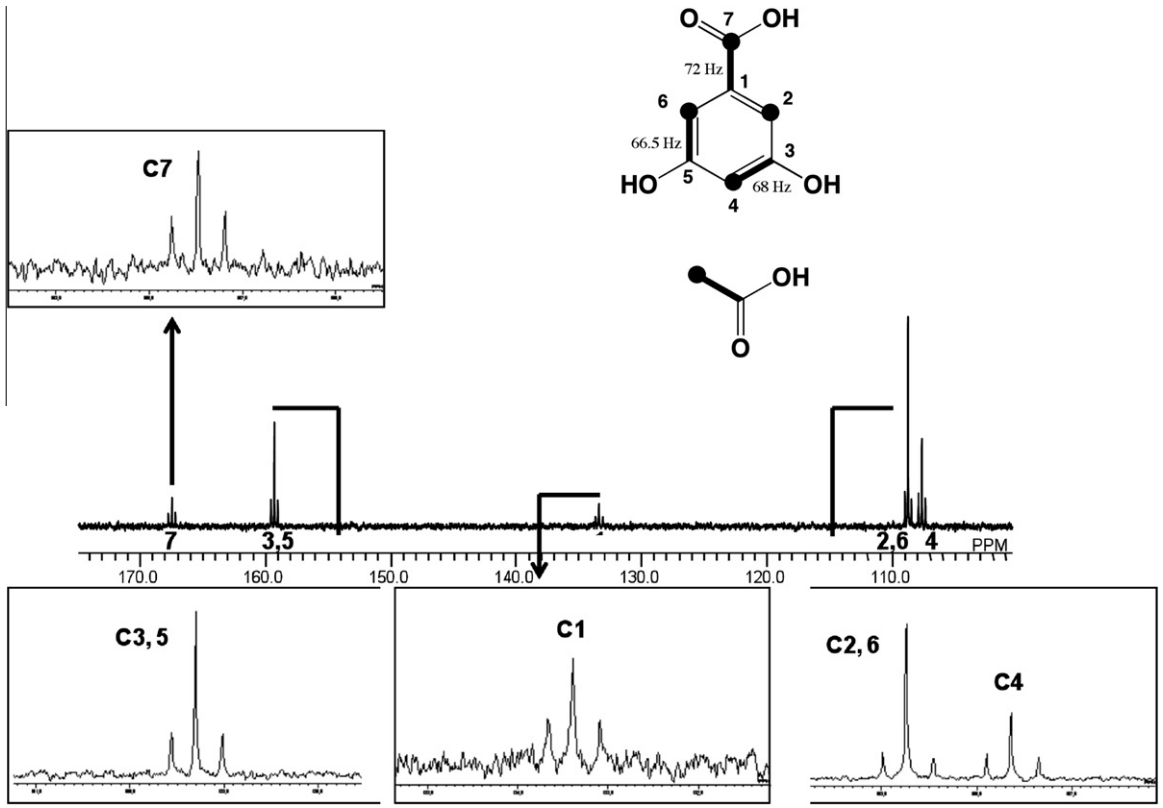

Figure 2. ^{13}C NMR analysis of 3,5-dihydroxybenzoic acid fed with [1,2-$^{13}C_2$] acetate. Fifty mg of sodium [1,2-$^{13}C_2$] acetate (99 atom % enriched, Isotec) mixed with 100 mg of non-labeled sodium acetate was fed to the *A. oryzae* pTA-csyA transformant culture (1 L) during the maltose induction for 4 days. After isolation of labeled DHBA, ^{13}C NMR was measured on ECA-500 spectrometer (125 MHz, JEOL, Japan) in acetone-d_6. Enlarged spectra are shown in boxes. Incorporations of acetate units are shown on the DHBA structure as bold lines, and those of methyl carbons of acetate units are shown as closed circles. ^{13}C–^{13}C coupling constants are shown in Hz.

and the eluate was then applied onto a ODS-80TM column (7.8 × 300 mm, Tosoh, Japan), and finally eluted with 10% aq acetonitrile containing 1% acetic acid at a flow rate of 2.0 ml/min to afford about 25 mg of the CsyA product A1. By physicochemical analysis including ^{1}H and ^{13}C NMR (Supplementary data), product A1 was identified to be 3,5-dihydroxybenzoic acid (DHBA). Comparison with the authentic compound (Tokyo Chemical Industry, Japan) confirmed its identity. This result indicated that *cysA* is involved in the production of DHBA in *A. oryzae*. Since structures of product A1 (DHBA), A2, and A3 could be informative for metabolic relationship among them, isolation and characterization of product A2 and A3 are now underway in our laboratory.

The involvement of type III PKS in DHBA biosynthesis was reported in kendomycin biosynthesis in *Streptomyces violaceoruber*.[13] Heterologous expression of a gene set including Ken2 PKS confirmed the production of DHBA in *Streptomyces coelicolor*. Ken2 PKS together with Ken3 and Ken7, enoyl CoA hydratase/isomerase, is considered to catalyze the formation of 3,5-dihydroxyphenylacetyl-CoA from four malonyl-CoAs, which is then converted by oxidation and decarboxylation to DHBA in kendomycin biosynthesis. In contrast to this bacterial DHBA formation, overexpression of CsyA in *A. oryzae* yielded DHBA as the apparent direct product.

To identify the polyketide nature of DHBA produced by the CsyA overexpressing transformant, feeding experiments with ^{13}C-labeled acetates were carried out. The initial feeding experiment with sodium [2-^{13}C] acetate gave ^{13}C-enrichments at C-2, -4, -6, and -7 indicated that DHBA was formed by the condensation of four acetate units, excluding an involvement of shikimate pathway. More information on the possible ring closure pattern was obtained by the feeding of [1,2-$^{13}C_2$] acetate. ^{13}C NMR analysis of the DHBA fed with [1,2-$^{13}C_2$] acetate indicated that all signals were accompanied by doublets due to incorporation of intact acetate units. The C-7 signal was coupled with C-1 signal with a coupling constant of $J = 72$ Hz. Two coupled C–C pairs with $J = 66.5$ and 68 Hz indicated the presence of two acetate units in DHBA benzene ring. The higher intensity of non-coupled ^{13}C signal of C-2 (or C-6) was ascribed to the incorporation of methyl carbon of acetate and then followed by decarboxylation, whose signal appeared as an enriched singlet (Fig. 2). This acetate labeling pattern corresponded well with tetraketide nature and is similar to that of orcinol derived from orsellinic acid. Also, this labeling pattern is different from that of DHBA derived from 3,5-dihydroxyphenylacetyl-CoA.[13]

Thus, CsyA is considered to be a tetraketide alkyl-resorcinol/resorcylic acid synthase, and the oxidation of its side chain resulted in the production of DHBA in *A. oryzae* transformant. Orsellinic acid is the smallest tetraketide methylresorcylic acid and the biosynthetic precursor of penicillic acid in *Penicillium* fungi.[14] Bacterial orsellinic acid synthase (OAS) gene *aviM* was identified in *Streptomyces viridochromogenes* Tü57 involved in the biosynthesis of avilamycins as the first iterative type I PKS gene in 1997,[15] which belongs to a partially reducing type PKS clade in phylogenetic analysis.[16] On the other hand, genes for fungal OAS were reported quite recently, which belongs to a non-reducing type I PKSs clade.[17,18] As a fungal type III PKS, CsyA could function as OAS or orcinol synthase though no orsellinic acid nor orcinol was detected in the *A. oryzae* pTA-csyA transformant (Fig. 3).

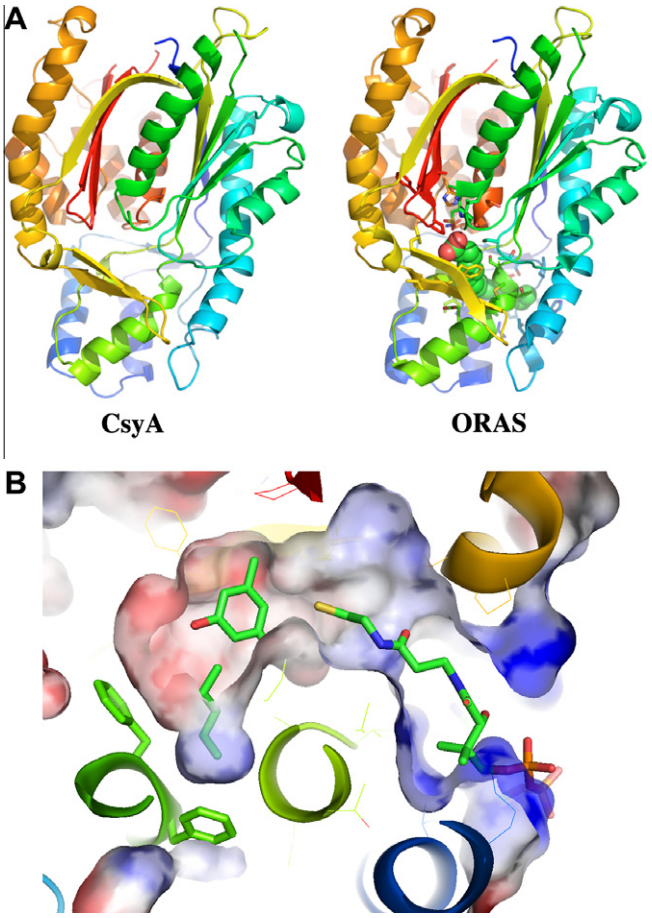

Figure 4. Homology modeling of CsyA and its comparison with ORAS crystal structure. (A) Homology model of CsyA and crystal structure of ORAS. Homology model of CsyA was constructed using Modeller based on ORAS crystal structure (PDB ID: 3EUT). Each protein molecule is shown as a ribbon diagram and the bound eicosanoic acid within the static acyl-binding tunnel of ORAS is shown as a CPK model. Side chains in the near vicinity of the ligand are shown as stick models. (B) Active site cavity of CsyA. Plausible product orcinol, CoA, and a short carbon chain are placed in the active site cavity (shown as half-transparent surface) of the homology model. Side chains of Phe^{204} and Phe^{205} (shown as stick models), respectively, form the side and bottom wall of the acyl-binding pocket. Of the corresponding Met^{189}-Val^{190} residues of ORAS, Met^{189} is considered to guide the binding of hydrocarbon tail of fatty acid substrate. The figures were drawn by PyMOL (DeLano, W.L. The PyMOL Molecular Graphics System (2002) DeLano Scientific, Palo Alto, CA, USA.)

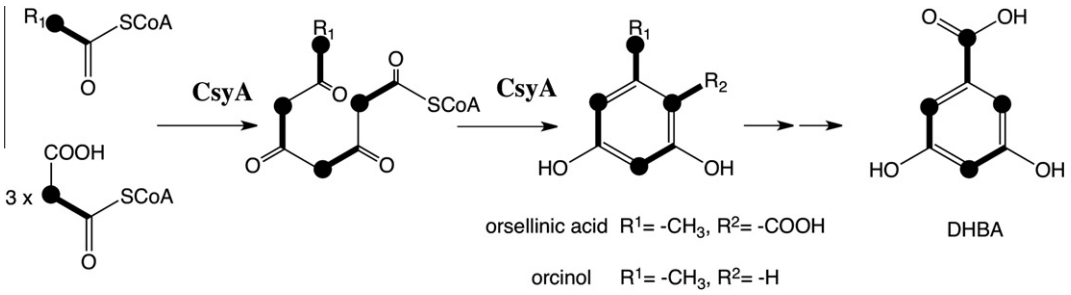

Figure 3. Proposed CsyA reaction and 3,5-dihydroxybenzoic acid formation in *A. oryzae*. CsyA catalyzes tetraketide formation with a short-chain acyl-CoA such as acetyl-CoA ($R_1 = -CH_3$) and 3 malonyl-CoAs. Then, aldol cyclization of tetraketide in CsyA active center cavity forms orsellinic acid ($R_1 = -CH_3$, $R_2 = -COOH$), and/or orcinol ($R_1 = -CH_3$, $R_2 = -H$) by the following decarboxylation. Further oxidation of the starter side chain by the host fungus gives 3,5-dihydroxybenzoic acid formation.

Related type III PKS for pentaketide alkylresorcylic acid synthesis from *N. crassa* was reported by Horinouchi and co-workers.[6] This enzyme named 2′-oxoalkylresorcylic acid synthase (ORAS) prefers stearoyl-CoA as starter substrate and condenses four malonyl-CoAs to give pentaketide products. Our feeding experiments indicated that *A. oryzae* CsyA is a tetraketide synthase for orsellinic acid and/or orcinol. CsyA showed 38% identity with ORAS which is the highest among type III PKSs of which 3D structures are reported. Thus homology modeling of CsyA was carried out using Modeller[19] based on the structure of ORAS[20] (Fig. 4A). From 3D structures of several type III PKSs, it was suggested that their active site cavity sizes control carbon chain-lengths of their products.[21,22] In *N. crassa* ORAS which utilizes long-chain fatty acid CoA ester as a starter, the presence of a static acyl-binding tunnel for the starter is observed.[20] However, structural model of CsyA indicated that side chains of Phe^{204} and Phe^{205} (shown as stick models in Fig. 4B), respectively, form the side and bottom wall of the acyl-binding pocket. Thus binding of long acyl chain could be blocked by these Phe^{204}-Phe^{205} residues. Of the corresponding Met^{189}-Val^{190} residues of ORAS, Met^{189} is considered to guide the binding of hydrocarbon tail of fatty acid substrate. The cavity size of CsyA catalytic center is similar to that of ORAS to accommodate a single aromatic ring which is significantly smaller than that of plant CHS.[23] These modeling studies strongly indicate that CsyA is a tetraketide alkyl-resorcinol/resorcylic acid synthase using short-chain acyl-CoA starter though site-directed mutation and crystallographic studies should be carried out to confirm the above mentioned assumption.

Independent study on CsyA was carried out by Zhan and co-workers.[24] They expressed His-tagged CsyA in *Escherichia coli* and analyzed its catalytic activity in vitro. Products they observed were all pyrone compounds with fatty acyl starters including long chain C_{10}–C_{18} CoAs. None of aromatic compounds were detected. This contradictory result from ours might indicate that proper expression system is required for functional analysis of PKSs, even in the relatively small type III PKSs.

Acknowledgments

This work was supported in part by the Grant-in-Aid for Young Scientists (Start-up) (No. 19810018) to Y.S., by Grant-in-Aid for Scientific Research (B) (No. 19310139) to I.F. from the Japan Society for the Promotion of Science, and by a Grant-in-Aid for Scientific Research on Priority Areas 'Applied Genomics' to I.F. from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.06.119.

References and notes

1. Staunton, J.; Weissman, K. J. *Nat. Prod. Rep.* **2001**, *18*, 380.
2. Hopwood, D. A. *Chem. Rev.* **1997**, *97*, 2465.
3. Cox, R. J. *Org. Biomol. Chem.* **2007**, *5*, 2010.
4. Seshime, Y.; Juvvadi, P. R.; Fujii, I.; Kitamoto, K. *Biochem. Biophys. Res. Commun.* **2005**, *331*, 253.
5. Austin, M. B.; Noel, J. P. *Nat. Prod. Rep.* **2003**, *20*, 79.
6. Funa, N.; Awakawa, T.; Horinouchi, S. *J. Biol. Chem.* **2007**, *282*, 14476.
7. Machida, M.; Asai, K.; Sano, T.; Tanaka, T.; Kumagai, T., et al *Nature* **2005**, *438*, 1157.
8. Seshime, Y.; Juvvadi, P. R.; Kitamoto, K.; Ebizuka, Y.; Fujii, I. *Bioorg. Med. Chem.* **2010**, *18*, 4542.
9. Fujii, T.; Yamaoka, H.; Gomi, K.; Kitamoto, K.; Kumagai, C. *Biosci., Biotechnol., Biochem.* **1995**, *59*, 1869.
10. Fujii, I. *J. Antibiot.* **2010**, *63*, 207.
11. Gomi, K.; Iimura, Y.; Hara, S. *Agric. Biol. Chem.* **1987**, *51*, 2549.
12. Kitamoto, K. *Adv. Appl. Microbiol.* **2002**, *51*, 129.
13. Wenzel, S. C.; Bode, H. B.; Kochems, I.; Müller, R. *ChemBioChem* **2008**, *9*, 2711.
14. Turner, W. B.; Aldridge, D. C. *Fungal Metabolites II*; Academic Press: London, 1983.
15. Gaisser, S.; Trefzer, A.; Stckert, S.; Kirshning, A.; Bechthold, A. *J. Bacteriol.* **1997**, *179*, 6271.
16. Fujii, I.; Watanabe, A.; Ebizuka, Y. In *Advances in Fungal Biotechnology for Industrial, Agriculture, and Medicine*; Tkacz, J. S., Lange, L., Eds.; Kluwer Academic/Plenum Publishers: New York, 2004; p 97.
17. Schroeckh, V.; Scherlach, K.; Nützmann, H.-W.; Shelest, E.; Schmidt-Heck, W.; Schuemann, J.; Martin, K.; Hertwck, C.; Brakhage, A. A. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 14558.
18. Sanchez, J. F.; Chiang, Y.-M.; Szeczyk, E.; Davidson, A. D.; Ahuja, M.; Oakley, C. E.; Bok, J. W.; Keller, N.; Oakley, B. R.; Wang, C. C. C. *Mol. Biosyst.* **2010**, *6*, 587.
19. Marti-Renom, M. A.; Stuart, A.; Fiser, A.; Sánchez, R.; Melo, F.; Sali, A. *Annu. Rev. Biophys. Biomol. Struct.* **2000**, *29*, 291.
20. Rubin-Pitel, S. B.; Zhang, H.; Vu, T.; Brunzelle, J. S.; Zhao, H.; Nair, S. K. *Chem. Biol.* **2008**, *15*, 1079.
21. Abe, I.; Oguro, S.; Utsumi, Y.; Sano, Y.; Noguchi, H. *J. Am. Chem. Soc.* **2005**, *127*, 12709.
22. Abe, I.; Utsumi, Y.; Oguro, S.; Morita, H.; Sano, Y.; Noguchi, H. *J. Am. Chem. Soc.* **2005**, *127*, 1362.
23. Ferrer, J.-L.; Jez, J. M.; Bowman, M. E.; Dixon, R. A.; Noel, J. P. *Nat. Struct. Biol.* **1999**, *6*, 775.
24. Yu, D.; Zeng, J.; Chen, D.; Zhan, J. *Enzyme Microb. Technol.* **2010**, *46*, 575.