Enzymatic Halogenation

Deutsche Ausgabe: DOI: 10.1002/ange.201604516
Internationale Ausgabe: DOI: 10.1002/anie.201604516

Regioselective Dichlorination of a Non-Activated Aliphatic Carbon Atom and Phenolic Bismethylation by a Multifunctional Fungal Flavoenzyme

*Pranatchareeya Chankhamjon, Yuta Tsunematsu, Mie Ishida-Ito, Yuzuka Sasa, Florian Meyer, Daniela Boettger-Schmidt, Barbara Urbansky, Klaus-Dieter Menzel, Kirstin Scherlach, Kenji Watanabe, and Christian Hertweck**

Dedicated to Prof. Dr. Dr. h.c. mult. Gerhard Bringmann on the occasion of his 65th birthday

Abstract: *The regioselective functionalization of non-activated carbon atoms such as aliphatic halogenation is a major synthetic challenge. A novel multifunctional enzyme catalyzing the geminal dichlorination of a methyl group was discovered in Aspergillus oryzae (Koji mold), an important fungus that is widely used for Asian food fermentation. A biosynthetic pathway encoded on two different chromosomes yields mono- and dichlorinated polyketides (diaporthin derivatives), including the cytotoxic dichlorodiaporthin as the main product. Bioinformatic analyses and functional genetics revealed an unprecedented hybrid enzyme (AoiQ) with two functional domains, one for halogenation and one for O-methylation. AoiQ was successfully reconstituted in vivo and in vitro, unequivocally showing that this $FADH_2$-dependent enzyme is uniquely capable of the stepwise gem-dichlorination of a non-activated carbon atom on a freestanding substrate. Genome mining indicated that related hybrid enzymes are encoded in cryptic gene clusters in numerous ecologically relevant fungi.*

In organic molecules, halogen substituents play eminent roles in altering the electronic, steric, and reactive properties that facilitate their binding to biological targets. Thus it is not surprising that among organohalogen compounds, there is a high propensity for biological activity.[1] This correlation also holds true for the many known halogenated natural products, including the important antibiotics chlorotetracycline, chloramphenicol, and vancomycin and toxic compounds such as epibatidine and ochratoxin.[1] In light of the harsh reaction conditions and toxic reagents often required for halogenation reactions in organic synthesis, it is remarkable that fluorine, chlorine, bromine, and iodine substituents are introduced into biomolecules under physiological conditions. During the past two decades, many meticulous studies at the genetic, biochemical, and chemical level have revealed a large number of halogenating enzymes and three major avenues for halogen incorporation in living cells.[2] Mainly for fluorination, an *S*-adenosylmethionine (SAM) dependent enzyme mediates the attack of a nucleophilic halide anion (X^-). In contrast, for chlorination, bromination, and iodination, oxidative strategies predominate. Either a halogen radical ($X^\bullet$) equivalent is provided by a halogen-bound high-valent Fe^{IV} oxo species generated by non-heme-iron-dependent halogenases, or electrophilic hypohalite (XO^-) species that are equivalent to X^+ are used in processes that involve heme- or vanadium-dependent haloperoxidases or flavin-dependent halogenases.[2] As all of these versatile halogenating enzymes utilize halide anions as substrates, they are valuable catalysts for biotransformations.[3,4] In comparison to the many well investigated aromatic halogenases, only a limited number of aliphatic halogenases have been characterized biochemically. With only one exception,[5] these enzymes utilize aliphatic substrates that are covalently bound to the thiolation domains of non-ribosomal peptide synthetases and polyketide synthases,[6] which hampers their synthetic application. Notably, all aliphatic halogenases that were successfully reconstituted in vitro are Fe^{II} ketoglutarate dependent enzymes. Herein, we report the discovery of an unprecedented fungal flavoenzyme that catalyzes the highly regiospecific sequential aliphatic dichlorination of a freestanding polyketide substrate.

When searching for congeners of the halogenated mycotoxin aspirochlorine in the secondary metabolome of *Aspergillus oryzae* RIB40 (Koji mold), a fungus that is widely used for food fermentation in Asian countries,[7] we unexpectedly discovered several halogenated metabolites (**1**–**4**; Figure 1 A) that are unrelated to aspirochlorine. HPLC-DAD-HRESI-MS analysis revealed distinct isotope patterns for **1**–**4**, which clearly indicated that these molecules contain one or even two chlorine atoms. To fully elucidate the structure of the novel metabolites, we purified analytical samples of **1**–**4** from the extract of a large-scale (100 L) culture (2.65 mg **1**, 320 μg **2**, 590 μg **3**, 246 μg **4**). NMR and MS data showed that compound **1** is dichlorodiaporthin (or dichlorodiaportin).

[*] P. Chankhamjon, Dr. M. Ishida-Ito, F. Meyer, Dr. D. Boettger-Schmidt, B. Urbansky, K.-D. Menzel, Dr. K. Scherlach, Prof. Dr. C. Hertweck
Department of Biomolecular Chemistry and BioPilotPlant
Leibniz Institute for Natural Product Chemistry and Infection Biology (HKI), Beutenbergstrasse 11a, 07745 Jena (Germany)
E-mail: christian.hertweck@leibniz-hki.de
Homepage: http://www.leibniz-hki.de
Dr. Y. Tsunematsu, Y. Sasa, Prof. Dr. K. Watanabe
Department of Pharmaceutical Sciences
University of Shizuoka
Shizuoka 422-8526 (Japan)

Supporting information for this article can be found under: http://dx.doi.org/10.1002/anie.201604516.

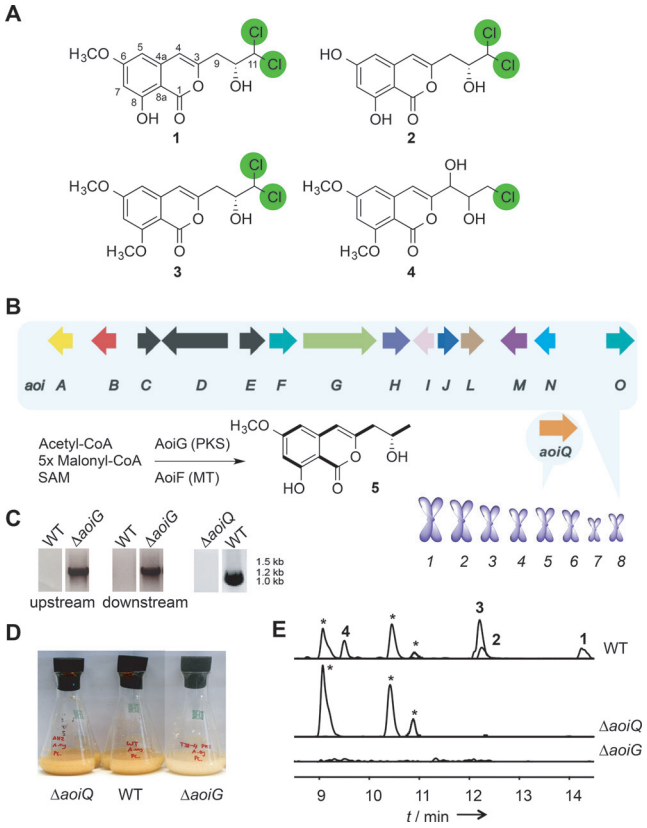

Figure 1. A) Structures of the mono- and dichlorinated diaporthins **1**–**4**. B) Organization of the diaporthin (**5**) biosynthesis gene cluster (*aoi*) in *A. oryzae* (chromosome 8) and the halogenase gene *aoiQ* (chromosome 5). MT = methyltransferase. C) PCR showing the successful deletion of *aoiG* and *aoiQ* genes in the genome of *A. oryzae*. D) Phenotypes of wild type (WT) and mutant variants. E) HPLC-MS profiles of wild-type, Δ*aoiQ*, and Δ*aoiG* mutant cultures. Extracted mass traces; peaks marked with asterisks indicate tentative side products of the diaporthin biosynthetic pathway.

This unusual polyketide was first isolated from *Penicillium nalgiovense*, the most dominant fungus contamination in cheese.[8] According to 1D and 2D NMR data, congeners **2** and **3** are structurally related to **1**, differing solely in the O-methylation pattern. Originally, these halogenated natural products were found in cultures of *Ampelomyces* sp.[9] and of a lichen mycobiont (*Graphis* sp.),[10] respectively. From MS and NMR analyses, we inferred the structure of the new compound **4**, a C9-hydroxylated derivative of **3** that lacks one chlorine substituent.

To evaluate potential toxic properties of the halogenated metabolites, we subjected the predominant derivative (**1**) to a cell-based assay. We found that **1** is highly cytotoxic (HeLa cells $CC_{50} = 9.0\ \mu g\,mL^{-1}$). Moreover, under emulated typical Koji fermentation conditions using rice (20 g), 2.5 μg of **1** are produced. In light of the eminent importance of this fungus that is extensively used for food fermentation, it is remarkable that the chlorinated compounds **1**–**4** have thus far been overlooked.

Notably, only the parent compound, diaporthin (**5**), and some non-chlorinated congeners had been detected in *A. oryzae*, albeit only after genetic activation of a silent polyketide synthase (PKS) gene cluster (*aoi* gene locus; Figure 1B).[11] To investigate whether the biosynthesis of the chlorinated compounds **1**–**4** involves the same polyketide synthase, we deleted the gene encoding the diaporthin PKS (*aoiG*; Figure 1C). The resulting mutant showed an altered phenotype that is less pigmented (Figure 1D). HPLC-MS monitoring of the Δ*aoiG* mutant culture revealed that the formation of **1** and derivatives was completely abrogated (Figure 1E), thus confirming the biogenetic relationship of **1**–**4**. Most surprisingly, however, no putative halogenase-encoding gene could be found within or in vicinity to the *aoi* gene cluster. To pinpoint candidate genes for halogenases involved in the formation of **1**–**4**, we mined the whole genome of *A. oryzae* and identified four genes (*hal1*–*hal4*) that could potentially code for halogenases. We have recently shown that one of the genes (*hal1* = *aclH*) is involved in aspirochlorine biosynthesis.[7] To test the involvement of any of the remaining three genes (*hal2*, *hal3*, and *hal4)* in diaporthin halogenation, we deleted all genes individually in the *A. oryzae* RIB40 genome. HPLC-HRESI-MS monitoring of the mutant cultures showed that the formation of the chlorinated derivatives **1**–**4** was fully abolished in the Δ*hal2* (renamed to Δ*aoiQ*) culture extract (Figure 1E). Thus we have unequivocally confirmed that AoiQ is involved in the biosynthesis of all chlorinated diaporthin derivatives (**1**–**4**).

Surprisingly, *aoiQ* is located on chromosome 5 next to an orphan PKS gene cluster whereas the diaporthin biosynthesis gene cluster is located on chromosome 8 (Figure 1B). Split gene clusters or biosynthetic crosstalk in fungal biosynthetic pathways are highly unusual. Rare examples are the flavin adenine dinucleotide ($FADH_2$) dependent monooxygenase from the fumiquinazoline biosynthetic pathway[12] and the *S*-methyltransferase TmtA, which alkylates reduced gliotoxin.[13] It appears that we have discovered a rare case of biosynthetic crosstalk involving a halogenase.

Bioinformatic analysis of the deduced amino acid sequence of AoiQ gave a surprising result. First, we found two signature motifs for $FADH_2$-dependent halogenases; aside from the highly conserved GxGxxG motif, which is involved in the binding of the flavin cofactor, we detected a region featuring the characteristic WxWxIP tryptophan motif.[14] Second, we noted that AoiQ markedly differs from known halogenases as the encoded protein is substantially larger than typical flavoproteins (1014 vs. 550–580 amino acids; Figure 2A); its C-terminus harbors a conserved *S*-adenosylmethionine (SAM) binding domain, a hallmark for methyltransferases. Thus the architecture of AoiQ suggested that the enzyme represents a novel type of halogenase–methyltransferase hybrid.

To rule out a potential misannotation and to evaluate whether AoiQ is indeed a bifunctional protein, the 5′- and 3′-ends of the matured mRNA transcript of *aoiQ* were determined by 5′- and 3′-RACE (rapid amplification of cDNA ends). cDNA sequencing confirmed that AoiQ comprises two distinct functional domains with a total of 1014 amino acids (Figure 2B).

To confirm the biochemical function of AoiQ, a codon-optimized synthetic gene was expressed in *E. coli*, and the 113 kDa N-His_6-tagged AoiQ was purified with a Ni-NTA

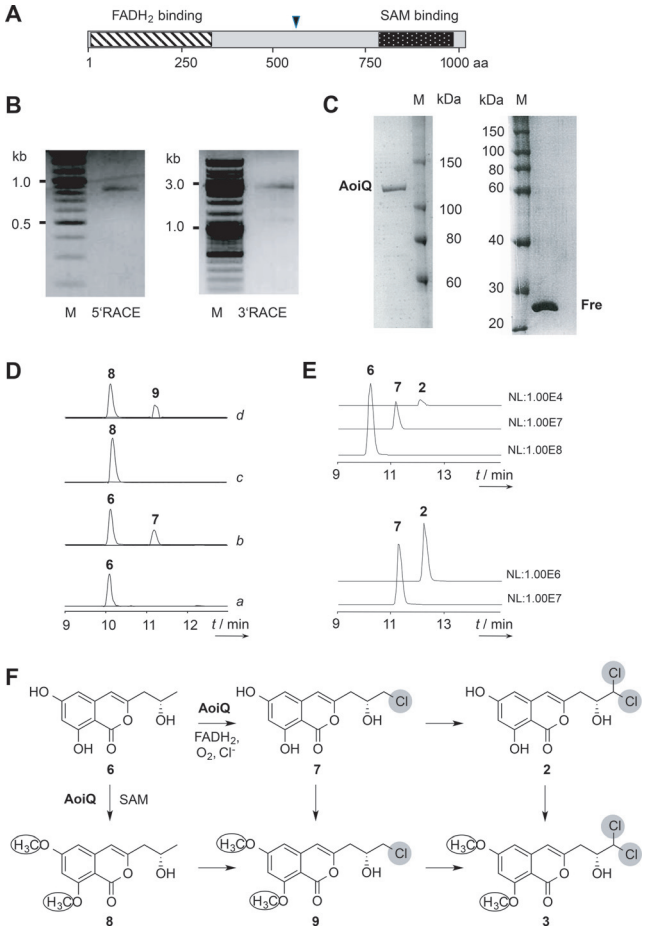

Figure 2. A) Schematic view of the bifunctional enzyme AoiQ with N-terminal halogenase and C-terminal methylase domains; the triangle indicates the average size of $FADH_2$-dependent halogenases. B) Determination of the 5′- and 3′-ends of *aoiQ* by RACE confirms the predicted transcript. C) Stained SDS-PAGE showing the purity of heterologously produced AoiQ and Fre. D) HPLC-MS profiles of biotransformation experiments of **6** using a) heat-inactivated AoiQ and Fre (negative control), b) active AoiQ and Fre, c) active AoiQ and SAM, and d) active AoiQ, Fre, and SAM. E) HPLC-MS profiles of up-scaled AoiQ enzyme assays for the conversion of synthetic **6** and **7** (monochlorinated) into dichlorinated **2**. F) Metabolic grid showing the structures of the starting materials, intermediates, and products formed by the dual action of AoiQ.

column (yield: 3.36 mg from a 1 L *E. coli* culture; Figure 2C). As a putative $FADH_2$-dependent halogenase AoiQ would require a flavin reductase to generate $FADH_2$ from FAD and NADH, we chose Fre from *E. coli* as the cofactor-regenerating enzyme. N-terminally His_6-tagged Fre was purified with a Ni-NTA column (yield: 4.26 mg from a 1 L culture; Figure 2C). To determine the cofactor in AoiQ, the heat-denatured enzyme was extracted with methanol and analyzed by LC-HRMS. A peak corresponding to FAD with *m/z* 784.1504 ($[M-H]^-$; calcd for $C_{27}H_{32}N_9O_{15}P_2^-$: 784.1499) was detected. The identity of FAD was confirmed by comparison with an authentic reference sample (Figure S4).

In the in vitro biotransformation assay, a solution of **6** (0.1 mM) was incubated with AoiQ and Fre in the presence of the three co-substrates, FAD, NADH, and O_2 (Figure 2D). In the negative controls, heat-inactivated enzymes were added. The course of the reaction was monitored by LC-HRMS. We observed that the active enzyme converted **6** into a new product **7** (Figure 2D, trace b) with *m/z* 269.0225 ($[M-H]^-$; calcd for $C_{12}H_{10}O_5Cl^-$: 269.0222), showing the characteristic isotope signature of a chloride atom (see the Supporting Information). In contrast, heat-inactivated AoiQ in the negative control was incapable of halogenating **6** (Figure 2D, trace a). The identity of the monochlorinated compound **7** was unequivocally confirmed by HPLC-MS comparison of the reaction product with a synthetic reference (Figure S5).

To confirm its predicted methyltransferase function, AoiQ was employed in an in vitro assay without Fre, but with added *S*-adenosylmethionine (SAM) as the methyl donor. Again, the course of the reaction was monitored by LC-MS. Whereas no transformation of **6** in the presence of inactivated AoiQ was observed, the active enzyme converted **6** into the bismethylated compound **8** (Figure 2D, trace c) with *m/z* 265.1066 ($[M+H]^+$: calcd for $C_{14}H_{17}O_5^+$: 265.1071). The structure of **8** was confirmed by $LC\text{-}MS^n$ and comparison with a synthetic reference sample. Next, we incubated compound **6** in the presence of AoiQ, Fre, and co-substrates FAD, NADH, SAM, and sodium chloride. LC-HRMS analyses revealed that **6** is converted into compound **8** and its monochlorinated derivative **9** (Figure 2D, trace d).

Initially, the dichlorinated products were not detectable in the enzyme assay, which may point to a low turnover number as is typical for halogenases.[2] Another plausible explanation would be that either two halogenases work together (as in the barbamide pathway)[6c] or additional enzymes are required (as in chloramphenicol biosynthesis).[15] To determine whether AoiQ is capable of introducing both chlorine atoms, we first established an in vivo biotransformation assay using *Aspergillus niger* A1179 as a heterologous expression host.[16] An expression vector harboring the *aoiQ* gene was constructed and introduced into *A. niger*, and **6** was administered to the culture of the transformant. LC-HRMS of the assay revealed the formation of dichlorinated **3** in the extract. Notably, the dichlorinated product **3** was not detectable in a control experiment using *A. niger* harboring the empty vector. These results indicate that AoiQ is responsible for attaching both chlorine atoms to the aliphatic polyketide side chain of diaporthins.

We then revisited the enzyme assay and scaled up the reaction mixture by using larger amounts of freshly prepared enzymes and synthetic **6** as the substrate. We detected not only monochlorinated **7**, but also small amounts of dichlorinated **2** in the in vitro assay mixture. In addition, to monitor the second halogenation step, we employed synthetic **7** in the enzyme assay, which led to substantially higher amounts of the dichlorinated compound **2**. Yet, the turnover proved to be very low, and kinetic analyses were hampered by the instability of the enzyme during the assay. Nonetheless, these results unequivocally confirm that AoiQ is a bifunctional flavin- and SAM-dependent halogenase/O-methyltransferase that catalyzes the sequential dichlorination of a non-activated methyl carbon atom followed by bis-O-methylation.

Aside from the unexpected multiple functions of the hybrid enzyme, it is most unusual that the site of halogenation is a non-activated methyl group, not the aromatic ring. All flavin-dependent halogenases that have been successfully reconstituted in vitro are involved in aromatic substitution reactions using a Cl^+ equivalent.[2] Accordingly, a non-aromatic substrate would be expected to react via a stabilized carbanion, for example, upon deprotonation of a C–H acidic carbon atom adjacent to a carbonyl group. Thus a thioester is considered to be a plausible intermediate in chloramphenicol biosynthesis.[2a] In contrast, our results unequivocally show that the diaporthin dihalogenase accepts a secondary alcohol as substrate. HPLC-MS monitoring of the enzyme reaction did not give any indication of a carbonyl intermediate. Consequently, the reaction mechanism seems to deviate from the typical scheme reported for aromatic halogenation, which indicates that alternative mechanisms, including a radical pathway, may have to be considered for such non-canonical flavoenzymes.

Surprisingly, a BLAST homology search revealed that orthologous enzymes are encoded in the genomes of at least a dozen other fungi. To shed light on their phylogenetic relationship, we constructed a neighbor-joining tree based on the amino acid sequences of AoiQ and other characterized and non-characterized halogenases/methyltransferases (Figure 3). AoiQ and yet uncharacterized bifunctional homologues encoded in various other fungal biosynthesis gene clusters form two new clades that are distinct from functionally related enzymes in bacteria and other eukaryotes. It is remarkable that the sources are highly diverse and include the pathogen *Aspergillus flavus*, the biocontrol fungus *Trichoderma harzianum*, the saprophyte *Chaetomium globosum*, and the blue stain sap fungus *Grosmannia clavigera*. The function of these putative halogenase/O-methyltransferase genes is enigmatic, and nothing is known about the putative halogenated metabolites encoded in these orphan gene clusters.

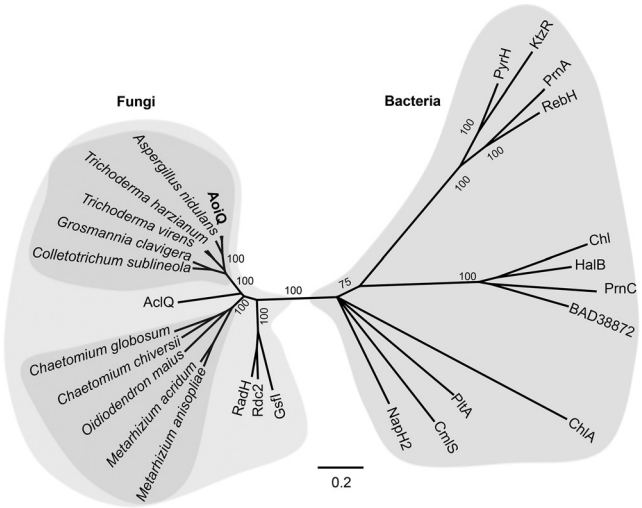

Figure 3. Phylogenetic analysis of AoiQ and related $FADH_2$-dependent halogenases from bacteria and fungi. The clades of (putative) halogenase/O-methyltransferase bifunctional proteins are highlighted in dark gray.

In conclusion, we have revealed that an important fungus used in food fermentation (*A. oryzae*) produces unusual aliphatic geminal dichloro compounds, of which the major component is cytotoxic. Through functional gene analyses, in vitro biochemical assays, and biotransformation experiments, we discovered and characterized an enzyme (AoiQ) that is unique because of the unprecedented combination of a methyltransferase and a halogenase in one gene product. AoiQ also represents the first characterized fungal aliphatic halogenase, a novel flavoprotein that introduces halogens at a non-activated aliphatic carbon atom, and the first halogenase that catalyzes a regioselective dihalogenation of a free-standing substrate. Surprisingly, many genes for related hybrid enzymes were detected in the genomes of diverse ecologically relevant fungi. Our findings may thus encourage and guide the search for encoded cryptic natural products.

Acknowledgements

We thank A. Perner for MS measurements, H. Heinecke for NMR analysis, M. Poetsch for MALDI measurements, Dr. Keishi Ishida for helpful advice, and H. M. Dahse for cytotoxicity measurements.

Keywords: Aspergillus oryzae · chlorination · enzyme catalysis · genome mining · halogenase

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 11955–11959
Angew. Chem. **2016**, *128*, 12134–12138

[1] G. W. Gribble, *Naturally Occurring Organohalogen Compounds*, Springer, Wien/New York, **2010**.
[2] a) F. H. Vaillancourt, E. Yeh, D. A. Vosburg, S. Garneau-Tsodikova, C. T. Walsh, *Chem. Rev.* **2006**, *106*, 3364–3378; b) C. S. Neumann, D. Galonic Fujimori, C. T. Walsh, *Chem. Biol.* **2008**, *15*, 99–109; c) K. H. van Pée, *Curr. Org. Chem.* **2012**, *16*, 2583–2597; d) A. Butler, M. Sandy, *Nature* **2009**, *460*, 848–854; e) C. Wagner, M. El Omari, G. M. König, *J. Nat. Prod.* **2009**, *72*, 540–553.
[3] D. R. M. Smith, S. Grüschow, R. J. M. Goss, *Curr. Opin. Chem. Biol.* **2013**, *17*, 276–283.
[4] a) W. S. Glenn, E. Nims, S. E. O'Connor, *J. Am. Chem. Soc.* **2011**, *133*, 19346–19349; b) K. H. van Pée, *ChemBioChem* **2011**, *12*, 681–683; c) J. T. Payne, M. C. Andorfer, J. C. Lewis, *Angew. Chem. Int. Ed.* **2013**, *52*, 5271–5274; *Angew. Chem.* **2013**, *125*, 5379–5382; d) S. Brown, S. E. O'Connor, *ChemBioChem* **2015**, *16*, 2129–2135; e) Q. Zhu, M. L. Hillwig, Y. Doi, X. Liu, *ChemBioChem* **2016**, *17*, 466–470.
[5] M. L. Hillwig, X. Liu, *Nat. Chem. Biol.* **2014**, *10*, 921–923.
[6] a) F. H. Vaillancourt, E. Yeh, D. A. Vosburg, S. E. O'Connor, C. T. Walsh, *Nature* **2005**, *436*, 1191–1194; b) F. H. Vaillancourt, J. Yin, C. T. Walsh, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 10111–10116; c) D. P. Galonić, F. H. Vaillancourt, C. T. Walsh, *J. Am. Chem. Soc.* **2006**, *128*, 3900–3901; d) D. P. Galonić, E. W. Barr, C. T. Walsh, J. M. J. Bollinger, C. Krebs, *Nat. Chem. Biol.* **2007**, *3*, 113–116; e) K. Podzelinska, R. Latimer, A. Bhattacharya, L. C. Vining, D. L. Zechel, Z. Jia, *J. Mol. Biol.* **2010**, *397*, 316–331.
[7] P. Chankhamjon, D. Boettger-Schmidt, K. Scherlach, B. Urbansky, G. Lackner, D. Kalb, H. M. Dahse, D. Hoffmeister, C.

Hertweck, *Angew. Chem. Int. Ed.* **2014**, *53*, 13409 – 13413; *Angew. Chem.* **2014**, *126*, 13627 – 13631.
[8] T. O. Larsen, J. Breinholt, *J. Nat. Prod.* **1999**, *62*, 1182 – 1184.
[9] A. H. Aly, R. Edrada-Ebel, V. Wray, W. E. Müller, S. Kozytska, U. Hentschel, P. Proksch, R. Ebel, *Phytochemistry* **2008**, *69*, 1716 – 1725.
[10] T. Tanahashi, Y. Takenaka, N. Nagakura, N. Hamada, H. Miyawaki, *Heterocycles* **2000**, *53*, 723 – 728.
[11] T. Nakazawa, K. Ishiuchi, A. Praseuth, H. Noguchi, K. Hotta, K. Watanabe, *ChemBioChem* **2012**, *13*, 855 – 861.
[12] Y. Tsunematsu, N. Ishikawa, D. Wakana, Y. Goda, H. Noguchi, H. Moriya, K. Hotta, K. Watanabe, *Nat. Chem. Biol.* **2013**, *9*, 818 – 825.
[13] D. H. Scharf, A. Habel, T. Heinekamp, A. A. Brakhage, C. Hertweck, *J. Am. Chem. Soc.* **2014**, *136*, 11674 – 11679.
[14] S. Zehner, A. Kotzsch, B. Bister, R. D. Sussmuth, C. Mendez, J. A. Salas, K. H. van Pée, *Chem. Biol.* **2005**, *12*, 445 – 452.
[15] M. Piraee, R. L. White, L. C. Vining, *Microbiology* **2004**, *150*, 85 – 94.
[16] V. Meyer, M. Arentshorst, A. El-Ghezal, A.-C. Drews, R. Kooistra, C. A. M. J. J. van der Hondel, A. F. J. Ram, *J. Biotechnol.* **2007**, *128*, 770 – 775.

Received: May 9, 2016
Revised: June 22, 2016
Published online: August 25, 2016

