VIP *Very Important Paper*

N-Heterocyclization in Gliotoxin Biosynthesis is Catalyzed by a Distinct Cytochrome P450 Monooxygenase

Daniel H. Scharf,*[a, b, c] Pranatchareeya Chankhamjon,[d] Kirstin Scherlach,[d] Jan Dworschak,[d] Thorsten Heinekamp,[a] Martin Roth,[e] Axel A. Brakhage,[a, f] and Christian Hertweck*[d, f]

Gliotoxin and related epidithiodiketopiperazines (ETP) from diverse fungi feature highly functionalized hydroindole scaffolds with an array of medicinally and ecologically relevant activities. Mutation analysis, heterologous reconstitution, and biotransformation experiments revealed that a cytochrome P450 monooxygenase (GliF) from the human-pathogenic fungus *Aspergillus fumigatus* plays a key role in the formation of the complex heterocycle. *In vitro* assays using a biosynthetic precursor from a blocked mutant showed that GliF is specific to ETPs and catalyzes an unprecedented heterocyclization reaction that cannot be emulated with current synthetic methods. *In silico* analyses indicate that this rare biotransformation takes place in related ETP biosynthetic pathways.

Medium-sized nitrogen heterocycles are important components in biologically active molecules, and often these moieties are integrated into polycyclic frameworks of alkaloids. A highly substituted dihydroindole-derived scaffold is a hallmark of the important epidithiodiketopiperazines (ETP) family of natural products.[1] The ETP prototype, gliotoxin (**1**, Figure 1A), has been implicated as a virulence factor of the infamous human-pathogenic fungus *Aspergillus fumigatus* and is considered as a pro-apoptotic agent.[2] Other relevant examples are the histamine-release inhibitor emethallicin E (**2**),[3] the antibiotic epicorazine A (**3**),[4] the cytotoxic rostratin A (**4**),[5] the antiviral agent aranotin (**5**),[6] and the phytotoxin sirodesmin PL (**6**),[7] which are produced by fungi occurring in highly diverse niches. Because of their eminent roles in ecology and medicine, ETPs like **1**–**6** have been in the focus of many total synthesis programs.[1b,8] The stereoselective synthesis of their hydroindole scaffolds with tertiary or even quaternary stereocenters, however, poses a particular challenge.[9] Thus, it would be intriguing to gain insight into the natural route towards this complex heterocyclic system.

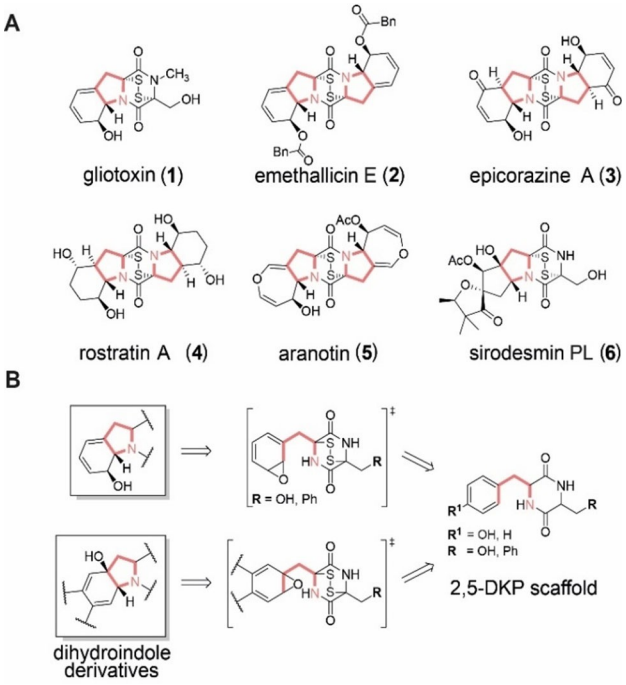

Figure 1. A) Structures of selected ETP toxins (**1**–**6**) bearing fused pyrrolidine rings. B) Retro-biosynthetic analysis: aryl epoxidation could set the stage for N-heterocyclizations (top route, for **1**–**5**, bottom route, for **6**).

The gliotoxin biosynthetic pathway has proven to be an excellent model for functional analyses that shed light on many aspects of the biological synthesis of ETPs. Genetic and biochemical investigations have shown that the 2,5-diketopiperazine (DKP) core of **1** is assembled by a non-ribosomal peptide synthetase,[10] and modified by an *N*-meth-

[a] *D. H. Scharf, T. Heinekamp, Prof. A. A. Brakhage*
Department of Molecular and Applied Microbiology
Leibniz Institute for Natural Product Research and Infection Biology (HKI)
Beutenbergstrasse 11a, 07745 Jena (Germany)
[b] *D. H. Scharf*
Department of Microbiology, Zhejiang University School of Medicine
Yuhangtang Road 866, Hangzhou 310058 (P. R. China)
E-mail: dhscharf@zju.edu.cn
[c] *D. H. Scharf*
The Children's Hospital, Zhejiang University School of Medicine
National Clinical Research Center for Child Health
Binsheng Road 3333, Hangzhou 310052 (P. R. China)
[d] *P. Chankhamjon, Dr. K. Scherlach, J. Dworschak, Prof. Dr. C. Hertweck*
Department of Biomolecular Chemistry
Leibniz Institute for Natural Product Research and Infection Biology (HKI)
Beutenbergstrasse 11a, 07745 Jena (Germany)
E-mail: christian.hertweck@leibniz-hki.de
[e] *M. Roth*
Bio Pilot Plant
Leibniz Institute for Natural Product Research and Infection Biology (HKI)
Beutenbergstrasse 11a, 07745 Jena (Germany)
[f] *Prof. A. A. Brakhage, Prof. Dr. C. Hertweck*
Faculty of Biological Sciences, Friedrich Schiller University Jena
07743 Jena (Germany)

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbic.202000550

yltransferase.[11] The pharmacophoric disulfide bridge is introduced by hydroxylation,[12] glutathione transfer,[13] sequential side-chain cleavage,[14] and oxidation.[15] Up until now, however, how the central hydroindole ring is formed has remained an enigma. Here we report that a single cytochrome monooxygenase, GliF, is essential and sufficient for an unprecedented heterocyclization to generate the ETP tetrahydroindole framework. We also show that homologues of this enzyme are widespread in fungi producing ETPs with related ring-fused pyrrolidine scaffolds.

Considering the constitution and hydroxyl substitution of the phenyl-derived cyclohexadiene residues, a retro-biosynthetic analysis suggests that a formal aryl epoxidation could set the stage for the N-heterocyclization (Figure 1B).[1] Depending on the precise position of the intermediary oxirane the cyclization would either lead to a 7-hydroxy-2,3,7,7a-tetrahydroindole ring (gliotoxin type) or to a regioisomer that has been proposed as a plausible intermediate in the sirodesmin biosynthetic pathway.[7]

To identify candidates for the proposed phenyl oxygenation, we first compared the biosynthesis gene clusters for diverse ETP biosynthetic pathways. A phylogenetic analysis revealed that the encoded cytochrome P450 monooxygenases fall into three clades, the GliC group, which is shared by all pathways, AtaY from aranotin biosynthesis, and the GliF group, which is restricted to the pathways leading to gliotoxin (**1**), aranotin (**5**), and sirodesmin (**6**). The latter, which is more distantly related, features a similar, yet contracted, pyrrolidine ring system.[1b,7] GliC has been shown to catalyze DKP bishydroxylation to initiate C–S bond formation, which is conserved in all ETP biosynthetic pathways, while AtaY is implicated in the formation of the oxacycloheptadiene ring of aranotin,[16] as the corresponding gene is exclusively found in the *ara* gene locus. Clade I comprises CYPs from fungi producing ETPs with pyrrolidine heterocycles. Thus, GliF and its homologues were considered as best candidates for enzymes catalyzing the heterocyclization.

To elucidate the role of GliF in gliotoxin biosynthesis, we created a *gliF* deletion strain by homologous recombination. The identity of the mutant was confirmed by Southern blot (Figure 2C). HPLC-MS monitoring of the Δ*gliF* mutant culture (3 L) showed that gliotoxin biosynthesis was completely abolished. In lieu of the pyrrolidine-containing product, we observed the accumulation of a new compound (**7**) in low amount with *m/z* 295.0217 ([*M*−H]$^-$; Figure 2D). From ESI-HRMS data, we deduced a molecular formula of $C_{12}H_{11}N_2O_3S_2$ for **7**. To obtain sufficient amounts of **7** for a full structure elucidation we scaled up the fermentation. From the extracts of a total of 120 L mutant culture broth, we isolated 5.78 mg of pure **7** by size-exclusion and silica gel chromatography, and repeated preparative HPLC. 1D and 2D NMR data of compound **7** indicated the presence of the diketopiperazine core found in gliotoxin precursors. In addition, the chemical shifts of 72.5 and 73.3 ppm for C-3 and C-6, respectively, indicated that two quaternary carbons are adjacent to heteroatoms as in the ETP scaffold. The diketopiperazine substitution pattern was inferred from HMBC couplings of H-8 to C-6 and H-7 to C-3, which was confirmed by

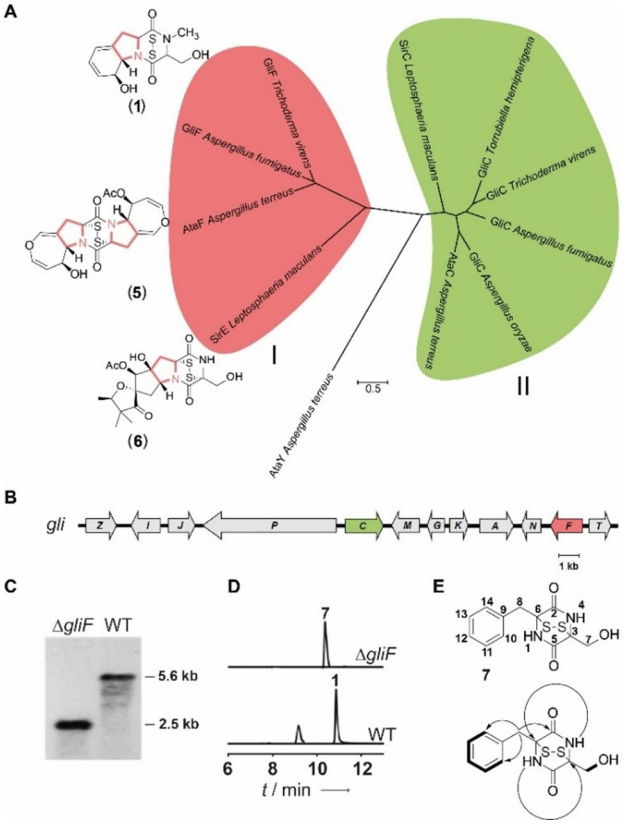

Figure 2. Molecular basis of pyrrolidine formation in ETP pathways. A) Phylogenetic analysis of cytochrome P450 monooxygenases encoded in ETP biosynthesis gene clusters, and structures of the corresponding natural products. Clade I: GliF-like enzymes; clade II: GliC-like enzymes, and AtaY from *Aspergillus terreus*. B) Organization of the gliotoxin (*gli*) biosynthesis gene cluster. C) Southern blot analysis of gDNA of wild type (WT) and △*gliF* mutant. D) Metabolic profiles (extracted ion chromatograms) of wild type (WT) and △*gliF* mutant. E) Structure of compound **7**, and key HMBC (curved arrows) and COSY (bold lines) correlations.

the H,H correlation (H,H COSY) of the hydroxy proton at C-7 to H-7, and the typical COSY and HMBC correlations of the benzyl moiety (Figure S1 and Table S1 in the Supporting Information). Thus, NMR and HRMS data fully support the proposed structure of **7** (Figure 2E).

Disulfide **7** from the Δ*gliF* mutant extract represents a plausible intermediate of gliotoxin biosynthesis. To test this model, we established an *in vitro* assay with GliF. We first attempted to obtain recombinant protein from *Escherichia coli*, yet the heterologous expression of *gliF* in this host only yielded inactive protein. An *in silico* analysis of the GliF amino acid sequence suggested that the hydrophobic N terminus of GliF causes the protein to be membrane-associated. To address this issue, we overproduced GliF in yeast. In addition to providing the heterologously produced enzyme in microsomes this host has the added benefit to also provide the native yeast CYP450 reductase that is vital for CYP450 reaction cycle.[17] With the biosynthetic intermediate and the working biocatalyst at hand, we performed the *in vitro* assay. Specifically, disulfide **7** was incubated with microsomal fraction with GliF and without GliF (negative control), and the reaction was monitored by LC/MS.

Whereas no biotransformation of **7** could be detected without GliF (Figure 3), we observed that active GliF readily converted **7** into three compounds, **10**, **11** and **12**, with *m/z* 311 $[M-H]^-$, *m/z* 342 $[M-H]^-$ and *m/z* 374 $[M-H]^-$, respectively (Figure 3).

The identity of **10** as desmethylgliotoxin was verified by comparison with an authentic reference obtained from a Δ*gliN* (*N*-methyltransferase) mutant.[11] The mass spectra with Δ*m/z* of 32 and 64 compared to **10** as well as isotope and fragmentation patterns of **11** and **12** (Figure S3) indicated that the congeners represent the tri- and tetrasulfide homologues of **10**, *N*-desmethylgliotoxin E (**11**) and G (**12**), respectively. As side products, we detected the tri- and tetrasulfide derivatives **11** and **12**, which were identified by comparison of retention time and MS^n fragmentation pattern of reference compounds. The tri- and tetrasulfides (**8** and **9**, **11** and **12**) result from the disproportionation of the corresponding disulfides (**7** and **10**), which is promoted by aerobic conditions and light (Supporting Information).[18] Previous studies suggested that the epoxidation of the phenyl ring is the prerequisite for the ring-closure reaction. This model is supported by the observation of spiro compounds in gliotoxin,[19] acetylaranotin,[16] and peniciadametizine A[20] biosynthesis, and an epoxidated phenyl ring in gliovirin pathway.[21]

These compounds are likely derived from epoxide intermediates. Although the epoxidation reaction is plausible, it is also conceivable that the reaction is initiated through radical formation at the amide nitrogen, followed by heterocyclization and quenching of the radical by water (Figure 4B).

The pyrrolidine ring is a common motif in many biologically active natural products from bacterial, fungal or plant origins. Typically, the five-membered N-heterocycles originate from cyclodehydration of amines, as in the well-studied nicotine and tropane biosynthetic pathways.[22] Mechanistically related transformations have been elucidated in bacterial natural product formation,[23] and variations of this theme, in part involving methylation or prenylation, lead to fungal pyrroloindole ring systems.[24] Several oxidative processes towards pyrrolidine-containing ring systems have been reported, involving an α-ketoglutarate-dependent enzyme,[25] a monoamine oxidase,[26] a flavoenzyme,[27] and a cytochrome P450 monooxygenase.[28] Yet, the pathway to the pyrrolidine ring of the ETP hydroindole scaffold differs from all previously reported routes as it involves a cytochrome P450 monooxygenase GliF that promotes a dearomatization-heterocyclization sequence. This unusual transformation is an addition to the growing number of known enzyme-catalyzed cascade reactions.[29] The enzyme phylogeny indicates that similar biotransformations take place in related ETP biosynthetic pathways. From a translational point of view, GliF and its orthologues could be harnessed for pathway engineering and biotransformations in organic synthesis. Elucidation of these heterocycle-forming enzymes is also relevant as they are involved in the biosynthesis of virulence factors and toxins that play roles in medicine and ecology.

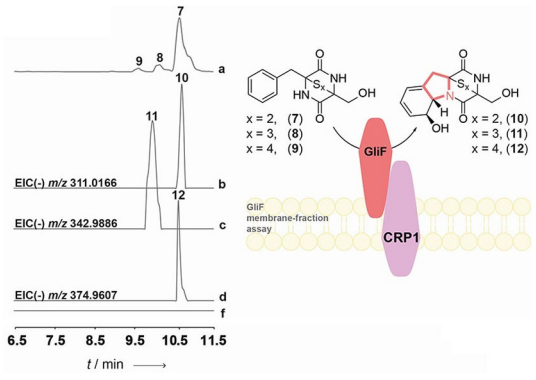

Figure 3. In vitro enzyme assays and biotransformation experiments. LC–MS monitoring as extracted ion chromatograms of the microsomal GliF fraction-mediated transformation of isolated substrate **7**–**9** (a) into **10** (b), **11** (c), and **12** (d); as well as the same using microsomal fraction without GliF, which shows no production of **10**–**12** (f).

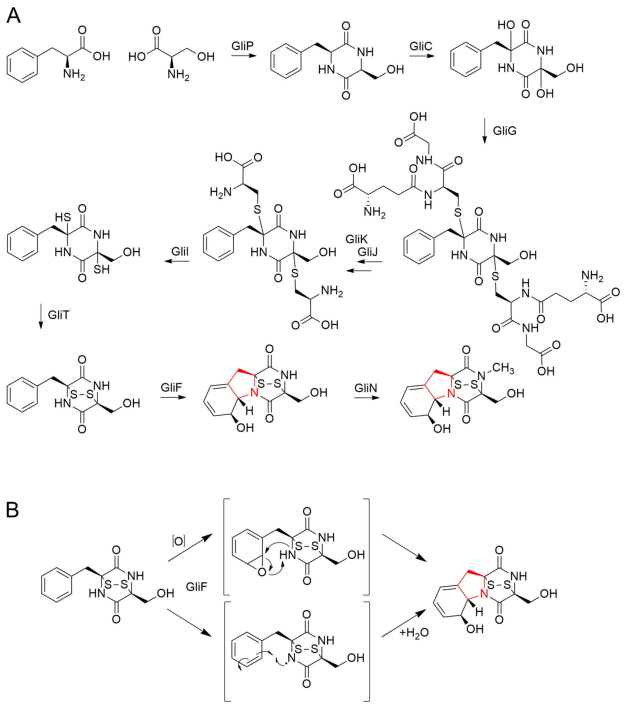

Figure 4. Model of gliotoxin biosynthesis and possible reaction mechanism for GliF. A) DKP formation by a nonribosomal peptide synthetase (GliP), bis-hydroxylation of DKP (GliC), addition of glutathione (GliG), sequential degradation of glutathione (GliK, GliJ), C–S bond cleavage (GliI), disulphide formation (GliT), *N*-heterocyclization (GliF), and N-methylation (GliN). B) Two possible mechanistic routes for the heterocyclization: route involving an epoxide intermediate vs. radical pathway.

Acknowledgements

We thank A. Perner for MS measurements, H. Heinecke for NMR measurements and S. Fricke for technical support. Financial support by the Federal Ministry of Education and Research (InfectControl2020, DrugBioTune, for C.H. and A.A.B.), the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) - SFB 1127/2 ChemBioSys – 239748522, and start-up funding from Zhejiang University talent introduction program, Zhejiang

Provincial Key Laboratory of Immunity and Inflammatory diseases, Children's hospital research fund and NSFC (for D.H.S.) is gratefully acknowledged. Open access funding enabled and organized by Projekt DEAL.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: *Aspergillus fumigatus* · cytochrome P450 monoxygenase · epidithiodiketopiperazines · heterocycles · mycotoxins

[1] a) D. M. Gardiner, P. Waring, B. J. Howlett, *Microbiology* **2005**, *151*, 1021–1032; b) T. R. Welch, R. M. Williams, *Nat. Prod. Rep.* **2014**, *31*, 1376–1404.
[2] a) D. H. Scharf, T. Heinekamp, N. Remme, P. Hortschansky, A. A. Brakhage, C. Hertweck, *Appl. Microbiol. Biotechnol.* **2012**, *93*, 467–472; b) D. H. Scharf, A. A. Brakhage, P. K. Mukherjee, *Environ. Microbiol.* **2015**, *18*, 1096–1109.
[3] N. Kawahara, S. Nakajima, M. Yamazaki, K.-i. Kawai, *Chem. Pharm. Bull.* **1989**, *37*, 2592–2595.
[4] G. Deffieux, M.-A. Baute, R. Baute, M.-J. Filleau, *J. Antibiot.* **1978**, *31*, 1102–1105.
[5] R. X. Tan, P. R. Jensen, P. G. Williams, W. Fenical, *J. Nat. Prod.* **2004**, *67*, 1374–1382.
[6] N. Neuss, R. Nagarajan, B. B. Molloy, L. L. Huckstep, *Tetrahedron Lett.* **1968**, *9*, 4467–4471.
[7] D. M. Gardiner, A. J. Cozijnsen, L. M. Wilson, M. S. C. Pedras, B. J. Howlett, *Mol. Microbiol.* **2004**, *53*, 1307–1318.
[8] a) U. Gross, M. Nieger, S. Bräse, *Chem. Eur. J.* **2010**, *16*, 11624–11631; b) K. C. Nicolaou, M. Lu, S. Totokotsopoulos, P. Heretsch, D. Giguère, Y.-P. Sun, D. Sarlah, T. H. Nguyen, I. C. Wolf, D. F. Smee, C. W. Day, S. Bopp, E. A. Winzeler, *J. Am. Chem. Soc.* **2012**, *134*, 17320–17332; c) J. A. Codelli, A. L. A. Puchlopek, S. E. Reisman, *J. Am. Chem. Soc.* **2012**, *134*, 1930–1933; d) T. Fukuyama, Y. Kishi, *J. Am. Chem. Soc.* **1976**, *98*, 6723–6724; e) U. Gross, M. Nieger, S. Bräse, *Org. Lett.* **2009**, *11*, 4740–4742.
[9] a) T. C. Henninger, M. Sabat, R. J. Sundberg, *Tetrahedron* **1996**, *52*, 14403–14418; b) M. Feng, X. Jiang, *Chem. Commun.* **2014**, *50*, 9690–9692; c) S. Zhong, P. F. Sauter, M. Nieger, S. Bräse, *Chem. Eur. J.* **2015**, *21*, 11219–11225.
[10] a) C. J. Balibar, C. T. Walsh, *Biochemistry* **2006**, *45*, 15029–15038; b) C. Kupfahl, T. Heinekamp, G. Geginat, T. Ruppert, A. Härtl, H. Hof, A. A. Brakhage, *Mol. Microbiol.* **2006**, *62*, 292–302.
[11] D. H. Scharf, A. Habel, T. Heinekamp, A. A. Brakhage, C. Hertweck, *J. Am. Chem. Soc.* **2014**, *136*, 11674–11679.
[12] a) S.-L. Chang, Y.-M. Chiang, H.-H. Yeh, T.-K. Wu, C. C. C. Wang, *Bioorg. Med. Chem. Lett.* **2013**, *23*, 2155–2157; b) D. H. Scharf, J. D. Dworschak, P. Chankhamjon, K. Scherlach, T. Heinekamp, A. A. Brakhage, C. Hertweck, *ACS Chem. Biol.* **2018**.
[13] a) D. H. Scharf, N. Remme, A. Habel, P. Chankhamjon, K. Scherlach, T. Heinekamp, P. Hortschansky, A. A. Brakhage, C. Hertweck, *J. Am. Chem. Soc.* **2011**, *133*, 12322–12325; b) C. Davis, S. Carberry, M. Schrettl, I. Singh, J. C. Stephens, S. M. Barry, K. Kavanagh, G. L. Challis, D. Brougham, S. Doyle, *Chem. Biol.* **2011**, *18*, 542–552.
[14] a) D. H. Scharf, P. Chankhamjon, K. Scherlach, T. Heinekamp, M. Roth, A. A. Brakhage, C. Hertweck, *Angew. Chem. Int. Ed.* **2012**, *124*, 10211–10215; b) D. H. Scharf, P. Chankhamjon, K. Scherlach, T. Heinekamp, K. Willing, A. A. Brakhage, C. Hertweck, *Angew. Chem. Int. Ed.* **2013**, *52*, 11092–11095; *Angew. Chem.* **2013**, *125*, 11298–11301; c) A. Marion, M. Groll, D. H. Scharf, K. Scherlach, M. Glaser, H. Sievers, M. Schuster, C. Hertweck, A. A. Brakhage, I. Antes, E. M. Huber, *ACS Chem. Biol.* **2017**, *12*, 1874–1882.
[15] a) D. H. Scharf, N. Remme, T. Heinekamp, P. Hortschansky, A. A. Brakhage, C. Hertweck, *J. Am. Chem. Soc.* **2010**, *132*, 10136–10141; b) D. H. Scharf, M. Groll, A. Habel, T. Heinekamp, C. Hertweck, A. A. Brakhage, E. M. Huber, *Angew. Chem. Int. Ed.* **2014**, *53*, 2221–2224; *Angew. Chem.* **2014**, *126*, 2253–2256.
[16] C.-J. Guo, H.-H. Yeh, Y.-M. Chiang, J. F. Sanchez, S.-L. Chang, K. S. Bruno, C. C. C. Wang, *J. Am. Chem. Soc.* **2013**, *135*, 7205–7213.
[17] J. Hausjell, H. Halbwirth, O. Spadiut, *Biosci. Rep.* **2018**, *38*, BSR20171290.
[18] a) J. Kim, M. Movassaghi, *Acc. Chem. Res.* **2015**, *48*, 1159–1171; b) J. Kim, M. Movassaghi, *J. Am. Chem. Soc.* **2010**, *132*, 14376–14378; c) M.-H. Wang, Y.-C. Hu, B.-D. Sun, M. Yu, S.-B. Niu, Z. Guo, X.-Y. Zhang, T. Zhang, G. Ding, Z.-M. Zou, *Org. Lett.* **2018**, *20*, 1806–1809.
[19] R. R. Forseth, E. M. Fox, D. Chung, B. J. Howlett, N. P. Keller, F. C. Schroeder, *J. Am. Chem. Soc.* **2011**, *133*, 9678–9681.
[20] Y. Liu, A. Mándi, X.-M. Li, L.-H. Meng, T. Kurtán, B.-G. Wang, *Mar. Drugs* **2015**, *13*, 3640–3652.
[21] a) R. D. Stipanovic, C. R. Howell, P. A. Hedin, *J. Antibiot.* **1994**, *47*, 942–944; b) P. D. Sherkhane, R. Bansal, K. Banerjee, S. Chatterjee, D. Oulkar, P. Jain, L. Rosenfelder, S. Elgavish, B. A. Horwitz, P. K. Mukherjee, *ChemistrySelect* **2017**, *2*, 3347–3352; c) M. Zhu, X. Zhang, H. Feng, J. Dai, J. Li, Q. Che, Q. Gu, T. Zhu, D. Li, *J. Nat. Prod.* **2016**, *80*, 71–75.
[22] J. Ziegler, P. J. Facchini, *Annu. Rev. Plant Biol.* **2008**, *59*, 735–769.
[23] a) D. Reimer, E. Luxenburger, A. O. Brachmann, H. B. Bode, *ChemBioChem* **2009**, *10*, 1997–2001; b) X. Zheng, Q. Cheng, F. Yao, X. Wang, L. Kong, B. Cao, M. Xu, S. Lin, Z. Deng, Y.-H. Chooi, D. You, *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 4135–4140.
[24] a) J. Liu, T. Ng, Z. Rui, O. Ad, W. Zhang, *Angew. Chem. Int. Ed.* **2014**, *53*, 136–139; *Angew. Chem.* **2014**, *126*, 140–143; b) C. García-Estrada, R. V. Ullán, S. M. Albillos, M. Á. Fernández-Bodega, P. Durek, H. von Döhren, J. F. Martín, *Chem. Biol.* **2011**, *18*, 1499–1512; c) H. Ali, M. I. Ries, J. G. Nijland, P. P. Lankhorst, T. Hankemeier, R. A. L. Bovenberg, R. J. Vreeken, A. J. M. Driessen, *PLoS One* **2013**, *8*, e65328.
[25] J. K. Brunson, S. M. K. McKinnie, J. R. Chekan, J. P. McCrow, Z. D. Miles, E. M. Bertrand, V. A. Bielinski, H. Luhavaya, M. Oborník, G. J. Smith, D. A. Hutchins, A. E. Allen, B. S. Moore, *Science* **2018**, *361*, 1356–1358.
[26] P.-K. Chang, K. C. Ehrlich, I. Fujii, *Toxin Rev.* **2009**, *1*, 74–99.
[27] C.-Y. Lai, I. W. Lo, R. T. Hewage, Y.-C. Chen, C.-T. Chen, C.-F. Lee, S. Lin, M.-C. Tang, H.-C. Lin, *Angew. Chem. Int. Ed.* **2017**, *56*, 9478–9482; *Angew. Chem.* **2017**, *129*, 9606–9610.
[28] a) S. Kishimoto, M. Sato, Y. Tsunematsu, K. Watanabe, *Molecules* **2016**, *21*, 1078; b) T. Saruwatari, F. Yagishita, T. Mino, H. Noguchi, K. Hotta, K. Watanabe, *ChemBioChem* **2014**, *15*, 656–659.
[29] C. T. Walsh, B. S. Moore, *Angew. Chem. Int. Ed.* **2018**, *58*, 6846–6879.

Manuscript received: August 5, 2020
Revised manuscript received: August 24, 2020
Accepted manuscript online: August 24, 2020
Version of record online: October 5, 2020