Natural Products

DOI: 10.1002/anie.201204540

Symbiotic Cooperation in the Biosynthesis of a Phytotoxin**

*Kirstin Scherlach, Benjamin Busch, Gerald Lackner, Uta Paszkowski, and Christian Hertweck**

Natural products play a key role in symbiotic interactions between microorganisms and higher organisms, covering all kingdoms of life.[1–3] The function of these secondary metabolites may range from signaling compounds in mutualism to virulence factors and antibiotics in parasitic relationships.[4] In many cases the interactions involve multiple partners and thus the biogenetic basis of chemical mediators can be quite complex. This complexity is well exemplified by the unparalleled tripartite relationship among the rice-seedling-blight fungus *Rhizopus microsporus*, its host plant *Oryza sativa* and endosymbiotic bacteria that reside in the fungal cytosol.[5] The bacterial symbionts (*Burkholderia* species) produce a phytotoxin complex to assist the phytopathogenic fungus in colonizing rice seedlings.[6] In turn the bacteria profit from a safe niche and access to nutrients released from the decaying plant. Initially, the macrolide rhizoxin (**1**, Figure 1) and various congeners such as WF-1360F (**2**) were isolated from cultures of *R. microsporus* van Tieghem var. *chinensis* and identified as the causative agent of rice seedling blight.[7–9] Rhizoxin efficiently inhibits eukaryotic cell proliferation by binding to β-tubulin and thus blocking the formation of the mitotic spindle.[10] Notably, the pure compound alone evokes the typical symptoms of seedling root swelling. Only recently, through detection, isolation, and cultivation of the endosymbionts we could unequivocally prove that actually associated bacteria are the true producers of the toxin complex.[6,11] The importance of this metabolic capability has been underlined by the finding that fungal reproduction depends entirely on the presence of the bacterial symbionts. Survival of the toxinogenic symbiosis is warranted by the strict sporulation control and exclusive dispersal of spores harboring endosymbionts.[12] Moreover, the unusual mutualism has been fine-tuned through symbiosis factors such as a type-III secretion system[13] and a novel lipopolysaccharide O-antigen

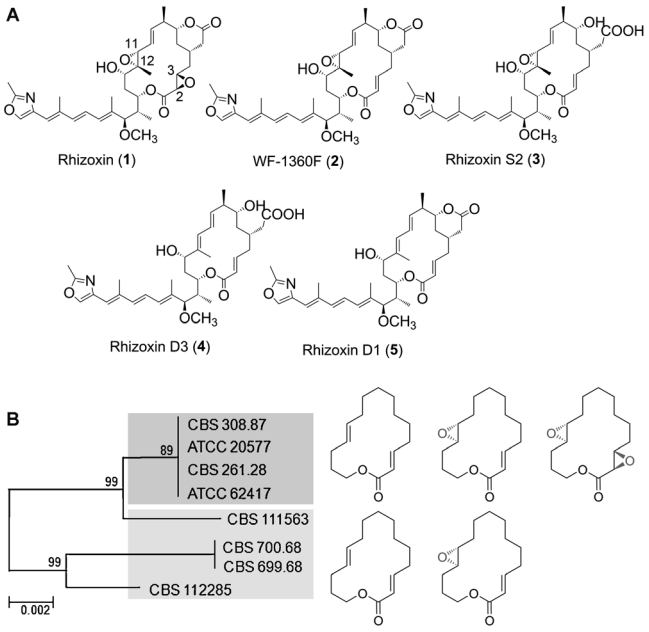

Figure 1. A) Structures of rhizoxin and congeners. B) Phylogenetic relationship of *Rhizopus microsporus* strains; structures of the corresponding metabolites indicate which strains can produce bisepoxides. The numbers on top of the branches indicate the clade probability values; the scale on the left site relates the length of a branch to the distance (number of changes that have taken place along a branch).

that sets the symbionts into a "stealth mode" by decorating the outer membrane of the endosymbionts.[14] The host, on the other hand, acquired resistance towards rhizoxin by mutation of the β-tubulin.[15] Because of its ecological and medicinal relevance as an antimitotic agent,[16] the biosynthesis of rhizoxin has been studied. Cloning, sequencing, and molecular analyses of the rhizoxin (*rhi*) biosynthetic gene cluster in the genome of *Burkholderia rhizoxinica*[17] revealed the molecular basis for a complex polyketide assembly line required for the biosynthesis of the virulence factor.[18] Whereas the biosynthesis of the macrolide backbone has been decoded by mutational analyses,[19,20] polyketide tailoring mechanisms and the biological role of the bis(epoxidation) have remained elusive. Herein we elucidate the dual epoxidation of rhizoxin and its impact on rice seedling blight and report an unprecedented case for symbiotic cooperation in the biosynthesis of an ecologically relevant natural product.

In a broader survey on rhizoxin-positive *Rhizopus* species we discovered that the unusual bacterial–fungal association is not restricted to a single isolate but has spread worldwide. We have identified eight related *Burkholderia–Rhizopus* associations from geographically highly different regions on five continents; these findings underlign the ecological impor-

[*] Dr. K. Scherlach,[+] B. Busch,[+] G. Lackner, Prof. Dr. C. Hertweck
Biomolecular Chemistry, Leibniz Institute for Natural Product Research and Infection Biology, HKI
Beutenbergstr. 11a, 07745 Jena (Germany)
E-mail: christian.hertweck@hki-jena.de
Homepage: http://www.hki-jena.de
Prof. Dr. C. Hertweck
Chair of Natural Product Chemistry, Friedrich Schiller University Jena (Germany)
Prof. Dr. U. Paszkowski
Department of Plant Molecular Biology, University of Lausanne Biophore, 4419, 1015 Lausanne (Switzerland)
[+] These authors contributed equally to this work.

[**] Financial support by the ILRS and the JSMC is gratefully acknowledged.
Supporting information (including experimental details) for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201204540.

tance of the mutualism.[21] All isolated endobacteria are able to synthesize rhizoxin analogues when grown in pure culture. According to extensive metabolic profiling the endosymbionts seem to solely produce macrolides bearing only a single epoxide moiety. In contrast, the metabolite originally isolated from *Rhizopus* features two oxirane rings.[9] To distinguish between this metabolic difference being either a specific trait of *R. microsporus* van Tieghem var. *chinensis* or a common characteristic of all endosymbiont containing *Rhizopus* species, we cultured all available fungal strains under conditions required for rhizoxin production and analyzed the extracts by HPLC–DAD–MS (DAD = diode array detector). This comparative metabolic profiling revealed that only four out of eight strains investigated were able to produce the bisepoxide **1**, whereas the others generate monoepoxides (Figure 1 and Figure 2, lanes A and B). The corresponding endosymbionts, however, do not show any significant difference in metabolite production with only monoepoxide rhizoxin derivatives (**2**, **3**) being produced (Figure 2, lanes C and D). A plausible explanation for this observation would be that bacteria produce bisepoxides only when the fungus provides a specific trigger. To unravel the molecular basis for the different chemotypes we selected two model symbioses, A) the bisepoxide-producing symbiosis *R. microsporus* van Tieghem var. *chinensis* ATCC62417 + *B. rhizoxinica,* and B) the monoepoxide-producing symbiosis *R. microsporus* CBS112285 + *B. endofungorum*. The contribution of putative cytochrome P450 monooxygenases (CYP) in the epoxidation reaction was suggested by our previous observation that oxirane formation can be efficiently blocked by addition of CYP inhibitors.[11] Indeed, the *rhi* gene cluster from *B. rhizoxinica* harbors a putative CYP gene (*rhiH*),[18] and the deduced gene product shows significant similarity to various bacterial epoxidases involved in polyketide tailoring.[22] In principle, RhiH could have a dual function and mediate a tandem epoxidation of both the C2–C3 and C11–C12 double bonds. To prove the function of RhiH we constructed a Δ*rhiH* mutant using a targeted gene deletion. Through HPLC–MS analysis of the resulting mutant culture we found that exclusively didesepoxy rhizoxin derivatives (**4**, **5**) are produced (Figure 2, lane E). Consequently, RhiH is clearly involved in the epoxidation of the rhizoxin scaffold and responsible at least for the epoxidation of the C11–C12 double bond. Further functional analyses of *rhiH* by heterologous expression and biotransformation experiments were severely hampered by the rapid decay of the monoepoxides **2** and **3** within the culture broth. Thus, no evidence for a dual function could be gleaned. To compare the symbiont genotypes we sequenced the homologous rhizoxin biosynthesis gene cluster of *B. endofungorum* (accession number: HE963103) and were surprised to find that the *rhi* gene loci of the symbionts are practically identical. The only difference was represented by a putative catechol 1,2-dioxygenase gene (*rhiJ*) flanking the *rhi* cluster in *B. rhizoxinica* (Figure 3). However, epoxidation by a catechol dioxygenase is highly improbable, because these enzymes typically catalyze the oxidative cleavage of catechols.

Furthermore, we could rule out an oxygen-dependent formation of mono- and bisepoxides by systematically varying

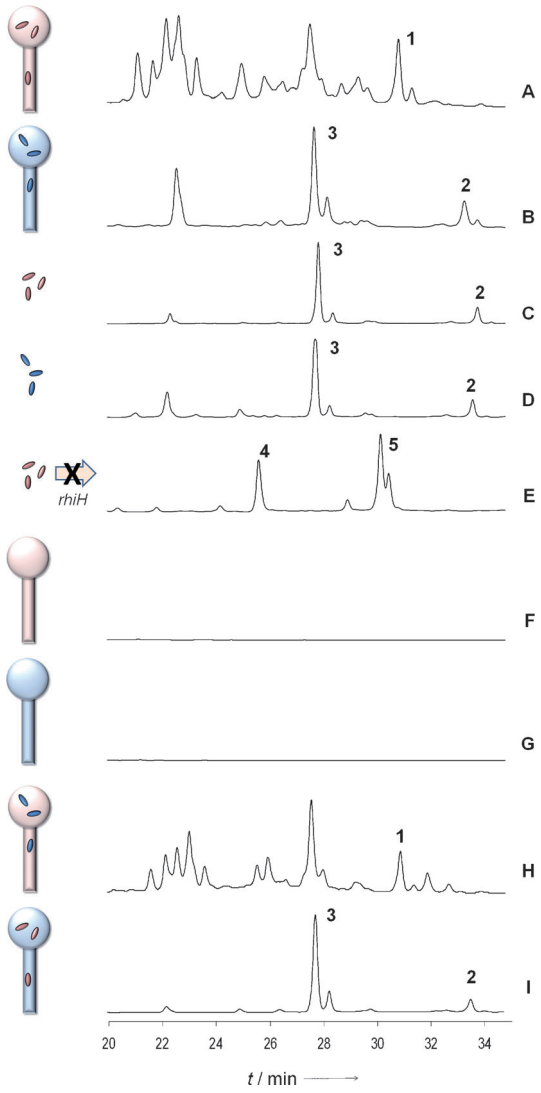

Figure 2. HPLC profiling of rhizoxin formation by A) *R. microsporus* ATCC62417 harboring *B. rhizoxininca*, B) *R. microsporus* CBS112285 harboring *B. endofungorum*, C) *B. rhizoxininca*, D) *B. endofungorum*, E) *B. rhizoxinica* Δ*rhiH*, F) cured *R. microsporus* ATCC62417/S, G) cured *R. microsporus* CBS112285/S, H) cross-infected *R. microsporus* ATCC62417 harboring *B. endofungorum*, and I) cross-infected *R. microsporus* CBS112285 harboring *B. rhizoxinic*a.

the culture conditions and aeriation (see the Supporting Information). Alternatively, the fungus might contribute the necessary enzymatic activity to produce the 2,3-oxirane ring of **1**. To investigate this option, we first cured the fungal strains *R. microsporus* van Tieghem var. *chinensis* ATCC62417 and *R. microsporus* CBS112285 from the endobacteria by constantly culturing these in the presence of ciprofloxacin.[6,12] The symbiont-free strains, *R. microsporus* ATCC62417/S and *R. microsporus* CBS112285/S, did not produce any rhizoxin derivatives (Figure 2, lanes F and G).

Next, we supplemented the cultures with isolated WF-1360F (**2**) and analyzed the metabolic products by LC–MS after four days of cultivation. Interestingly, whereas **2** was not at all transformed by *R. microsporus* CBS112285/S, we could observe small amounts of the corresponding bisepoxidized

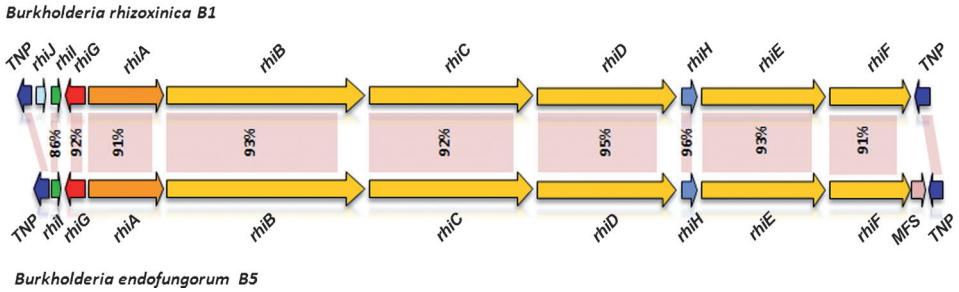

Figure 3. Organisation of the rhizoxin biosynthesis gene clusters in *B. rhizoxinica* and *B. endofungorum*. Percent values reflect relative identities of amino acid sequences computed by BlastP. TNP: transposase, MFS: major facilitator superfamily protein.

rhizoxin in the spiked culture of *R. microsporus* ATCC62417/S (Figure S1 in the Supporting Information). However, the majority of supplemented monoepoxides had not been converted, possibly owing to insufficient uptake of the macrolides by the fungal cells. To overcome this limitation, we initiated a cross-infection experiment.

The endosymbiont-free fungus *R. microsporus* ATCC62417/S, originally host to *B. rhizoxinica*, was infected with *B. endofungorum*. Likewise, an artificial symbiosis was generated by infecting endosymbiont-free *R. microsporus* CBS112285/S, originally host to *B. endofungorum*, with *B. rhizoxinic*a. The secondary metabolomes of the cross-infected strains were analyzed by HPLC–MS, revealing an intriguing consequence of switching hosts. Strain *R. microsporus* ATCC62417/S, now harboring *B. endofungorum*, is clearly able to produce rhizoxin and other bisepoxide derivatives in symbiosis, whereas strain *R. microsporus* CBS112285/S, now hosting *B. rhizoxinica*, exclusively forms monoepoxide analogues in association with the bacterium (Figure 2, lanes H and I). This clear-cut result unequivocally proved that the epoxide moiety at carbon atoms C2/3 of rhizoxin is introduced by the fungus and not by the endosymbiont.

Why does the host fungus specifically tailor the pathogenicity factor provided by its endosymbiont? To our knowledge, no case has been reported to date where a secondary metabolite is jointly biosynthesized by two partners in an endosymbiotic relationship. Cooperative metabolism has only been known from coculturing experiments, for example, for food technological processes[23] or in other artificial systems,[24–26] but not in a natural symbiosis observed in the field. As to the naturally evolved *Rhizopus–Burkholderia* symbiosis, one plausible explanation would be that some fungal hosts have to modify the bacterial metabolite to acquire resistance to the toxin as rhizoxin affects various eukaryotic cells. To test this hypothesis, we probed host sensitivity towards mono- and bisepoxide rhizoxin derivatives. However, agar diffusion assays showed that all *R. microsporus* strains are resistant towards rhizoxin derivatives irrespective of the substitution pattern (Figure S2 in the Supporting Information). This result is reasonable since the β-tubulin amino acid sequences of the hosts are practically identical, and all strains share a mutation at position 100 that confers resistance.[15,27] Another plausible explanation for the strain-specific polyketide tailoring would be an enhancement of rhizoxin bioactivity. However, no obvious advantage for the in vitro cytostatic activities could be deduced,[11] therefore, it was tempting to speculate that the fungal modification of a bacterial metabolite has an ecological function. Indeed, internally transcribed spacer (ITS) sequence analyses of the bisepoxide-producing host strains revealed that these fungi are phylogenetically related and belong to the "pacific branch" (top group in Figure 1).[21] Interestingly, this clade comprises *Rhizopus* species that were initially isolated from infected rice seedlings. To investigate whether the rhizoxin derivatives possess different phytotoxic properties, we studied the effect of the purified compounds on rice seedlings. This assay is viable since the *Rhizopus* does not invade plant tissue but secretes the phytotoxin to weaken or kill the rice seedling.[7] By binding to the β-tubulin of rice plant cells the phytotoxin prevents the seedlings from developing. The characteristic symptom of the pathogenic effect is a swelling of the seedling tip, which served as a read-out in the assay.

However, this assay proved to be challenging, because the macrolides are prone to undergo photooxidation[28] and hydrolytic decomposition when in solution for a longer incubation period. Nonetheless, the results of the rice-seedling swelling assay indicated that rhizoxin featuring two epoxide rings is more potent as a phytotoxin than its monoepoxide counterpart (Figure 4).

Whereas the additional epoxide ring does not seem to have a dramatic effect at higher concentrations, the impact on the phytotoxic efficacy is substantially higher at lower concentrations; this behavior may mirror the natural context during fungal infection. Thus, from an evolutionary perspective, our findings unveil a new facet of the toxinogenic bacterial–fungal alliance. During an early stage of the microbial interaction, the symbiosis has undergone a shift from

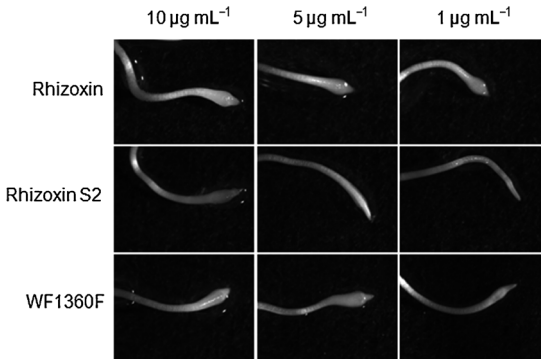

Figure 4. Phytotoxic effect of rhizoxin (**1**) and monoepoxy congeners rhizoxin S2 (**3**) and WF1360F (**2**): The toxins induce a characteristic swelling of the rice seedling tip, yet the bisepoxide **1** shows the strongest effect.

bacterial pathogenicity to mutualism owing to the host resistance towards the antimitotic agent and development of symbiosis factors. Finally, the fungus tailored the core structure of the bacterial polyketide to yield an even more potent phytotoxin in the context of rice seedling blight (Figure 5). This is a proof of concept for the evolution of a natural product in a symbiotic interaction.[29]

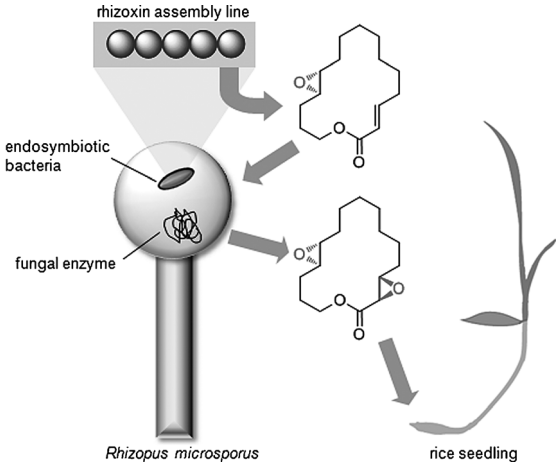

Figure 5. Flowchart of synergistic phytotoxin production. The bacterial endosymbiont produces the rhizoxin backbone, which is tailored by an oxygenase provided by the fungal host to render it more effective against rice seedlings.

In conclusion, through a combination of genetic and chemical analyses we have solved the riddle of the dual epoxidation in rhizoxin biosynthesis. Sequencing and comparison of rhizoxin biosynthesis gene clusters and engineering of a mutant producing didesepoxy variants of rhizoxin revealed that the macrolide is first epoxidized by the cytochrome P450 monooxygenase RhiH. By whole-cell biotransformation and cross-infection experiments we could unequivocally demonstrate that the 2,3-oxirane ring is introduced by the fungal host to specifically tailor the rhizoxin scaffold. According to the rice seedling swelling assays, the additional epoxide moiety substantially increases phytotoxic potency. From an ecological point of view, this finding is fully plausible, since the second epoxidation is a specific trait of fungi belonging to the clade of rice-seedling-blight fungi. We therefore report for the first time on symbiotic synergism in the biosynthesis of a secondary metabolite that has a biological significance.

Received: June 11, 2012
Published online: August 22, 2012

Keywords: *Burkholderia* · cytochrome P450 · epoxidation · *Rhizopus* · rhizoxin

[1] J. Piel, *Nat. Prod. Rep.* **2009**, *26*, 338 – 362.
[2] G. M. König, S. Kehraus, S. F. Seibert, A. Abdel-Lateff, D. Müller, *ChemBioChem* **2006**, *7*, 229 – 238.
[3] H. B. Bode, *Curr. Opin. Chem. Biol.* **2009**, *13*, 224 – 230.
[4] R. Dudler, L. Eberl, *Curr. Opin. Biotechnol.* **2006**, *17*, 268 – 273.
[5] G. Lackner, L. P. Partida-Martinez, C. Hertweck, *Trends Microbiol.* **2009**, *17*, 570 – 576.
[6] L. P. Partida-Martinez, C. Hertweck, *Nature* **2005**, *437*, 884 – 888.
[7] S. Iwasaki, H. Kobayashi, J. Furukawa, M. Namikoshi, S. Okuda, Z. Sato, I. Matsuda, T. Noda, *J. Antibiot.* **1984**, *37*, 354 – 362.
[8] S. Iwasaki, M. Namikoshi, H. Kobayashi, J. Furukawa, S. Okuda, *Chem. Pharm. Bull.* **1986**, *34*, 1387 – 1390.
[9] S. Kiyoto, Y. Kawai, T. Kawakita, E. Kino, M. Okuhara, I. Uchida, H. Tanaka, M. Hashimoto, H. Terano, *J. Antibiot.* **1986**, *39*, 762 – 772.
[10] M. Takahashi, S. Iwasaki, H. Kobayashi, S. Okuda, T. Murai, Y. Sato, *Biochim. Biophys. Acta Gen. Subj.* **1987**, *926*, 215 – 223.
[11] K. Scherlach, L. P. Partida-Martinez, H.-M. Dahse, C. Hertweck, *J. Am. Chem. Soc.* **2006**, *128*, 11529 – 11536.
[12] L. P. Partida-Martinez, S. Monajembashi, K. O. Greulich, C. Hertweck, *Curr. Biol.* **2007**, *17*, 773 – 777.
[13] G. Lackner, N. Moebius, C. Hertweck, *ISME J.* **2011**, *5*, 252 – 261.
[14] M. R. Leone, G. Lackner, A. Silipo, R. Lanzetta, A. Molinaro, C. Hertweck, *Angew. Chem.* **2010**, *122*, 7638 – 7642; *Angew. Chem. Int. Ed.* **2010**, *49*, 7476 – 7480.
[15] I. Schmitt, L. P. Partida-Martinez, R. Winkler, K. Voigt, A. Einax, J. Wöstemeyer, C. Hertweck, *ISME J.* **2008**, *2*, 632 – 641.
[16] G. Lackner, C. Hertweck, *PloS Pathog.* **2011**, *7*, e1002096.
[17] G. Lackner, N. Moebius, L. P. Partida-Martinez, S. Boland, C. Hertweck, *BMC Genomics* **2011**, *12*, 210.
[18] L. P. Partida-Martinez, C. Hertweck, *ChemBioChem* **2007**, *8*, 41 – 45.
[19] B. Kusebauch, B. Busch, K. Scherlach, M. Roth, C. Hertweck, *Angew. Chem.* **2009**, *121*, 5101 – 5104; *Angew. Chem. Int. Ed.* **2009**, *48*, 5001 – 5004.
[20] B. Kusebauch, B. Busch, K. Scherlach, M. Roth, C. Hertweck, *Angew. Chem.* **2010**, *122*, 1502 – 1506; *Angew. Chem. Int. Ed.* **2010**, *49*, 1460 – 1464.
[21] G. Lackner, N. Moebius, K. Scherlach, L. P. Partida-Martinez, R. Winkler, I. Schmitt, C. Hertweck, *Appl. Environ. Microbiol.* **2009**, *75*, 2982 – 2986.
[22] L. M. Podust, T. L. Poulos, M. R. Waterman, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 3068 – 3073.
[23] A. Corsetti, J. Rossi, M. Gobbetti, *Int. J. Food Microbiol.* **2001**, *69*, 1 – 10.
[24] S. Frases, S. Chaskes, E. Dadachova, A. Casadevall, *Appl. Environ. Microbiol.* **2006**, *72*, 1542 – 1550.
[25] S. Frases, A. Salazar, E. Dadachova, A. Casadevall, *Appl. Environ. Microbiol.* **2007**, *73*, 615 – 621.
[26] J. Gibson, A. Sood, D. A. Hogan, *Appl. Environ. Microbiol.* **2009**, *75*, 504 – 513.
[27] M. Takahashi, S. Matsumoto, S. Iwasaki, I. Yahara, *Mol. Gen. Genet.* **1990**, *222*, 169 – 175.
[28] K. Scherlach, N. Brendel, K. Ishida, H. M. Dahse, C. Hertweck, *Org. Biomol. Chem.* **2012**, *10*, 5756 – 5759.
[29] E. W. Schmidt, *Nat. Chem. Biol.* **2008**, *4*, 466 – 473.

