Natural Sulfur Compounds

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Epidithiol Formation by an Unprecedented Twin Carbon–Sulfur Lyase in the Gliotoxin Pathway**

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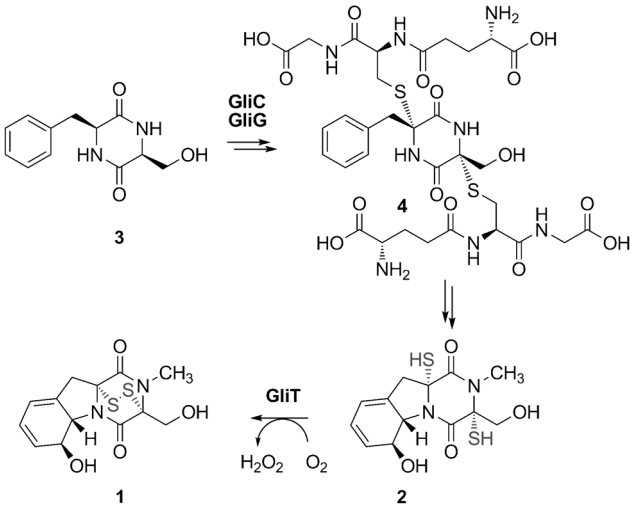
Sulfur, the tenth most abundant element in the universe, has been a spearhead in the development of life on earth. It was not only present in the first organic compounds that originated from hydrogen sulfide,[1] but, according to the autotrophic theory of the origin of life, sulfur was also crucial for mediating amino acid, peptide, and RNA synthesis.[2,3] Whereas cysteine- and methionine-derived thiols and thioethers are essential components of proteins and peptides,[4–6] other sulfur-containing natural products are much less abundant and only little is known about their biosynthesis. Even so, when present, sulfur is often the key to biological activity of secondary metabolites. Important examples are the highly reactive glucosinolate-derived isothiocyanates[7,8] and polysulfides that act as plant defense agents,[9,10] thiazole-bearing cytotoxic compounds,[11] and polythioamide pharmacophores in antibacterial *Clostridium* metabolites.[12,13] A structurally and functionally outstanding sulfur-containing moiety is the transannular disulfide bridge in epidithiodiketopiperazines (ETP), a family of microbial toxins.[14] The prototype of these compounds, gliotoxin (**1**; Scheme 1), is an infamous virulence factor of the human pathogenic fungus *Aspergillus fumigatus*, which is notable as a leading cause of death in immunocompromized patients.[15–17] Endowed with the reactive epidithio moiety, the toxin exerts deleterious effects in host cells through redox-cycling and inactivation of vital proteins by conjugation.[17–19] The cell-damaging impact of dithiol species **2** is illustrated by the mandatory presence of a disulfide forming oxidoreductase, GliT, to provide the gliotoxin producer with self-resistance.[20] A number of studies showed that the diketopiperazine core (**3**) of gliotoxin is assembled by a non-ribosomal peptide synthetase.[21–24] However, downstream pathway steps have remained elusive, mainly because of the scarcity and instability of pathway intermediates produced.[19] However, several gliotoxin congeners or shunt products could be detected in crude extracts.[25] Recently, we demonstrated by in vivo and in vitro studies that the diketopiperazine is activated by an oxygenase, GliC, to allow glutathione transfer catalyzed by a dedicated glutathione *S*-transferase, GliG, yielding bis-adduct **4**.[26] The function of GliG has also been studied by another group.[27] Herein, we elucidate the key step of epidithiol formation in gliotoxin biosynthesis. We unveil the structure of an unusual bis(cysteinyl) adduct and demonstrate that a specialized C–S lyase, GliI, promotes a dual carbon–sulfur cleavage to generate the notorious epidithiol moiety of the toxin.



Scheme 1. Structure of gliotoxin (**1**), a virulence factor of the human pathogen *Aspergillus fumigatus*, and its biosynthesis starting from diketopiperazine **3**.

Our previous discovery of the bis(glutathione) adduct **4** and deduced gene functions were highly suggestive of a scenario involving hydrolytic degradation of the peptide side chains of **4** followed by carbon–sulfur cleavage. A bioinformatic analysis of the gliotoxin biosynthesis gene cluster revealed a candidate gene, *gliI*, that could potentially code for an enzyme catalyzing the latter step. According to BLAST analyses, the deduced gene product of *gliI* was annotated as a 1-aminocyclopropane-1-carboxylic acid syn-

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thase. To elucidate the role of the putative enzyme we aimed at constructing a *gliI* deletion mutant (Δ*gliI*) of *A. fumigatus*. We succeeded in generating the targeted knockout by using a split-fragment PCR-based strategy. By this reaction, overlapping ends to the pyrithiamine resistance cassette were introduced at the 3′ end of the upstream flanking region and at the 5′ end of the downstream flanking region of the *gliI* gene, thus warranting the identity of the desired mutant (Figure 1 A). HPLC-MS monitoring of the Δ*gliI* mutant culture revealed that gliotoxin biosynthesis was completely abrogated (Figure 1 B). We carefully examined mutant broth and mycelium by HPLC-HRMS and detected trace amounts

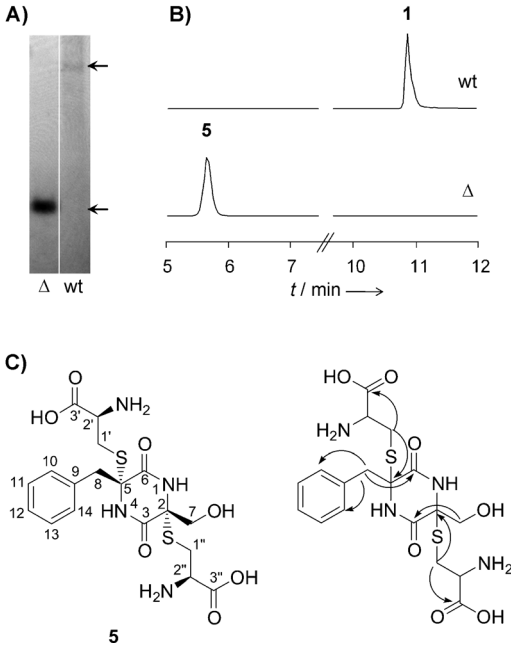


Figure 1. Genotype and phenotype of the Δ*gliI* mutant. A) Southern blot showing successful *gliI* gene deletion in the genome of *A. fumigatus*; Δ = Δ*gliI* mutant, wt = wild type. B) HPLC-MS profiles (SIM mode) of the wild type and the Δ*gliI* mutant. C) Structure of bis(cysteinyl) adduct **5** and key HMBC correlations. The absolute configuration was inferred from **1**.

of a new compound (**5**) with a molecular weight of *m*/*z* 473 $[M+H]^+$. A molecular formula of $C_{18}H_{24}N_4O_7S_2$ was deduced from HRESI-MS data, which is in full agreement with the proposed composition of the tentative pathway intermediate resulting from glutathione side chain cleavage of **4**. MS/MS fragmentation patterns and HRMS of daughter ions provided further support for the identity of **5**. To unequivocally confirm the structure of the bis adduct, a full characterization of **5** was desired. However, this proved to be a major challenge because of the extremely low amounts produced and the instability of the metabolite. Furthermore, we noted that **5** is highly water soluble and mainly retained in the fungal cytosol, which prevented its extraction from the fermentation broth. To obtain sufficient material of **5** we pooled, sheared, and extracted mycelia from a total of 140 L of mutant cultures. Through size exclusion chromatography and repeated preparative LC-MS, 1.49 mg of **5** could be isolated. ^{13}C NMR and DEPT135 spectra of **5** showed signals for two amide carbons, an aromatic ring system, two carboxyl functions, four methylene carbon atoms, and two methine carbon atoms. The chemical shifts of 28.2 and 28.6 ppm for C1″ and C1′, respectively, indicated the proximity of two methylene functions to the sulfur atoms. This was corroborated by marked HMBC couplings of H1″ to C2 and C3″, and H1′ to C5 and C3′. Moreover, we observed correlations of H8 to C9, C10, and C14 as well as to the amide carbon C6 (δ = 165.6 ppm), thus establishing the assignment of the phenylalanine partial structure. The hydroxymethyl protons (H7) were seen to have HMBC correlations to the amide carbon C3 and the quaternary carbon C2. Furthermore, the structure of the unprecedented bis(cysteinyl) diketopiperazine **5** was fully supported by high-resolution MS analyses of the daughter ions generated by tandem mass spectrometry employing an Exactive (Orbitrap) mass spectrometer. The molecular composition of each fragment ion is in full agreement with the proposed structure (Supporting Information, Figure S8).

To clarify the biochemical function of GliI in vitro, we PCR-amplified *gliI*, cloned the amplicon into an *E. coli* expression vector, and introduced the construct into *E. coli* BL21(DE3) cells for protein overproduction. MBP-tagged GliI, which had been harvested from the biomass of an *E. coli* culture (1 L), was purified using a dextrin column and treated with TEV protease for tag removal (Figure 2 A). Static light scattering experiments revealed the homodimeric status of GliI. The theoretical molecular mass of a GliI dimer is 97 kDa, which is in excellent accordance with the measured mass of 96.3 ± 2 kDa (Figure 2 C). To identify the cofactor of the putative C–S lyase we searched for consensus motifs in the amino acid alignment and identified a conserved pyridoxal 5′-phosphate (PLP) binding domain within the GliI amino acid sequence. This was supported by spectrophotometric characterization, which indicated a 1:1 stoichiometry for GliI–PLP (Figure 2 B). To elucidate the fate of the bis(cysteinyl) adduct in the presence of GliI, a solution of **5** (5 μM) was incubated with GliI-PLP and heat-inactivated GliI-PLP as a negative control. The course of the reaction was monitored by LC-HRMS. Whereas no biotransformation of **5** in the presence of inactivated GliI could be detected (Figure 2 D, trace a), we observed that active Gli-PLP readily converted **5** into two new products, **6** and **7** (Figure 2 D, trace b), with *m*/*z* $[M+H]^-$ 297.0379 (calcd. for $C_{12}H_{13}N_2O_3S_2$ 297.0373) and *m*/*z* 295.0220 $[M+H]^-$ (calcd. for $C_{12}H_{11}N_2O_3S_2$ 295.0217), respectively. Through analysis of an oxidation reaction, we determined that disulfide **7** is the oxidation product of the dithiol **6**, which explains the formation of **7** under the aerobic conditions of the assay. We could unequivocally assign the structure of **7** by HPLC-MS comparison with a fully characterized reference compound (Figure S10).

The finding that dithiol **6** is the immediate product of the GliI-mediated biotransformation of **5** is intriguing as it implies that both cysteinyl C–S bonds were cleaved enzymatically. A plausible reaction mechanism would involve a β-elimination to liberate the thiol with concomitant formation of an imine that would hydrolyze to yield pyruvate and ammonia. To verify the postulated release of NH_3 in the GliI-

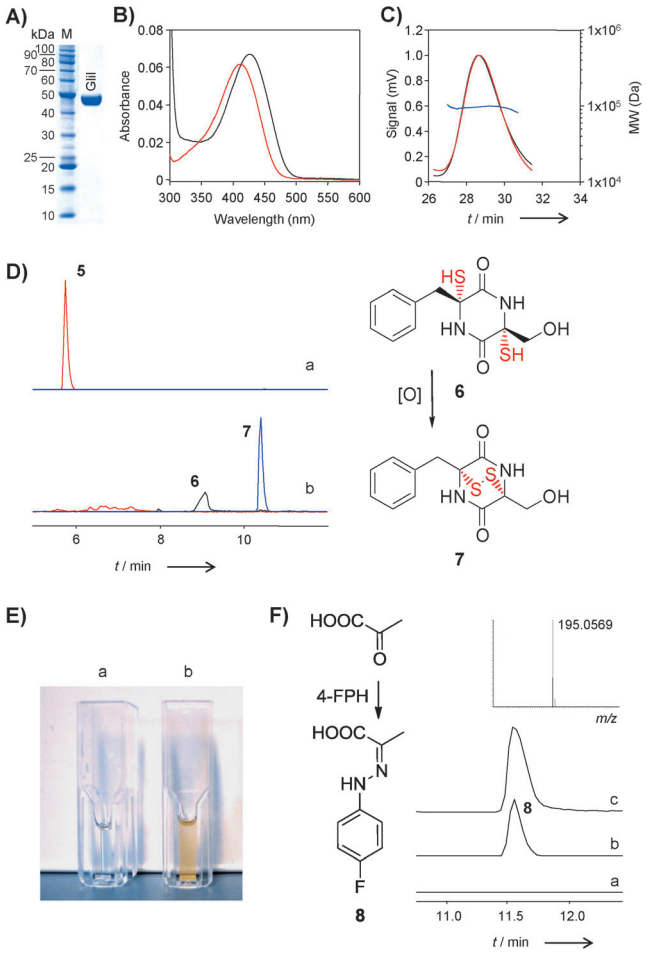
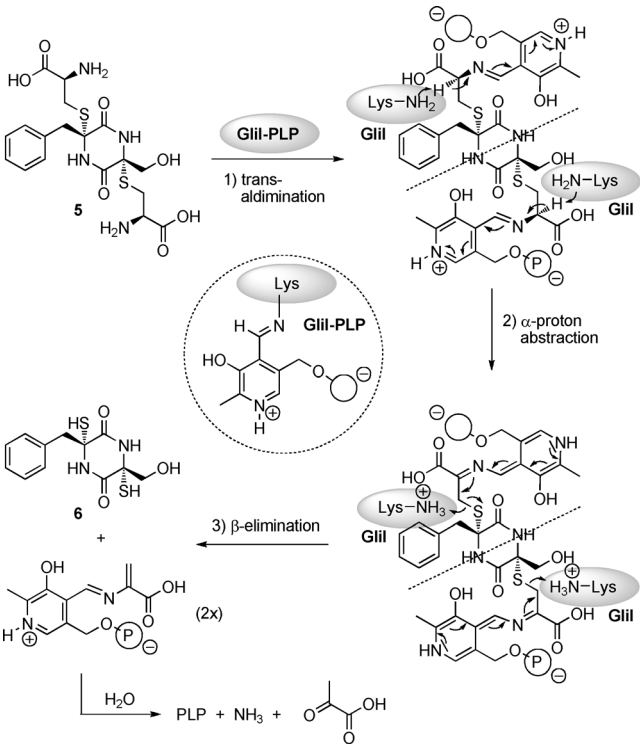


Figure 2. Characterization of GliI and results from in vitro assays. A) Purity of GliI analyzed by SDS-PAGE stained with Coomassie Brilliant Blue R-250. B) UV/Vis absorbance spectra of native (——) and denatured (——; NaOH, 95°) PLP-saturated GliI. C) Native absolute molecular mass determination by static light scattering; UV (——; ultraviolet light), LS (——; light scattering), MW (——; molecular weight). D) HPLC-MS profiles (SIM mode) of **5** (——; $m/z = 473$), **6** (——; $m/z = 297$), and **7** (——; $m/z = 295$) of biotransformation experiments using a) heat-inactivated GliI (negative control) and b) active GliI-PLP. Structures of reaction products **6** and **7** (reduced and oxidized forms, respectively) E) Detection of ammonia using Nessler's reagent. F) HPLC-MS analysis of pyruvate-derived hydrazone **8** (assay treated with 4-fluorophenylhydrazine); a) negative control, b) assay using active GliI-PLP, and c) synthetic reference.

catalyzed reaction we utilized Nessler's reagent (K_2HgI_4 in a KOH solution). Indeed, in the positive enzyme assay the occurrence of the typical brown-orange color verified the formation of ammonia (Figure 2E). To prove the formation of pyruvate as a side product we trapped the predicted oxo compound by addition of 4-fluorophenylhydrazine (4-FPH) to the enzyme assay. By HRESI-MS we detected a peak corresponding to the expected hydrazine **8** with m/z $[M+H]^- = 195.0569$ (calcd. for $C_9H_8N_2O_2F$ 195.0575). The identity of **8** was further corroborated through comparison with a synthetic reference (Figure 2F). These results unequivocally show that GliI functions as a C–S lyase to generate a dithiol with concomitant liberation of pyruvate and ammonia. In analogy to other PLP-dependent reactions the GliI-mediated transformation can be dissected into three stages: 1) transaldimination, 2) α-proton abstraction (both promoted by the ω-amino group of a Lys residue) and 3) β-elimination, followed by hydrolysis (Scheme 2). As no monocysteine intermediate was detected in the enzyme assay, and considering that GliI is a homodimer, one may conceive of a simultaneous C–S cleavage in both substrate halves.



Scheme 2. Model for the pyridoxal-dependent β-elimination reaction catalyzed by GliI.

From an evolutionary point of view it is remarkable that cysteine conjugate β-lyases have only been known from detoxification reactions and primary metabolism.[28] Whereas related C–S lyase activities have been implicated in a number of secondary metabolite pathways, so far only indirect evidence for their involvement has been given. In glucosinolate biosynthesis, a C–S lyase (Sur1) has been tentatively assigned to the pathway, but its activity has only been probed using an artificial surrogate substrate.[29] Another C–S lyase homologue, EtgE, seems to play a role in the biosynthesis of ergothioneine. However, the insolubility of the heterologously produced enzyme prevented the collection of firm biochemical evidence.[30] Thus, GliI represents the first functionally proven C–S lyase involved in a secondary metabolite pathway. Furthermore, this is the first twin C–S cleavage to yield an epidithiol. Another interesting observation is the relaxed substrate specificity of GliI. The enzyme mediates both C–S disconnections in **5**, irrespective of the different substitution pattern and configuration of the diketopiperazine substructures. To shed light on the phylogenetic relationship

of this unusual enzyme, we constructed a neighbor-joining tree based on the amino acid sequences of GliI and related enzymes (Figure 3). To our surprise, GliI and yet uncharacterized homologues encoded in various other fungal ETP biosynthesis gene clusters form a new clade (IV) that is clearly distinct from related enzymes. In particular, lyases that are involved in primary metabolism and xenobiotic detoxification in bacteria, fungi, plants, and humans fall into one clade (I) that is only distantly related. Furthermore, it is particularly noteworthy that the GliI clade (IV) is more closely related to alliinases, for example, in garlic (clade II), and 1-aminocyclopropane-1-carboxylate synthases from plants (clade III). It appears that the C–S lyase activity of GliI and its homologues has evolved independently in secondary metabolism. Finally, on biotechnological grounds, it will be interesting to study the specificities of the yet uncharacterized C–S lyases that appear to be functionally related to GliI (clade IV).

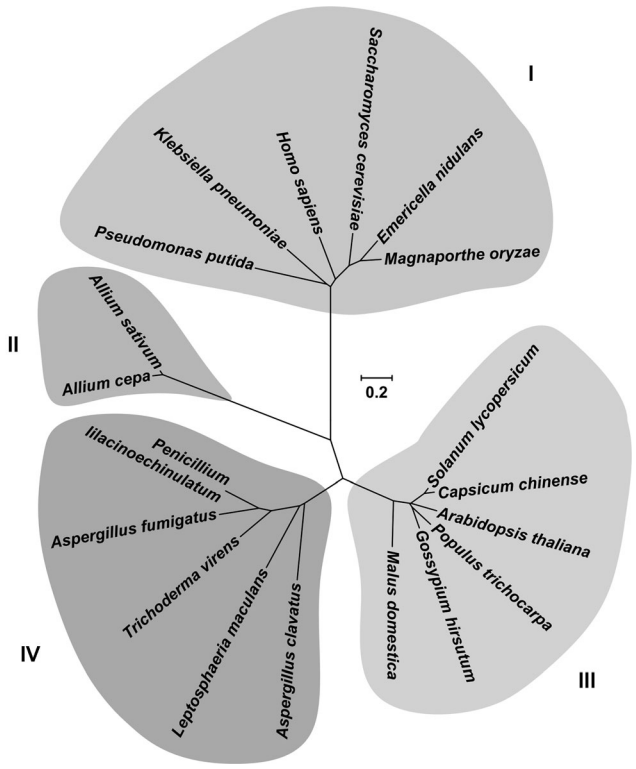


Figure 3. Phylogenetic analysis of GliI and related enzymes. Clade I: cysteine-conjugate lyases from different organisms. Clade II: alliinases from plants. Clade III: 1-aminocyclopropane-1-carboxylate synthases from plants. Clade IV: GliI homologues encoded in other fungal ETP clusters (for full details, see the Supporting Information).

In summary, we have elucidated a key step in the biosynthesis of gliotoxin, an infamous virulence factor of the human pathogen *A. fumigatus*. From the large-scale fermentation of an engineered Δ*gliI* mutant we isolated an unparalleled bis(cysteine) conjugate **5**, a missing link between the diketopiperazine precursor and the mycotoxin. We also succeeded in producing active GliI and verified through an in vitro assay that the PLP-dependent enzyme catalyzes a dual C–S cleavage of **5**, yielding the corresponding epidithiol. Moreover, through a series of further analyses we identified pyruvate and ammonia as side products of the lyase reaction. To the best of our knowledge, this is the first complete characterization of a thiol-forming C–S lyase reaction in a secondary metabolic pathway. Furthermore, the GliI-catalyzed reaction is unparalleled in forming two thiol groups concomitantly, possibly by the dual action of the GliI homodimer. A phylogenetic analysis granted first insights into the evolution of this unusual enzyme and revealed that GliI is the prototype of a new subfamily of C–S lyases that are encoded in ETP biosynthesis clusters. Thus, our findings not only hold lessons for a deeper understanding of gliotoxin biosynthesis, but will also facilitate the elucidation of many related ETP pathways. From a broader perspective the GliI-mediated C–S lyase reaction sheds new light on the formation of organosulfur compounds that have evolved as highly reactive biomolecules.

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[1] E. T. Parker, H. J. Cleaves, M. P. Callahan, J. P. Dworkin, D. P. Glavin, A. Lazcano, J. L. Bada, *Orig Life Evol. Biosph.* **2011**, *41*, 569 – 574.
[2] L. Leman, L. Orgel, M. R. Ghadiri, *Science* **2004**, *306*, 283 – 286.
[3] A. D. Keefe, S. L. Miller, G. McDonald, J. Bada, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 11904 – 11906.
[4] G. Bierbaum, H. G. Sahl, *Curr. Pharm. Biotechnol.* **2009**, *10*, 2 – 18.
[5] J. O. Melby, N. J. Nard, D. A. Mitchell, *Curr. Opin. Chem. Biol.* **2011**, *15*, 369 – 378.
[6] J. M. Willey, W. A. van der Donk, *Annu. Rev. Microbiol.* **2007**, *61*, 477 – 501.
[7] B. A. Halkier, J. Gershenzon, *Annu. Rev. Plant Biol.* **2006**, *57*, 303 – 333.
[8] I. E. Sønderby, F. Geu-Flores, B. A. Halkier, *Trends Plant Sci.* **2010**, *15*, 283 – 290.
[9] U. Münchberg, A. Anwar, S. Mecklenburg, C. Jacob, *Org. Biomol. Chem.* **2007**, *5*, 1505 – 1518.
[10] I. D. Nwachukwu, A. J. Slusarenko, M. C. Gruhlke, *Nat. Prod. Commun.* **2012**, *7*, 395 – 400.
[11] Z. Jin, *Nat. Prod. Rep.* **2011**, *28*, 1143 – 1191.
[12] S. Behnken, T. Lincke, F. Kloss, K. Ishida, C. Hertweck, *Angew. Chem.* **2012**, *124*, 2475 – 2478; *Angew. Chem. Int. Ed.* **2012**, *51*, 2425 – 2428.
[13] T. Lincke, S. Behnken, K. Ishida, M. Roth, C. Hertweck, *Angew. Chem.* **2010**, *122*, 2055 – 2057; *Angew. Chem. Int. Ed.* **2010**, *49*, 2011 – 2013.
[14] D. M. Gardiner, P. Waring, B. J. Howlett, *Microbiology* **2005**, *151*, 1021 – 1032.
[15] D. H. Scharf, T. Heinekamp, N. Remme, P. Hortschansky, A. A. Brakhage, C. Hertweck, *Appl. Microbiol. Biotechnol.* **2012**, *93*, 467 – 472.
[16] T. R. T. Dagenais, N. P. Keller, *Clin. Microbiol. Rev.* **2009**, *22*, 447 – 465.
[17] K. J. Kwon-Chung, J. A. Sugui, *Med. Mycol.* **2009**, *47*, S97 – S103.
[18] N. Möbius, C. Hertweck, *Curr. Opin. Plant Biol.* **2009**, *12*, 390 – 398.

[19] D. H. Scharf, T. Heinekamp, N. Remme, P. Hortschansky, A. A. Brakhage, C. Hertweck, *Appl. Microbiol. Biotechnol.* **2012**, *93*, 467 – 472.
[20] D. H. Scharf, N. Remme, T. Heinekamp, P. Hortschansky, A. A. Brakhage, C. Hertweck, *J. Am. Chem. Soc.* **2010**, *132*, 10136 – 10141.
[21] C. J. Balibar, C. T. Walsh, *Biochemistry* **2006**, *45*, 15029 – 15038.
[22] R. A. Cramer, M. P. Gamcsik, R. M. Brooking, L. K. Najvar, W. R. Kirkpatrick, T. F. Patterson, C. J. Balibar, J. R. Graybill, J. R. Perfect, S. N. Abraham, W. J. Steinbach, *Eukaryotic Cell* **2006**, *5*, 972 – 980.
[23] C. Kupfahl, T. Heinekamp, G. Geginat, T. Ruppert, A. Hartl, H. Hof, A. A. Brakhage, *Mol. Microbiol.* **2006**, *62*, 292 – 302.
[24] J. A. Sugui, J. Pardo, Y. C. Chang, K. A. Zarember, G. Nardone, E. M. Galvez, A. Muellbacher, J. I. Gallin, M. M. Simon, K. J. Kwon-Chung, *Eukaryotic Cell* **2007**, *9*, 1562 – 1569.
[25] 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.
[26] 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.
[27] 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.
[28] A. J. Cooper, B. F. Krasnikov, Z. V. Niatsetskaya, J. T. Pinto, P. S. Callery, M. T. Villar, A. Artigues, S. A. Bruschi, *Amino Acids* **2011**, *41*, 7 – 27.
[29] M. D. Mikkelsen, P. Naur, B. A. Halkier, *Plant J.* **2004**, *37*, 770 – 777.
[30] F. P. Seebeck, *J. Am. Chem. Soc.* **2010**, *132*, 6632 – 6633.

