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Opposed Effects of Enzymatic Gliotoxin *N*- and *S*-Methylations

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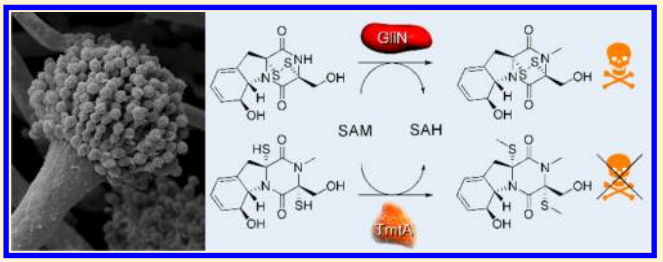
Supporting Information

ABSTRACT: Gliotoxin (**1**), a virulence factor of the human pathogenic fungus *Aspergillus fumigatus*, is the prototype of epipoly(thiodioxopiperazine) (ETP) toxins. Here we report the discovery and functional analysis of two methyl transferases (MTs) that play crucial roles for ETP toxicity. Genome comparisons, knockouts, and in vitro enzyme studies identified a new *S*-adenosyl-L-methionine-dependent *S*-MT (TmtA) that is, surprisingly, encoded outside the *gli* gene cluster. We found that TmtA irreversibly inactivates ETP by *S*-alkylation and that this detoxification strategy appears to be not only limited to ETP producers. Furthermore, we unveiled that GliN functions as a freestanding amide *N*-MT. GliN-mediated amide methylation confers stability to ETP, damping the spontaneous formation of tri- and tetrasulfides. In addition, enzymatic *N*-alkylation constitutes the last step in gliotoxin biosynthesis and is a prerequisite for the cytotoxicity of the molecule. Thus, these specialized alkylating enzymes have dramatic and fully opposed effects: complete activation or inactivation of the toxin.



INTRODUCTION

Pathogenic fungi produce a range of secondary metabolites to weaken or kill the host.[1,2] Structurally remarkable examples are found in the heterogeneous family of epipoly(thiodioxopiperazine)s (ETPs) that are produced by a phylogenetically diverse range of filamentous fungi.[3] ETP toxins are characterized by a diketopiperazine (DKP) core furnished with unusual transannular di- or polysulfide bridges,[4,5] which are indispensable for their deleterious effects. The epidithio residue enables ETPs not only to inactivate proteins by conjugation to cysteine residues, but also to generate reactive oxygen species through cycling between oxidized (disulfide) and reduced (dithiol) forms.[6] The prototype of ETP toxins, gliotoxin (**1**; Figure 1), is an infamous virulence factor of the human pathogenic fungus *Aspergillus fumigatus*.[7] It should be highlighted that invasive aspergillosis caused by this fungus is a leading cause of death in immunocompromised patients.[8,9] Self-resistance of the producer is, at least in part, conferred by the disulfide-forming oxidoreductase GliT.[10,11] Because of the eminent role of the toxin, gliotoxin (*gli*) biosynthesis has been studied at the synthetic,[12] genetic, biochemical, and structural levels. In brief, the DKP core is assembled by a nonribosomal peptide synthetase (GliP),[13−16] and the essential thiol residues are introduced by sequential oxygenation (GliC) and glutathione transfer (GliG),[17,18] followed by a cascade of unusual glutathione dissociation reactions catalyzed by GliK, GliJ,[19] and GliI.[20] Further enzymatic tailoring reactions such as methylation have remained elusive. Here we report the surprising finding that enzymatic *N*- and *S*-methylation reactions play crucial, yet opposing, roles in gliotoxin biosynthesis: ETP activation or irreversible inactivation.

RESULTS AND DISCUSSION

Enzymatic Methylation Reactions in Gliotoxin Biosynthesis. In the course of metabolic profiling of *A. fumigatus*, we noted the formation of bis(methylthio)gliotoxin (**2**) as a minor metabolite. This compound has been previously reported as a derivative of gliotoxin in *Trichoderma deliquescens* (previously *Gliocladium deliquescens*).[21] Interestingly, *S*-methylation leads to a complete loss of cytotoxic activity (Figure 1C). Moreover, in contrast to the reversible detoxification by disulfide bond formation, alkylation of the thiol moieties is irreversible. To elucidate the biosynthetic origin of the methyl groups, we performed stable isotope labeling experiments with ^{13}C-methionine. MS analyses of the *A. fumigatus* wild-type (Af293) broth extract showed that all three methyl groups of **2** (M + 3; *m*/*z* 360 $[M + H]^+$) and the *N*-methyl group of **1** (M + 1; *m*/*z* 328 $[M + H]^+$) are methionine-derived and are thus introduced enzymatically.

TmtA Catalyzes the *S*-Methylation of Gliotoxin. *S*-Methyltransferases are scarce and have not yet been characterized in fungi. The gliotoxin gene cluster of *A. fumigatus* harbors two candidate genes, *gliN* and *gliM*, for putative *S*-adenosyl-L-methionine (SAM)-dependent methyltransferases (MTs). A phylogenetic analysis showed that the deduced gene products fall into distinct new clades in an MT

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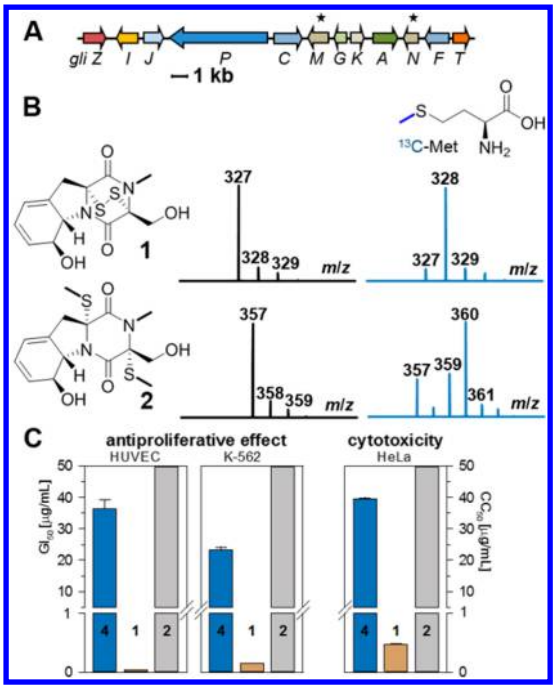

Figure 1. (A) Organization of the *gli* biosynthesis gene cluster. (B) Structures and mass spectra of gliotoxin (**1**) and bis(methylthio)-gliotoxin (**2**) compared to mass spectra of mono-^{13}C-methylated **1** and tri-^{13}C-methylated **2** formed after incubation of the *A. fumigatus* wild type (wt) with ^{13}C-methionine. (C) Antiproliferative and cytotoxic activities of **1** and its congeners (absolute values, Supporting Information, Table S3).

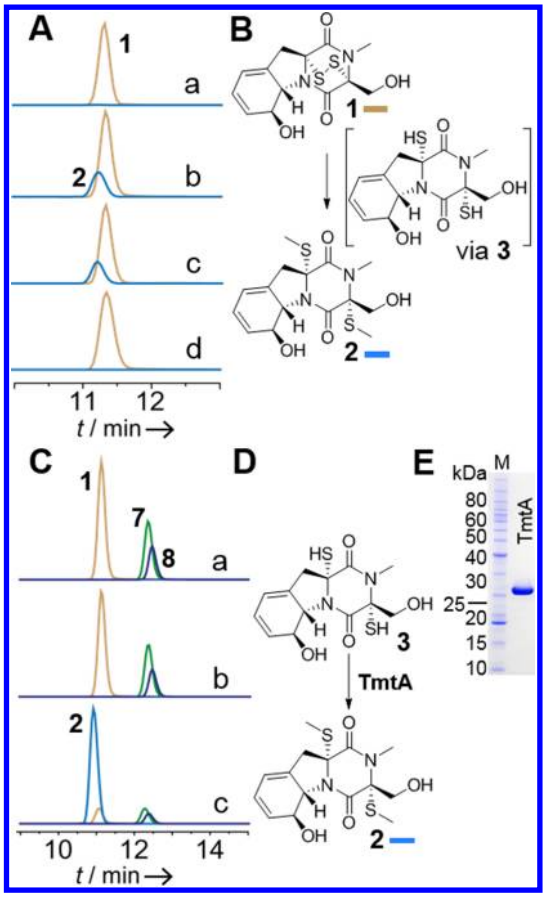

Figure 2. (A) HPLC monitoring of the biotransformation reaction. Extracted ion chromatograms are shown for (a) gliotoxin reference **1**, (b) incubation of **1** with the Δ*gliM* mutant, (c) incubation of **1** with the Δ*smtA* mutant, and (d) production of **1** by the Δ*tmtA* mutant. (B) Detoxification reaction of **1** to **2** via **3** as an intracellular intermediate. (C) Characterization and in vitro reconstitution of TmtA activity. HPLC monitoring of the biotransformation reaction. Extracted ion chromatograms are shown for (a) incubation of **3** in buffer showing degradation to **1**, **7**, and **8**, (b) incubation of **3** with heat-inactivated TmtA only showing degradation to **7** and **8** (Figure 4), and (c) TmtA-mediated transformation of **3** to **2**. (D) Scheme of TmtA-mediated biotransformation of **3** to **2**. (E) Molecular mass of His$_6$-tagged TmtA analyzed by SDS−PAGE.

dendrogram (Supporting Information, Figures S1 and S2), yet did not lend support for any candidate substrate. To investigate the functions of the putative MT genes, we constructed targeted *A. fumigatus* mutants lacking either *gliN* (Δ*gliN*) or *gliM* (Δ*gliM*). We succeeded in generating the desired mutants using a split-fragment polymerase chain reaction (PCR)-based strategy.[22] We verified successful gene deletion in both cases by PCR and Southern blot analysis (Supporting Information, Figure S4C). Notably, neither the Δ*gliN* mutant nor the Δ*gliM* mutant produced gliotoxin. It is known that disruption of essential gliotoxin biosynthesis genes, e.g., *gliP*, may result in the down-regulation of the entire pathway. Moreover, gene expression is typically restored by addition of gliotoxin,[14] indicating an autoregulation of the *gliZ* transcription factor. However, we could still detect **2** in Δ*gliM* (Figure 2A, trace b) and Δ*gliM*Δ*gliN* double-mutant (Figure S4B, trace b) cultures treated with pure exogenous **1**. To rule out the possibility of redundant enzyme functions in *A. fumigatus*, we searched for alternative *S*-MTs. Genome analysis led to the discovery of another, albeit cryptic and partial, gliotoxin biosynthesis gene cluster in *A. fumigatus* that consists of gene copies from *gliC′*, *gliZ′*, *gliP′*, *gliM′*, and *gliA′*[23] (Figure S4A). To test whether this *gliM*-like gene is responsible for gliotoxin detoxification, we constructed additional mutants lacking *gliM′*. Again, exogenous gliotoxin was applied to cultures of *A. fumigatus* mutants Δ*gliM′* and Δ*gliM*Δ*gliM′*, yet LC/MS analysis still revealed formation of **2** at a ratio similar to that of the single Δ*gliM* mutant (Figure S4B). Therefore, we could rule out any contribution of GliN, GliM, and GliM′ in gliotoxin detoxification by *S*-methylation. Notably, *gliM′* is not induced by addition of **1** as shown for the gliotoxin cluster genes (Supporting Information, Figure S3). We found that even *Aspergillus nidulans* and *Fusarium oxysporum*, which lack any ETP biosynthesis gene cluster.[24] are capable of ETP detoxification (Figure S4B). It thus appears that *S*-methylation for detoxification is a trait not limited to ETP producers and that the *S*-MT genes are not clustered with ETP biosynthesis genes. Considering the high number of putative MT genes present in the genomes of fungi, currently an in vitro analysis of all candidates is out of the question. However, comparative genome analyses of fungi known for ETP *S*-methylation and extensive data mining yielded two candidate MT genes, Afu4G13570 (*S*-methyltransferase, *smtA*) and Afu2G11120 (thiol methyltransferase, *tmtA*), a homologue of MT-II from *Aspergillus niger* (Figures S1 and S2) which is up-regulated after exposure to dithiothreitol (DTT).[25] Whereas the Δ*smtA* strain is still able to produce **2** (Figure 2A, trace c), the cultures of the Δ*tmtA* strain lost the ability of *S*-methylation and produced only **1** (Figure 2A, trace d). To verify the proposed enzyme function, we performed in vitro experiments using heterologously produced GliN, GliM, GliM′, SmtA, and TmtA. Therefore, we amplified the corresponding open reading

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frames (ORFs), cloned the generated PCR fragments into *Escherichia coli* expression vectors, and propagated the resulting plasmids in *E. coli* DH5α cells. After sequencing of the insets, the vectors were introduced into *E. coli* BL21(DE3) cells for protein overproduction. After the cells were harvested, His6-tagged GliN, GliM, GliM′, SmtA, and TmtA were purified using a Ni Sepharose column. Gliotoxin (in its reduced form, **3**) was individually incubated with the putative MTs in the presence of SAM. Whereas no *S*-alkylation could be observed for GliN, GliM, GliM′, and SmtA, the transformation of **1** into **2** could be observed only in the presence of TmtA (Figure 2C, trace c).

When using heat-inactivated TmtA, *S*-methylation does not take place. Taken together, we can rule out that the MT genes in the *gli* gene locus contribute to *S*-methylation. Instead, this reaction is carried out by a specialized thiol methyltransferase (TmtA) encoded elsewhere in the genome.

Role of TmtA for Gliotoxin Resistance. To clarify the in vivo function of TmtA, we performed an inhibition zone assay. In contrast to the Δ*gliT* strain, which is deficient of the resistance-conferring thiol−disulfide oxidoreductase, the *tmtA* mutant shows only slightly increased sensitivity to **1** compared to the wild type (Figure 3). A plausible explanation for this

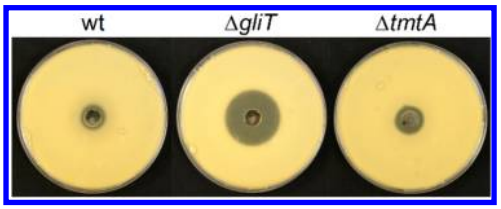

Figure 3. Sensitivity of *A. fumigatus* wild-type (wt) and mutant strains toward gliotoxin (**1**) (c = 4.5 mM) determined in an inhibition zone assay.

observation is that TmtA serves as the second line of protection against gliotoxin. In accordance with this, **2** is formed after prolonged incubation times. It thus appears that TmtA does not play the primary role in toxin self-resistance, but prevents self-poisoning of the producing organism when exposed to the toxin in higher titers or for longer periods. This model is also supported by the kinetics of TmtA (v_{max} = 1.2 μmol min^{-1}, K_m = 239.8 μM). Interestingly, detoxification by *S*-methylation was also observed in bacteria such as the holomycin producer *Streptomyces clavuligerus*; however, the *S*-MT in charge has remained elusive.[26] To our knowledge, microbial *S*-MTs that modify natural products are unknown.

GliN Plays a Key Role in Gliotoxin Biosynthesis. The surprising finding that *S*-alkylation is mediated by an enzyme encoded outside the *gli* gene locus raised questions about the roles of *gliM* and *gliN*. Thus, we more closely inspected the metabolic profiles of the corresponding mutants. Whereas the Δ*gliM* mutant did not show any new compound, the Δ*gliN* mutant culture accumulated a slightly more polar compound with m/z 313 $[M + H]^+$. Traces of the same compound were also detected in the wild type. Sufficient amounts of the new compound for a full characterization were obtained from an upscaled mutant culture. Purification by open column chromatography on silica and preparative HPLC yielded pure **4** with a molecular formula of $C_{12}H_{13}N_2O_4S_2$ as deduced from HR-ESI-MS data (m/z 313.0312 $[M + H]^+$, calcd for $C_{12}H_{13}N_2O_4S_2$ 313.0311). 1D and 2D NMR data showed that **4** has a spectrum similar to that of **1**, yet lacking a methyl carbon signal (at N2). Thus, **4** represents *N*-desmethylgliotoxin, a novel intermediate in the gliotoxin pathway.

GliN Is a Monomeric, SAM-Dependent *N*-Methyltransferase. To verify the function of GliN as an amide *N*-MT, we investigated the enzyme in vitro. Static light scattering experiments revealed the homodimeric status of GliN, and the theoretical molecular mass of a GliN monomer was found to be 33.6 kDa, which is in excellent agreement with the measured mass of 30.36 ± 0.75 kDa (Figure 4C). Using SAM

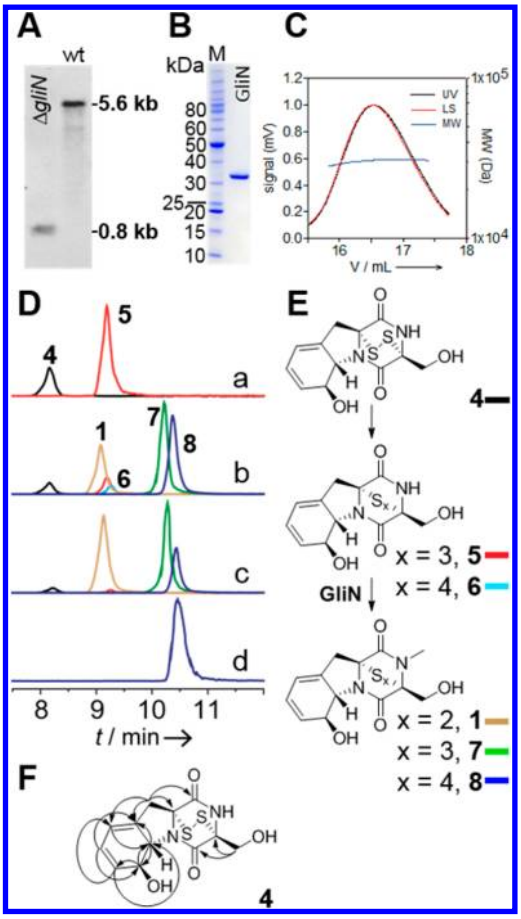

Figure 4. Characterization and in vitro reconstitution of GliN activity. (A) Southern blot analysis of gDNA of the wild type (wt) and the Δ*gliN* mutants. (B) Molecular mass of His_6-tagged GliN analyzed by SDS−PAGE. (C) Native molecular size determination by static light scattering. (D) HPLC monitoring of the biotransformation reaction. Extracted ion chromatograms are shown for (a) incubation of **4** with heat-inactivated GliN showing degradation to **5**, (b) GliN-mediated transformation of **4** to GT metabolites **1**, **7**, and **8**, (c) GliN-mediated transformation of **4** to GT metabolites **1**, **7**, and **8** in the presence of GliT, and (d) the synthetic reference of **8**. (E) Observed degradation pattern for **4** followed by GliN-mediated biotransformation. (F) Structure of *N*-desmethylgliotoxin (**4**) and key HMBC correlations.

as a cosubstrate, *N*-desmethylgliotoxin (**4**) was incubated with GliN and heat-inactivated GliN, and the course of reaction was monitored by LC/MS. Whereas no biotransformation of **4** could be detected with inactivated GliN (Figure 4D, trace a), we observed that active GliN readily converted **4** into two new products, **7** and **8** (Figure 4D, trace b), with m/z 359 $[M + H]^+$ and m/z 391 $[M + H]^+$, respectively. The increased $[M + 2]^+$ peak and the $\Delta m/z$ of 32 (64) compared to those of gliotoxin indicated the formation of gliotoxin E[27] and gliotoxin G,[28]

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respectively. The latter was confirmed by comparison with a synthetic reference.[29] We found that the polysulfide formation is reduced when GliT is included in the in vitro assay because the enzyme promotes the formation of the transannular disulfide bridge (Figure 4D, trace c). Furthermore, we observed the rapid decomposition of **4** during NMR measurements. Subsequent analysis of the decomposition products revealed the presence of the S3- and S4-analogues of *N*-desmethylgliotoxin, **5** (*m*/*z* 345.0033 $[M + H]^+$, calcd for $C_{12}H_{13}N_2O_4S_3$ 345.0032) and **6** (*m*/*z* 376.9754 $[M + H]^+$, calcd for $C_{12}H_{13}N_2O_4S_4$ 376.9753). In stark contrast to that of gliotoxin, which is stable over several hours, incubation of **4** leads to rapid degradation within minutes. It is well conceivable that *N*-methylation stabilizes ETP, and in fact free amide moieties are exceptional for known ETPs.[4,5] Most surprisingly, however, is the finding that the loss of the amide methyl leads to a complete loss of bioactivity: the antiproliferative and cytotoxic potency of **4** is at least 100-fold reduced compared to that of gliotoxin (Figure 1C).

A plausible explanation for this observation is that *N*-methylation substantially contributes to the membrane permeability of gliotoxin, as has been shown for other *N*-methylated peptides.[30] In addition, *N*-methylation might influence the integrity of the sulfur bridge as shown for the pentathiepin heterocycle.[31] From a biosynthetic viewpoint, it is noteworthy that GliN is uniquely positioned among enzymes that methylate amide nitrogens. Although about 23% of all known MTs are classified as *N*-MTs,[32,33] most of the well-characterized examples catalyze the methylation of amine nitrogens. Known amide *N*-MTs are either of bacterial origin or partial domains of larger enzymes.[34−36] Furthermore, the substrate specificity of GliN is unique among known fungal MTs.[37]

■ CONCLUSION

In summary, we have elucidated the roles of two new MTs that are crucially associated with gliotoxin tailoring. We found that enzymatic *S*-methylation is a strategy of ETP producers and some nonproducers to irreversibly inactivate ETP toxins. This reaction is catalyzed by TmtA, the first example of an ETP *S*-MT. In general, there are only a few examples of *S*-MTs from plants and animals,[38,39] and microbial *S*-MTs are unprecedented. Surprisingly, these *S*-MTs are not encoded in the ETP biosynthesis gene clusters, which hampered their discovery. From a biotechnological point of view, Δ*tmtA* strains may be employed as valuable sources of ETPs, preventing compound losses by *S*-alkylation. In this study, gene inactivation, isolation of a new pathway intermediate (**4**), and the successful in vitro biotransformation of **4** into **1** also elucidated the last step in gliotoxin biosynthesis. The SAM-dependent *N*-methylation of the diketopiperazine core not only confers stability to the molecule, but also turns a harmless precursor into a potent toxin. Thus, *N*- and *S*-methylations of gliotoxin represent an unusual case of turning on and off the toxicity of a virulence factor by simple, yet regiospecific and substrate-specific, methylation. Without doubt, this finding will have implications for related natural products of the large family of ETP toxins.

■ EXPERIMENTAL SECTION

General Analytical Procedures. All 1D (^{1}H, ^{13}C, DEPT) and 2D (^{1}H−^{1}H COSY, HSQC, NOESY, HMBC) NMR spectra were recorded in deuterated solvents on a Bruker AVANCE II 300 MHz or AVANCE III 500 or 600 MHz instrument equipped with a Bruker Cryo platform. The chemical shifts are reported in parts per million relative to the solvent residual peak ($\delta(CHCl_3)$ = 7.26 ppm, $\delta(CD_3OD)$ = 3.31 ppm). The following abbreviations are used for multiplicities of the resonance signals: s = singlet, d = doublet, t = triplet, q = quartet, qt = quintet, br = broad. HPLC/MS was conducted on a Thermo Surveyor Plus (LC) and Thermo LTQ Velos (MS), an ESI source operating in positive mode. Preparative HPLC purification was achieved by using a Gilson Abimed device with binary pump 321 and UV/vis 156 (column, Phenomenex Luna C18, 10 μm, 250 × 21.2 mm; eluent, water, acetonitrile (20−100%)). HR-ESI-MS was conducted on a Thermo Accela (LC) and Thermo Exactive (HRMS), an ESI source operating in positive mode and an orbitrap analyzer.

General Methods. All reagents were obtained from commercial suppliers (Sigma-Aldrich, TCI, etc.) and used without further purification unless otherwise explained. Reactions were carried out under inert gas (argon) by using the Schlenk technique in dried solvents. Open column chromatographic separations were executed on silica gel (Kieselgel 60, 15−40 μm, Merck KGaA). Reaction progress was monitored by thin-layer chromatography (TLC) (silica gel on aluminum sheets (20 × 20 cm) with fluorescent dye, 254 nm, Merck KGaA) or HPLC/MS.

General Strain Cultivation. Strains were grown in *Aspergillus* minimal medium (AMM) as described previously.[40] AMM agar was prepared by addition of 1.6% (w/v) Select agar (Invitrogen, Germany). For formation of conidia, *A. fumigatus* was cultivated on AMM agar plates at 37 °C for 5 days. Conidia were harvested in 0.9% (w/v) NaCl/0.1% (v/v) Tween 80 and counted using a CASY cell counter (model TT, Innovatis AG, Germany).

Mutant Strain Cultivation in the Presence of Gliotoxin. All *A. fumigatus* strains, Δ*gliM*, Δ*gliM′*, Δ*gliM*Δ*gliM′*, Δ*gliN*Δ*gliM*, Δ*tmtA*, and Δ*smtA*, were cultivated at 28 °C in 500 mL of Czapek Dox medium at 180 rpm for 6 days. Gliotoxin (2 × 5 mg) was added on days 2 and 3. Cultures of *A. nidulans* were handled equally. The cultures were extracted twice with 300 mL of ethyl acetate. The combined organic phases were dried over sodium sulfate and concentrated to dryness. For gliotoxin and bis(methylthio)gliotoxin determination, the residues were dissolved in 3 mL of MeOH and measured with HPLC.

Cultivation of the Wild Type in the Presence of ^{13}C-Methionine. *A. fumigatus* strain CEA17Δ*akuB* was cultivated at 28 °C in 500 mL of Czapek Dox medium at 180 rpm for 6 days. ^{13}C-Methionine (2 × 25 mg) was added on days 2 and 3. The cultures were extracted twice with 300 mL of ethyl acetate. The combined organic phases were dried over sodium sulfate and concentrated to dryness.

Isolation and Manipulation of Nucleic Acids. Isolation of chromosomal DNA of *A. fumigatus* was done using the Master Pure Yeast DNA Purification Kit (Epicentre Biotechnologies, United States). For Southern blot analysis, DNA fragments were separated on an agarose gel and blotted onto Hybond N+ nylon membranes (GE Healthcare Life-Sciences, Germany). Labeling of DNA probes, hybridization, and detection of DNA−DNA hybrids were performed as described previously.[41]

Generation of *A. fumigatus* Mutant Strains. Partial deletion of *gliN* was achieved by a PCR-based strategy. Upstream and downstream flanking regions of gene *gliN* (AFUA_6G09720) were amplified by PCR using primer pairs gliN5-for and gliN-ptrA-rev and gliN3-rev and gliN-ptrA-for, respectively. By this reaction, overlapping ends of 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 *gliN* gene. The *ptrA* resistance cassette was amplified from plasmid pSK275 with primers ptrA-for and ptrA-rev. All PCR fragments were purified by gel extraction. The final deletion construct was generated by a three-fragment PCR employing primers gliN5-for and gliN3-rev. All PCRs were performed with Phusion high-fidelity DNA polymerase (Finnzymes) according to the manufacturer's recommendations. The resulting 3.5 kb PCR product was purified and used for transformation of *A. fumigatus* wild-type protoplasts. Pyrithiamine (1 mg mL^{-1}) resistant transformants were analyzed for

partial deletion of gliN by Southern blot analysis. One positive transformant, designated Δ*gliN*, was chosen for further analysis. The gene *gliM* (AFUA_6G09680) was likewise deleted using primer pairs gliM5-for and gliM-ptrA-rev and gliM3-rev and gliM-ptrA-for. The resulting 3.6 kb PCR fragment was used for transformation. The gene *tmtA* (AFUA_2G11120) was likewise deleted using primer pairs tmtA5-for and tmtA-ptrA-rev and tmtA3-rev and tmtA-ptrA-for. The resulting 3.9 kb PCR fragment was used for transformation. The gene *gliM* (Afu3g12910) was likewise deleted using primer pairs gliMlike5-for and gliMlike-hph-rev and gliMlike3-rev and gliMlike-hph-for. The hygromycin resistance cassette was amplified from plasmid pUChph with primers hph-for and hph-rev. The resulting 4.8 kb PCR fragment was used for transformation. Hygromycin (250 μg mL^{-1}, InvivoGen, France) resistant transformants were analyzed for partial deletion of *gliM* by Southern blot analysis. The gene *smtA* (Afu4G13570) was likewise deleted using primer pairs smtA5-for and smtA-hph-rev and smtA3-rev and smtA-hph-for. The resulting 5 kb PCR fragment was used for transformation.

Heterologous Production and Purification of Proteins. The *gliN* ORF (849 bp) was amplified by PCR using primer pair gliN forward and gliN reverse. The generated PCR fragment was cloned into the *Bam*HI/*Hin*dIII sites of pET43.1H6GliT to create pET43.1H6GliN. The resulting plasmid was propagated in *E. coli* DH5α cells. The DNA sequence was verified by sequencing, and the vector was introduced into *E. coli* BL21(DE3) cells for protein overproduction. Recombinant GliN was produced by autoinduction in Overnight Express instant TB medium (Novagen). Cells were harvested after 24 h of incubation at 30 °C. His6-GliN-containing cleared cellular extracts in 20 mM Tris/HCl, 150 mM NaCl, pH 7.5, were applied to a 25 mL Ni Sepharose 6 FF column (all columns purchased from GE Healthcare Bio-Sciences, Freiburg, Germany). GliN was eluted with 200 mM imidazole. GliN was applied to a HiLoad 26/60 Superdex 200 pg column. The GliN-containing fraction was identified by SDS−PAGE and stored in 50% glycerol at −20 °C. Protein concentrations were determined according to Bradford using the CoomassiePlus protein assay reagent (Pierce). The gliM ORF (1257 bp) was amplified by PCR using primer pair gliM forward and gliM reverse. The generated PCR fragment was cloned into the *Bam*HI/*Not*I sites of pET43.1H6GliT to create pET43.1H6GliM. Protein production and purification of GliM were similar to GliN purification. All other proteins were cloned and purified as described for GliN.

Determination of Physical and Biochemical Properties of GliN. The absolute molecular mass was determined by static light scattering experiments using a miniDawn Treos monitor (Wyatt). Therefore, pure GliN was applied to a HiLoad 16/60 Superdex 200 pg column (GE Healthcare), and identified peaks were processed by ASTRA 6 software (Wyatt). The absolute molecular mass of native GliN was calculated from both the UV and light scattering responses.

Isolation of Desmethylgliotoxin from the Δ*gliN* Mutant. The Δ*gliN* mutant was cultivated in 2 L (4 × 500 mL) of Czapek Dox medium in a 2 L Erlenmeyer flask for 5 days (28 °C, 220 rpm). The broth was filtered (pleated filters), the mycelium was washed with water, and the combined culture filtrate (2 L) was extracted twice with a half volume of EtOAc. The crude extract of the Δ*gliN* mutant was subjected to silica gel chromatography using cyclohexane/EtOAc (starting from 80:20 with increasing amount of EtOAc). Fractions containing **4** were combined and further purified by preparative HPLC. HPLC conditions: column, Macherey Nagel (C18 Nucleodur Polartec, 250 × 10 mm, 5 μm); flow rate, 4.8 mL min^{-1}; water (solvent A), acetonitrile (solvent B); gradient, 1 min 10% B, 1−18 min 10−100% B, 18−20 min 100% B, 20−24 min 10% B. Compound **4** eluted after 12.0 min. The yield was 4.2 mg.

In Vitro Biotransformation Assay Using GliN. The enzymatic activity of purified GliN was measured by incubation of **3** with GliN or heat-inactivated GliN as the control for 2 h and *S*-adenosylmethionine as a cosubstrate. After EtOAc extraction (2 × 500 μL) the organic solvent was dried under a stream of nitrogen, and the residues were redissolved in 100 μL of methanol and analyzed by HPLC/MS. HPLC conditions: column, Thermo Betasil (C18, 150 × 2.1 mm, 5 μm); flow rate, 200 μL min^{-1}; 0.1% formic acid in water (solvent A), 0.1% formic acid in acetonitrile (solvent B); gradient, 1 min 5% B, 1−16 min 5−98% B, 16−19 min 98% B, 20−28 min 5% B.

In Vitro Biotransformation Assay Using GliM, GliM′, SmtA, and TmtA. The enzymatic activity of purified proteins was measured by incubation of reduced gliotoxin with pure protein or heat-inactivated protein as the control for 2 h and *S*-adenosylmethionine as a cosubstrate. After EtOAc extraction (2 × 250 μL) the organic solvent was dried under a stream of nitrogen, and the residues were redissolved in 100 μL of methanol and analyzed by HPLC/MS. HPLC conditions: column, Thermo Betasil (C18, 150 × 2.1 mm, 5 μm); flow rate, 200 μL min^{-1}; 0.1% formic acid in water (solvent A), 0.1% formic acid in acetonitrile (solvent B); gradient, 1 min 5% B, 1−16 min 5−98% B, 16−19 min 98% B, 20−28 min 5% B. The kinetic characterization of TmtA was carried out with the SAM methyltransferase assay (G-Biosciences). The concentrations of protein (1 μM) and SAM (200 μM) were kept constant, while the concentration of reduced gliotoxin was varied.

Isolation of Gliotoxin from *A. fumigatus*. *A. fumigatus* was cultivated in Czapek Dox medium for 5 days (28 °C, 180 rpm). The broth was filtered (pleated filters), the mycelium was washed with water, and the combined culture filtrate was extracted twice with half its volume of chloroform. The crude extract was resuspended in a minimal volume of methanol and extracted three times with hexane to remove all lipids. The methanol fraction was evaporated and the crude material resuspended in ethanol, treated with charcoal, and stored at 2 °C overnight for crystallization. The crystallized gliotoxin was filtered and recrystallized from ethanol.

Synthesis of Gliotoxin G Reference. Synthesis was performed according to a published method.[29] Briefly, to a solution of gliotoxin 6 (10 mg, 31 μmol) and rhombic sulfur (100 mg) (recrystallized from toluene) in carbon disulfide (1 mL) was added lithium phenylmethanethiolate (3.1 μmol, 0.1 equiv) (prepared from benzenethiol and nBuLi in THF) at room temperature. After being stirred for 15 h, the suspension was poured onto a silica plug, which was eluted by dichloromethane to remove excess sulfur, followed by ethyl acetate. The crude fraction was subjected to preparative HPLC. HPLC conditions: flow rate, 21 mL min^{-1}; water (solvent A), acetonitrile (solvent B); gradient, 1 min 10% B, 1−18 min 10−100% B, 18−20 min 100% B, 20−24 min 10% B. Compound **8** eluted after 15.1 min. The yield was 4.8 mg (48%) as a white solid. The spectroscopic data agreed fully with those reported.

Reduction of Gliotoxin. Gliotoxin (20 mg, 61.3 μmol) was suspended in 2-propanol (2 mL), cooled to 0 °C, and treated successively with $NaBH_4$ (18.5 mg, 0.49 mmol, 8 equiv). After being stirred for 30 min at 0 °C, the reaction mixture was poured into 1 M HCl (20 mL) and extracted twice with EtOAc (20 mL). The combined EtOAc fractions were dried over Na_2SO_4 and evaporated, yielding bis(methylthio)gliotoxin (18.2 mg, 90.4%).

Inhibition Zone Assays for Gliotoxin Sensitivity. The sensitivity of various *A. fumigatus* strains to gliotoxin was determined in an inhibition zone assay. Of the test strains, 2 × 10^7 conidia were mixed with 20 mL of YAG agar (0.5% (w/v) yeast extract, 2% (w/v) agar, 2% (w/v) glucose, trace elements). Next, 100 μL of 4.5 mM gliotoxin was added in a hole in the center of the plate, and the diameter of the inhibition zone was determined after 24 h of incubation at 37 °C. The experiment was repeated in triplicate.

■ ASSOCIATED CONTENT

Supporting Information

Detailed phylogenetic tree, Northern blot, accession numbers, originating species, and function of the proteins used for phylogenetic analysis, oligonucleotides used in this study, acitivity testing of **1**, **2**, and **4**, physiochemical data for **4**, full spectra for **4**, HR-ESI-MS spectra of **5** and **6**, kinetic analysis of *S*-methylation by TmtA, and UV and MS traces from the *S*-methylation assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Möbius, N.; Hertweck, C. *Curr. Opin. Plant Biol.* **2009**, *12*, 390.
(2) Scharf, D. H.; Heinekamp, T.; Brakhage, A. A. *PLoS Pathog.* **2014**, *10*, e1003859.
(3) Scharf, D. H.; Heinekamp, T.; Remme, N.; Hortschansky, P.; Brakhage, A. A.; Hertweck, C. *Appl. Microbiol. Biotechnol.* **2012**, *93*, 467.
(4) Borthwick, A. D. *Chem. Rev.* **2012**, *112*, 3641.
(5) Gardiner, D. M.; Waring, P.; Howlett, B. J. *Microbiology* **2005**, *151*, 1021.
(6) Waring, P.; Sjaarda, A.; Lin, Q. H. *Biochem. Pharmacol.* **1995**, *49*, 1195.
(7) Kwon-Chung, K. J.; Sugui, J. A. *Med. Mycol.* **2009**, *47* (Suppl.1), S97.
(8) Brakhage, A. A.; Langfelder, K. *Annu. Rev. Microbiol.* **2002**, *56*, 433.
(9) Dagenais, T. R.; Keller, N. P. *Clin. Microbiol. Rev.* **2009**, *22*, 447.
(10) Scharf, D. H.; Remme, N.; Heinekamp, T.; Hortschansky, P.; Brakhage, A. A.; Hertweck, C. *J. Am. Chem. Soc.* **2010**, *132*, 10136.
(11) Scharf, D. H.; Groll, M.; Habel, A.; Heinekamp, T.; Hertweck, C.; Brakhage, A. A.; Huber, E. M. *Angew. Chem., Int. Ed.* **2014**, *53*, 2221.
(12) Kim, J.; Movassaghi, M. *Chem. Soc. Rev.* **2009**, *38*, 3035.
(13) Balibar, C. J.; Walsh, C. T. *Biochemistry* **2006**, *45*, 15029.
(14) Cramer, R. A., Jr.; Gamcsik, M. P.; Brooking, R. M.; Najvar, L. K.; Kirkpatrick, W. R.; Patterson, T. F.; Balibar, C. J.; Graybill, J. R.; Perfect, J. R.; Abraham, S. N.; Steinbach, W. J. *Eukaryot. Cell* **2006**, *5*, 972.
(15) Kupfahl, C.; Heinekamp, T.; Geginat, G.; Ruppert, T.; Härtl, A.; Hof, H.; Brakhage, A. A. *Mol. Microbiol.* **2006**, *62*, 292.
(16) Sugui, J. A.; Pardo, J.; Chang, Y. C.; Zarember, K. A.; Nardone, G.; Galvez, E. M.; Mullbacher, A.; Gallin, J. I.; Simon, M. M.; Kwon-Chung, K. J. *Eukaryot. Cell* **2007**, *6*, 1562.
(17) Scharf, D. H.; Remme, N.; Habel, A.; Chankhamjon, P.; Scherlach, K.; Heinekamp, T.; Hortschansky, P.; Brakhage, A. A.; Hertweck, C. *J. Am. Chem. Soc.* **2011**, *133*, 12322.
(18) Davis, C.; Carberry, S.; Schrettl, M.; Singh, I.; Stephens, J. C.; Barry, S. M.; Kavanagh, K.; Challis, G. L.; Brougham, D.; Doyle, S. *Chem. Biol.* **2011**, *18*, 542.
(19) Scharf, D. H.; Chankhamjon, P.; Scherlach, K.; Heinekamp, T.; Willing, K.; Brakhage, A. A.; Hertweck, C. *Angew. Chem., Int. Ed.* **2013**, *125*, 11298.
(20) Scharf, D. H.; Chankhamjon, P.; Scherlach, K.; Heinekamp, T.; Roth, M.; Brakhage, A. A.; Hertweck, C. *Angew. Chem., Int. Ed.* **2012**, *124*, 10211.
(21) Kirby, G. W.; Robins, D. J.; Sefton, M. A.; Talekar, R. R. *J. Chem. Soc., Perkin Trans. 1* **1980**, 119.
(22) Szewczyk, E.; Nayak, T.; Oakley, C. E.; Edgerton, H.; Xiong, Y.; Taheri-Talesh, N.; Osmani, S. A.; Oakley, B. R. *Nat. Protoc.* **2006**, *1*, 3111.
(23) Patron, N. J.; Waller, R. F.; Cozijnsen, A. J.; Straney, D. C.; Gardiner, D. M.; Nierman, W. C.; Howlett, B. J. *BMC Evol. Biol.* **2007**, *7*, 174.
(24) Andersen, M. R.; Nielsen, J. B.; Klitgaard, A.; Petersen, L. M.; Zachariasen, M.; Hansen, T. J.; Blicher, L. H.; Gotfredsen, C. H.; Larsen, T. O.; Nielsen, K. F.; Mortensen, U. H. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, E99.
(25) MacKenzie, D. A.; Guillemette, T.; Al-Sheikh, H.; Watson, A. J.; Jeenes, D. J.; Wongwathanarat, P.; Dunn-Coleman, N. S.; van Peij, N.; Archer, D. B. *Mol. Genet. Genomics* **2005**, *274*, 410.
(26) Li, B.; Forseth, R. R.; Bowers, A. A.; Schroeder, F. C.; Walsh, C. T. *ChemBioChem* **2012**, *13*, 2521.
(27) Waring, P.; Eichner, R.; Tiwari-Palni, U.; Mullbacher, A. *Aust. J. Chem.* **1987**, *40*, 991.
(28) Waring, P.; Eichner, R. D.; Palni, U. T.; Mullbacher, A. *Tetrahedron Lett.* **1986**, *27*, 735.
(29) Kirby, G. W.; Rao, G. V.; Robins, D. J.; Stark, W. M. *Tetrahedron Lett.* **1986**, *27*, 5539.
(30) Chatterjee, J.; Gilon, C.; Hoffman, A.; Kessler, H. *Acc. Chem. Res.* **2008**, *41*, 1331.
(31) Brzostowska, E. M.; Greer, A. *J. Am. Chem. Soc.* **2003**, *125*, 396.
(32) Liscombe, D. K.; Louie, G. V.; Noel, J. P. *Nat. Prod. Rep.* **2012**, *29*, 1238.
(33) Liscombe, D. K.; Usera, A. R.; O'Connor, S. E. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 18793.
(34) Giessen, T. W.; von Tesmar, A. M.; Marahiel, M. A. *Chem. Biol.* **2013**, *20*, 828.
(35) Hornbogen, T.; Riechers, S. P.; Prinz, B.; Schultchen, J.; Lang, C.; Schmidt, S.; Mugge, C.; Turkanovic, S.; Sussmuth, R. D.; Tauberger, E.; Zocher, R. *ChemBioChem* **2007**, *8*, 1048.
(36) Wu, Y.; Kang, Q.; Shang, G.; Spiteller, P.; Carroll, B.; Yu, T. W.; Su, W.; Bai, L.; Floss, H. G. *ChemBioChem* **2011**, *12*, 1759.
(37) Rigbers, O.; Li, S. M. *J. Biol. Chem.* **2008**, *283*, 26859.
(38) Coiner, H.; Schroder, G.; Wehinger, E.; Liu, C. J.; Noel, J. P.; Schwab, W.; Schroder, J. *Plant J.* **2006**, *46*, 193.
(39) Weisiger, R. A.; Jakoby, W. B. *Arch. Biochem. Biophys.* **1979**, *196*, 631.
(40) Weidner, G.; d'Enfert, C.; Koch, A.; Mol, P. C.; Brakhage, A. A. *Curr. Genet.* **1998**, *33*, 378.
(41) Grosse, C.; Heinekamp, T.; Kniemeyer, O.; Gehrke, A.; Brakhage, A. A. *Appl. Environ. Microbiol.* **2008**, *74*, 4923.