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A Dedicated Glutathione *S*-Transferase Mediates Carbon−Sulfur Bond Formation in Gliotoxin Biosynthesis

Daniel H. Scharf,[†] Nicole Remme,[†] Andreas Habel,[†] Pranatchareeya Chankhamjon,[†] Kirstin Scherlach,[†] Thorsten Heinekamp,[†] Peter Hortschansky,[†] Axel A. Brakhage,[†,‡] and Christian Hertweck*[,†,‡]

[†]Leibniz Institute for Natural Product Research and Infection Biology — Hans Knöll Institute, Beutenbergstr. 11a, 07745 Jena, Germany

[‡]Friedrich Schiller University, Jena, Germany

Supporting Information

ABSTRACT: Gliotoxin is a virulence factor of the human pathogen *Aspergillus fumigatus*, the leading cause of invasive aspergillosis. Its toxicity is mediated by the unusual transannular disulfide bridge of the epidithiodiketopiperazine (ETP) scaffold. Here we disclose the critical role of a specialized glutathione *S*-transferase (GST), GliG, in enzymatic sulfurization. Furthermore, we show that bishydroxylation of the diketopiperazine by the oxygenase GliC is a prerequisite for glutathione adduct formation. This is the first report of the involvement of a GST in enzymatic C−S bond formation in microbial secondary metabolism.

Gliotoxin (**1**) is the prototype of the infamous family of epidithiodiketopiperazine (ETP) toxins that are produced by a wide range of microorganisms.[1,2] A hallmark of ETPs is the cyclopeptide scaffold equipped with a transannular disulfide bridge. This outstanding structural unit is indeed responsible for deleterious effects of the toxins, as it readily mediates intracellular redox cycling and inactivates vital proteins by conjugation.[1−4] Endowed with these adverse properties, gliotoxin significantly contributes to the virulence of the human pathogen *Aspergillus fumigatus*. Notably, invasive aspergillosis caused by this fungus is the leading cause of death in immunocompromised patients.[5,6] Since knowledge of the molecular basis and enzymology of ETP biosynthesis could greatly aid in developing diagnostics and antifungal therapy, this area has been the focus of intense research over the past years. The full genome sequencing of *A. fumigatus* has set the stage for identifying the gliotoxin (*gli*) biosynthesis gene cluster (Figure 1A). Various work groups have since independently demonstrated that the diketopiperazine core [*cyclo*-L-Phe-L-Ser (**2**)] is assembled by GliP, a bimodular nonribosomal peptide synthetase (NRPS).[7−11] More recently, we unveiled by in vivo and in vitro studies that an FAD-dependent oxidoreductase, GliT, generates the intramolecular disulfide bond from the corresponding dithiol **3** (Scheme 1).[12] Nevertheless, it has remained fully enigmatic how the sulfur is incorporated into the cyclopeptide framework. Here we report that a CYP450 monooxygenase and a specialized glutathione *S*-transferase (GST) play a key role in gliotoxin C−S bond formation.

Through bioinformatic analysis of the *gli* biosynthesis gene cluster, we identified a gene (*gliG*) that could code for a GST. Enzymes belonging to the diverse GST family are best known for their ability to form glutathione (GSH) conjugates with xenobiotics and leucotrienes and to harness reactive oxygen species and radicals.[13] However, to our knowledge, despite their widespread occurrence,[14] they have not been implicated in fungal biosynthetic pathways. This is particularly remarkable as we noted that orthologues of GliG are encoded in all presently known ETP biosynthesis gene clusters.[15] Furthermore, a phylogenetic analysis revealed that the deduced gene products even represent a new clade (I) in the GST dendrogram (Figure 1D).

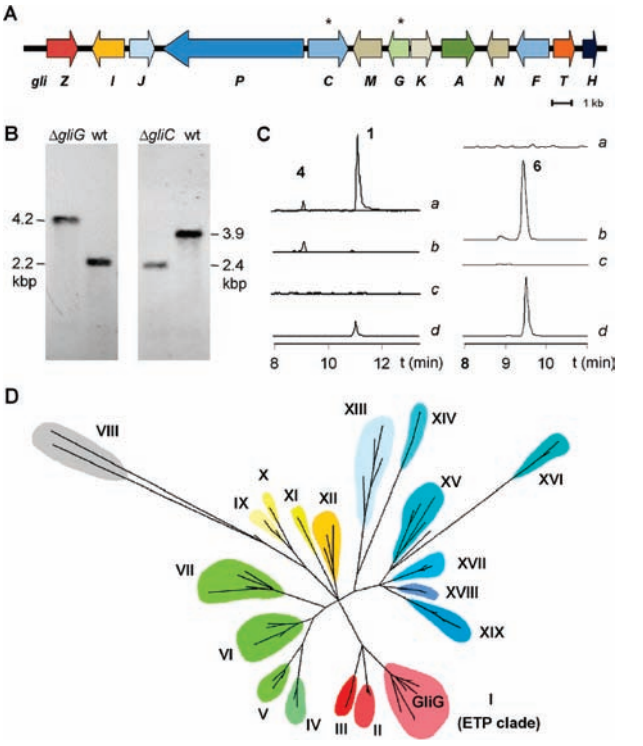

Figure 1. (A) Organization of the *gli* biosynthesis gene cluster. (B) Southern blot analysis of gDNA of the wild type (wt) and the Δ*gliG* and Δ*gliC* mutants. (C) Metabolic profiles (extracted ion chromatograms) of (a) wt, (b) Δ*gliG* mutant, (c) Δ*gliC* mutant, and (d) references of **1** and **6**. (D) Cladogram of GliG and homologous GST sequences (the detailed phylogeny is given in the Supporting Information).

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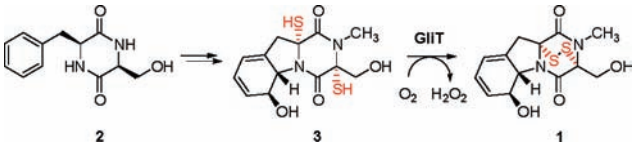
Scheme 1. Structures of Gliotoxin (1) and Its Known Biosynthetic Precursors, Dithiol 3 and Diketopiperazine 2



We thus set out to investigate the role of GliG in gliotoxin biosynthesis and deleted the corresponding gene in the *A. fumigatus* genome through homologous recombination (Figure 1). We noted that the growth of the Δ*gliG* mutant is comparable to that of the wild type upon addition of **1**. The lack of sensitivity of the mutant toward the toxin shows that GliG is not involved in detoxification of toxins as other GSTs are. HPLC−MS profiling of the resulting mutant broth indicated that the lack of GliG led to a complete abrogation of gliotoxin biosynthesis (Figure 1C).

While this result unequivocally showed that *gliG* is essential for gliotoxin formation, in vitro studies were required to gain insight into its exact function. Therefore, we amplified the *gliG* open reading frame (720 base pairs) by PCR and cloned the amplicon into an *Escherichia coli* expression vector. After propagation of the plasmid and sequencing of the insert, the vector was introduced into *E. coli* BL21(DE3) cells for protein overproduction. After the cells were harvested, His_6-tagged GliG was purified using a NiSepharose column (Figure 2A). Size-exclusion chromatography (SEC) revealed the homodimeric status of GliG (Figure 2B). To identify conditions favoring the stability of GliG, we performed melting temperature analysis (Figure 2C). On the basis of this, we optimized the starting purification conditions of the protein and the storage buffer and eventually established a one-step purification that led to pure, soluble His_6-tagged GliG. Interestingly, like the phylogenetically related theta-class proteins of the GST superfamily, GliG lacks the ability to bind to GSH sepharose. Furthermore, it does not employ standard GST substrates such as 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-epoxy-3-(nitrophenoxy)propane (EPNPP). The alignment of GliG with several homologues shows a division of the protein into two conserved domains, as shown for other GSH-binding proteins. The N-terminal domain should be involved in GSH binding, whereas the second domain might be involved in binding of the cosubstrate and protein dimerization.

Apparently, GliG does not employ any of the standard substrates. To identify a candidate that could have accumulated in the Δ*gliG* mutant, we investigated the culture extract of the Δ*gliG* mutant. Using HPLC−MS in the positive-ion mode, we noted the accumulation of a new compound, **6**, that forms an ion with *m/z* 263 ($[M + H]^+$). The compound was isolated from an upscaled mutant culture and then purified by open column chromatography on silica, SEC, and preparative HPLC, after which its structure was elucidated by high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) and 1D and 2D NMR measurements. From the HR-ESI-MS data, a molecular composition of $C_{13}H_{14}N_2O_4$ was deduced, which was corroborated by the 1D NMR data. The ^{13}C NMR and 135° distortionless enhancement by polarization transfer (DEPT135) spectra showed the presence of two amide carbons, an aromatic ring system, and an *O*-methyl function as well as two methylene carbon atoms. H,H correlation spectroscopy (H,H-COSY) and heteronuclear multiple-bond correlation (HMBC) measurements established

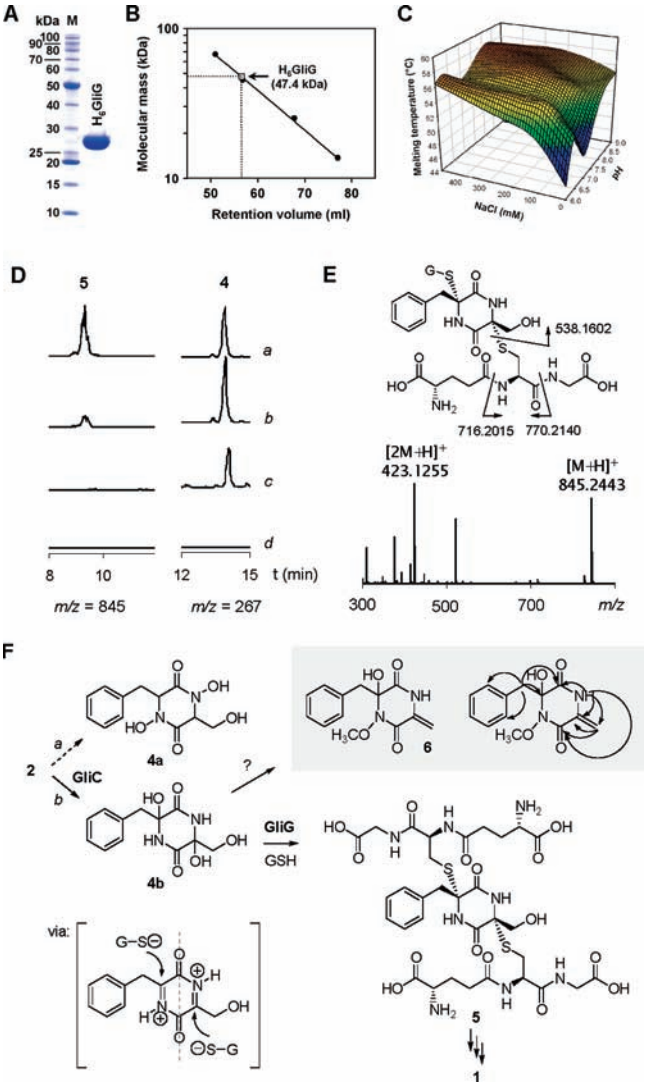


Figure 2. Characterization and in vitro reconstitution of GliG activity. (A) Molecular mass of His_6-tagged GliG analyzed by SDS-PAGE. (B) Native molecular size determination by gel filtration. (C) Three-dimensional plot of GliG melting temperature as a function of pH and NaCl concentration. (D) HLPC monitoring of the biotransformation reaction. Extracted ion chromatograms are shown for (a) GliG-mediated transformation of **4** from the Δ*gliG* mutant; (b) same as (a) but using heat-inactivated GliG (residual activity after 10 min; longer heating led to complete loss of activity); (c) negative control lacking GliG; (d) negative control using the crude extract from the Δ*gliC* mutant. (E) HR-MS^n analysis of GT conjugate. (F) Structures of the proposed bishydroxylated intermediates (**4a** and **4b**) and shunt product **6**, HMBC correlations for **6**, and a model of the enzyme-mediated hydroxylation−sulfurization sequence.

the structure of **6** as a diketopiperazine with an *exo*-methylene group. A chemical shift of 82.4 ppm for C6 disclosed two neighboring heteroatoms. Since no coupling of the *O*-methyl function was observed, its connectivity to an amide nitrogen atom was deduced. H,H-COSY coupling of H4 and H7α/β revealed the position of the NH function and thus established the structure of **6** (Figure 2E). While compound **6** is obviously derived from the gliotoxin precursor **2**, its substitution pattern indicates that it would rather be a shunt product than a substrate for GliG. However, the finding of **6** points toward an oxygenated

metabolite upstream of C−S bond formation. Thus, we monitored the metabolic profiles of the wild type and mutant by LC−HR-MS (Exactive) and detected $[M + H]^+$ peaks at m/z 267.0970 (calcd for $C_{12}H_{15}N_2O_5$: 267.0981) that could well correspond to a bishydroxylated diketopiperazine, **4** (Figure 1C, traces a and b). This assumption was supported by the observed MS^n fragmentation pattern, which corresponds with the sequential loss of 2 equiv of water (see the Supporting Information). Unfortunately, the scarcity and instability of **4** hampered its isolation to clarify whether the hydroxyl groups are attached to the amide nitrogen (**4a**) or the α-carbon (**4b**). To address this, we prepared a synthetic reference of **4a** and compared it with the metabolite produced by the Δ*gliG* mutant. Interestingly, both the HPLC retention times and MS^n spectra fragmentation patterns for these samples differed, indicating that the diketopiperazine is bishydroxylated at the α-carbon (**4b**), not at the nitrogen. This conclusion is also in line with the structure of the shunt product **6**.

We next tested whether the bishydroxylated intermediate (**4b**) is a key intermediate in the gliotoxin pathway. First, we surmised that **4** would result from enzymatic bishydroxylation, a reaction that could be catalyzed by the *gliC* gene product, a putative cytochrome P450 monooxygenase. To verify this, we generated a mutant lacking *gliC* and monitored its metabolite profile. In fact, neither **6** nor **4** were detectable in the broth of the Δ*gliC* mutant, unequivocally proving that GliC acts upstream of GliG in the gliotoxin pathway (Figure 1C, trace c). Furthermore, we found that the second putative cytochrome P450 monooxygenase (GliF) encoded in the *gli* gene cluster is not involved in the hydroxylation of the diketopiperazine, since deletion of *gliF* did not affect the production of the bishydroxylated intermediate (data not shown). In this way, it could be ruled out that GliF plays a role upstream of C−S bond formation. Finally, we found that **4b** is indeed a precursor of gliotoxin, as it was consumed in the GliG in vitro assay with formation of a novel sulfur-containing adduct (**5**) (Figure 2D, trace a). Specifically, a freshly prepared crude extract from the Δ*gliG* mutant was incubated with GSH and GliG. Using LC−HRMS, we monitored the conversion of the bishydroxylated intermediate into a new compound, **5**, whose $[M + H]^+$ peak appeared at m/z 845.2443 (calcd for $C_{32}H_{45}N_8O_{15}S_2$: 845.2446). This was in perfect agreement with the structure of a bis-GSH adduct, and the HR-MS^n fragmentation pattern confirmed the presence of two GSH units loaded onto the diketopiperazine core of **5** (Figure 2E). In negative control experiments without GliG or with heat-inactivated GliG, none or only traces of the conjugate could be detected (Figure 2D, traces b and c). The nonzero transformation of **4b** into **5** could be due to spontaneous dehydration and subsequent GSH addition, which is known for strong electrophiles,[16] but this does not take place under physiological conditions in the Δ*gliG* mutant. Instead, we found that GliG is quite thermostable (see Figure 2C) and that there is residual enzymatic activity after standard heat treatment (for 10 min), with longer heating periods leading to a completely inactive enzyme. Finally, in another control experiment, we demonstrated that **5** was not formed using the crude extract of the Δ*gliC* mutant broth (Figure 2D, trace d), thus excluding the possibility that another precursor would undergo GSH conjugation. In sum, these findings revealed that a hydroxylase and a new type of GST are required in order to produce an unusual bis-GSH adduct. A mechanistically reasonable scenario would be that the bishydroxylated intermediate undergoes sequential elimination of water, thus yielding intermediary imine species, which would represent suitable electrophiles for the attacking thiolate (Figure 2F). The observed retention of configuration in gliotoxin is remarkable and suggests that hydroxylation and the downstream elimination/GSH addition take place from the same side of the molecule. On another note, it is interesting that an oxygenation reaction mediated by a cytochrome P450 enzyme (GliC) is the prerequisite of gliotoxin C−S bond formation, a scenario that is strikingly similar to what is observed in phase I/II detoxification reactions. Finally, we reason that the ultimate steps for formation of the epidithio bridge involve the degradation of the bis-GSH conjugate by the putative dipeptidase (GliJ) and an ACC-synthase-like enzyme (GliI) to yield the dithiol, which is eventually converted by GliT into the canonical ETP disulfide bridge.

The topic of enzymatic C−S bond formation has been the focus of various excellent recent studies, for example in the context of His−Cys cross-linking[17] and lantibiotic cyclization.[18] It has been shown that enzymatic sulfurization involves γ-glutamylcysteine in ergothioneine biosynthesis[19] and cysteine in ovothiol biosynthesis.[20] GSH has been identified as the sulfur donor in the biosynthesis of glucosinolates,[21] yet a dedicated GST for its incorporation has not been characterized to date. Also, except for their contribution to double-bond isomerization in hypothemycin biosynthesis,[22] to the best of our knowledge, GSTs have not been implicated to date in microbial pathways yielding a sulfur-containing metabolite. Through the targeted knockouts of *gliG* and *gliC*, metabolic profiling, and in vitro reconstitution of GST activity, we have now provided the first insight into the sulfurization steps in gliotoxin biosynthesis. These results are likely to be significant for a broad range of microorganisms, as our phylogenetic analysis revealed that genes coding for GliG homologues are widespread in the genomes of ETP producers. GliG is related to theta-class GSTs but appears to be the first representative of a new family of biosynthetic enzymes. Thus, our work has not only unveiled key steps in the pathway of a virulence factor of a severe human pathogen but also outlined a new role of a microbial GST. Future studies will shed light on the structure and exact mechanism of GliG.

■ ASSOCIATED CONTENT

Supporting Information. Detailed phylogenetic tree, sequence alignment, HR-MS^n data for **4** and **5**, full spectra for **6** and synthetic compounds, HPLC comparison of synthetic **4a** with **4b**, and full experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
Christian.Hertweck@hki-jena.de

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