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Diketopiperazine Formation in Fungi requires Dedicated Cyclization and Thiolation Domains

Joshua A. Baccile[a]†, Henry H. Le[a], Brandon T. Pfannenstiel[b], Jin Woo Bok[b], Christian Gomez[a], Eileen Brandenburger[c], Dirk Hoffmeister[c], Nancy P. Keller[b]*, and Frank C. Schroeder[a]*

Abstract: Cyclization of linear dipeptidyl precursors derived from nonribosomal peptide synthetases (NRPSs) into 2,5-diketopiperazines (DKPs) is a crucial step in the biosynthesis of a large number of bioactive natural products. However, the mechanism of DKP formation in fungi has remained unclear, despite extensive studies of their biosyntheses. Here we show that DKP formation *en route* to the fungal virulence factor gliotoxin requires a seemingly extraneous couplet of condensation (C) and thiolation (T) domains in the NRPS GliP. *In vivo* truncation of GliP to remove the CT couplet or just the T domain abrogated production of gliotoxin and all other *gli* pathway metabolites. Point mutation of conserved active sites in the C and T domains diminished cyclization activity of GliP *in vitro* and abolished gliotoxin biosynthesis *in vivo*. Verified NRPSs of other fungal DKPs terminate with similar CT domain couplets, suggesting a conserved strategy for DKP biosynthesis by fungal NRPSs.

NRPS-derived DKPs form a large class of natural products with diverse biological activities.[1-5] Gliotoxin (**1**) is the best-known member of the epipolythiodiketopiperazines (ETPs), a family of toxic DKPs produced by a variety of filamentous fungi.[1-5] The biosynthesis of **1** has been extensively studied, due to its significant contribution to the virulence of the devastating human pathogen, *A. fumigatus*[6-12] and as a model system for fungal NRPS pathways. Production of **1** is accomplished via the *gli* biosynthetic gene cluster (BGC) in *A. fumigatus* and related fungi (**Figure 1a**). The 13-gene *gli* BGC encodes the transcriptional regulator, GliZ, one transporter (GliA), several backbone tailoring enzymes, and the core NRPS, GliP (**Figure 1a**).[13] Whereas, the majority of *gli*-cluster tailoring enzymes have previously been characterized extensively *in vitro* and *in vivo* (blue arrows, **Figure 1a**), functional analysis of the core enzyme, GliP, is incomplete (**Figure 1b**).[14]

Homology-based annotation of GliP uncovers two adenylation (A), two condensation (C), and three thiolation (T) domains, referred to as A_1-T_1-C_1-A_2-T_2-C_2-T_3.[14] Previous work showed that recombinant GliP converts L-Phe and L-Ser into the DKP cyclo(L-Phe-L-Ser) (**2**), which was speculated to result from spontaneous cyclization of a GliP-T_2-tethered-L-Phe-L-Ser intermediate (**Figure 1b**).[7,14] Although **2** could plausibly be derived from non-enzymatic cyclization of T_2-tethered L-Phe-L-Ser, the presence of the C_2 and T_3 domains, which in this model have no function, may suggest an alternative mechanism. In 2012, Gao *et al*. reported that macrocyclization of linear peptidyl precursors produced by a variety of fungal multi-module NRPSs is catalyzed by conserved terminal condensation-like (C_T) domains,[15] and that C_T domain activity was dependent on a conserved histidine within the amino acid sequence SHXXXDXXS/T. We noted that this C_T-conserved sequence exists in the GliP-C_2 domain (**Figure S1**), suggesting that the C_2 domain in GliP may be involved in cyclization of a tethered L-Phe-L-Ser dipeptide (**Figure 1b**). We further hypothesized that the seemingly extraneous T_3 domain also plays a role in DKP formation.

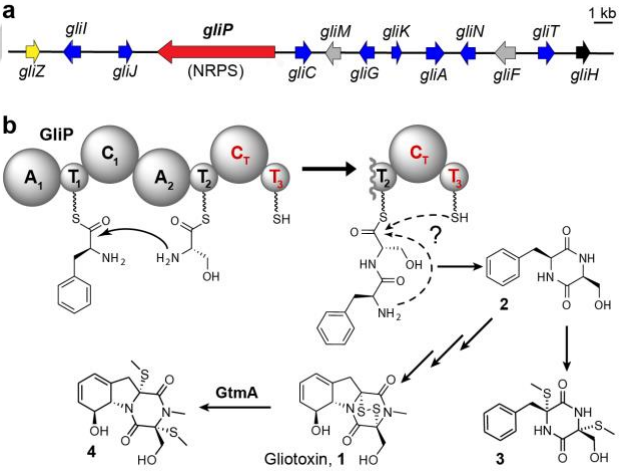

Figure 1. Gliotoxin biosynthesis in *A. fumigatus*. (a) *gli*-cluster gene annotations, including characterized tailoring enzymes (blue), tailoring enzymes with inferred functions (grey), and genes without predicted function (black). *gliZ*: transcription factor; *gliI*: pyridoxal phosphate dependent desulfurase; *gliC, gliF*: cytochrome P450 oxidases; *gliM*: *O*-methyltransferase; *gliG*: glutathione-*S*-transferase; *gliK*: glutamate cyclase; *gliA*: transporter; *gliN*: *N*-methyltransferase; *gliT*: oxidase; (b) Putative function of GliP and abbreviated biosynthesis of **1** showing the most abundant intermediates or shunt metabolites **2** and **3**, as well as the detoxification product, **4**.[6,9,16-19]

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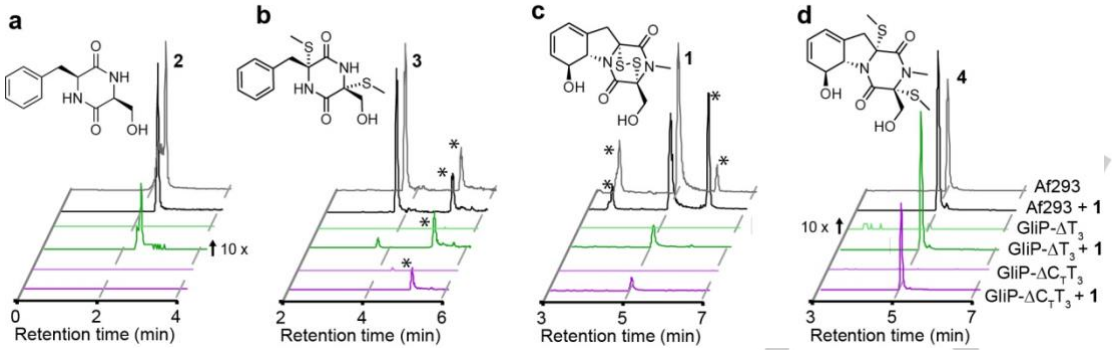

Figure 2. LC-HRMS analysis of whole-metabolome extracts from WT and mutant *A. fumigatus* strains. (a-d) Representative overlaid extracted-ion-chromatograms (EICs) for **1-4** in Af293 (black), GliP-ΔT_3 (green), and GliP-$\Delta C_T T_3$ (purple). Darker lines representative overlaid EICs for **1-4** in the indicated strains with added exogenous **1**. "10 x" indicates scaling of weak signals applied for comparison. "*" = unrelated peaks.

To investigate the function of the putative GliP-C_T and the GliP-T_3 domains *in vivo,* we constructed two truncation mutants in *A. fumigatus*, GliP-ΔT_3 and GliP-$\Delta C_T T_3$, and assessed the impact of these truncations on the biosynthesis of **1** and *gli*-pathway metabolites (**1-4**, **Figure 2a-d**). Comparison of whole-metabolome extracts from WT (Af293), GliP-ΔT_3, and GliP-$\Delta C_T T_3$ revealed a complete loss of **1-4** in the GliP-$\Delta C_T T_3$ strain (**Figure 2a-d**). In extracts from the GliP-ΔT_3 strain we were unable to detect **1** and the most abundant *gli*-pathway shunt metabolites **2** and **3**, however, we observed trace quantities of **4** (99 % less than Af293), the detoxification product of **1** (**Figure 2a-d**).[20] These data suggested that normal biosynthesis of **1** requires the T_3 domain of GliP.

Gliotoxin (**1**) serves as a positive feedback loop for its own production through regulation of *gli*-cluster expression.[21] Accordingly, the inability of the GliP-$\Delta C_T T_3$ mutant strain to produce **1** resulted in almost complete loss of *gli*-cluster expression (**Figure S2**), which could have affected our results. Therefore, we repeated the experiment by growing cultures supplemented with exogenous **1**, which largely rescued *gli*-cluster expression (**Figure S3**).[21] LC-HRMS comparison of extracts from gliotoxin-supplemented cultures showed that rescue of cluster gene expression did not recover production of *gli*-pathway metabolites. As in the case without supplementation of **1**, the GliP-$\Delta C_T T_3$ mutant did not produce any **1-3** (**Figure 2a-d**), whereas the GliP-ΔT_3 strain produced small quantities of **2** (96 % less than Af293) and the tailored metabolite, **3** (96 % less than Af293) (**Figure 2a-d**). These results indicate that normal biosynthesis of **1** requires the T_3 domain of GliP, whereas in the absence of T_3, only small amounts of **1** and other *gli*-pathway metabolites are produced, possibly via cyclization of a T_2-tethered intermediate.

We further considered the possibility that the GliP-$\Delta C_T T_3$ and GliP-ΔT_3 variants may have reduced adenylation activity and do not load L-Phe and L-Ser as efficiently as the WT GliP. To address this concern, we heterologously produced and purified GliP-WT, GliP-$\Delta C_T T_3$ and GliP-ΔT_3 proteins from *E. coli* (**Figure S4**) and conducted an ATP-[^{32}P]pyrophosphate exchange assay. We found that adenylation activity of the A_1- and A_2–domains is not reduced in the truncated proteins, GliP-$\Delta C_T T_3$ and GliP-ΔT_3, relative to GliP-WT (**Figure S5**).[14,22] Notably, the heterologously expressed GliP truncations remained largely functional. Production of cyclo-Phe-Ser (**2**) was only slightly reduced with the GliP-ΔT_3 mutant and still about one third of WT with the GliP-$\Delta C_T T_3$ mutant (**Figure S6**). Residual production of **2** by these mutants likely results from spontaneous or C_T-catalyzed cyclization of the T_2-tethered L-Phe-L-Ser (**Figure 1b**), which does not appear to occur *in vivo* in the corresponding *A. fumigatus* mutants (compare **Figures 2a** and **S6**).

Taken together, these results indicate that the GliP-T_3 domain is required for DKP formation *in vivo*, since in the absence of T_3, only small amounts of **1** and other *gli*-pathway metabolites are produced, possibly via cyclization of a T_2-tethered intermediate. Therefore, we hypothesized that in full length GliP the L-Phe-L-Ser dipeptide is transferred from the T_2 domain to T_3 prior to cyclization via C_T.

To assess whether the T_3 domain is functional, we tested for appropriate post-translational decoration of the predicted active site residue, serine 2095, with a phosphopantetheinyl-(ppant-) moiety. LC-HRMS/MS analysis of tryptic digests of GliP-WT showed diagnostic ppant-fragmentation of the peptide containing S2095, confirming ppant attachment to the active site serine in T_3 (**Figure S7**).[24]

To further probe the roles of the T_3 and C_T domains for DKP formation, we constructed *A. fumigatus* strains carrying point mutations at the T_3 and C_T active sites. For this purpose, we first created a new ΔGliP strain, then reintroduced either GliP(WT), a point mutant of the T_3 active site, GliP-S2095A, or a point mutant lacking the putative catalytic histidine of the C_T domain, GliP-H1754A (see **Figure S2** and **Supporting Methods** for details). The ΔGliP+GliP(WT) strain produced a distribution of pathway metabolites (**Figure 3a**) very similar to that of WT *A. fumigatus*, proving that the reintroduced GliP is fully functional. In contrast, production of *gli* pathway metabolites was almost completely abolished in the S2095A and H1754A mutant strains (**Figure 3b,c**). To rescue potentially suppressed expression of *gli*-cluster genes in the absence of gliotoxin (**1**), we repeated the experiment with exogenously added **1**, which resulted in a production of a small amount (<2% of WT) of cyclo-Phe-Ser in the S2095A mutant while cyclo-Phe-Ser remained undetectable in the H1754A mutant (**Figure 3b,c**).

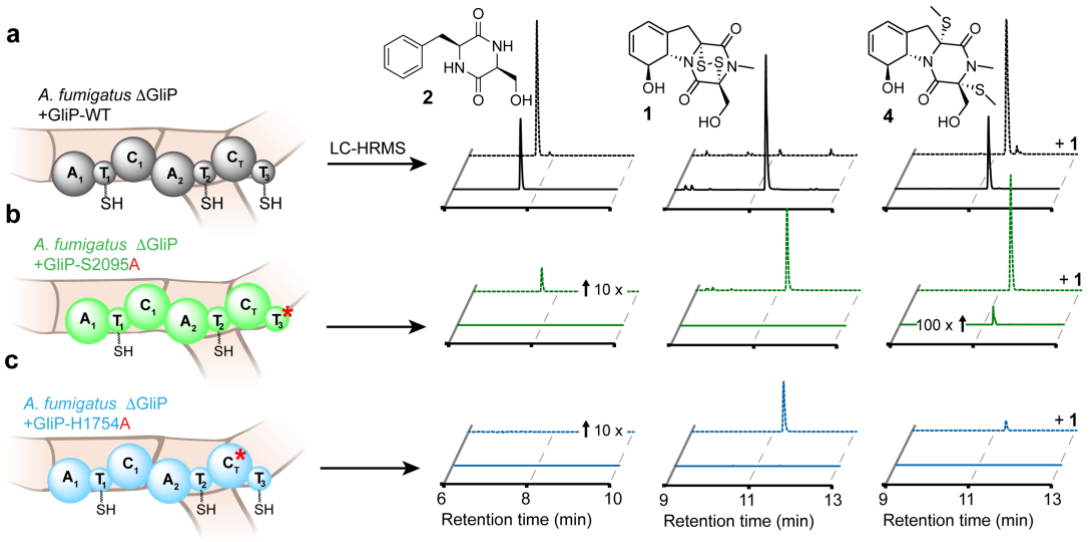

Figure 3. LC-HRMS analysis of whole-metabolome extracts from mutant *A. fumigatus* strains. Representative EICs for compounds **1, 2 and 4** in (a) *A. fumigatus* ΔGliP +GliP-WT, (b) *A. fumigatus* ΔGliP +GliP-S2095A, and (c) *A. fumigatus* ΔGliP +GliP-S2095A. Dashed chromatograms representative EICs for **1, 2, and 4** in the indicated strains with added exogenous gliotoxin, **1**. Note that the extent of conversion of gliotoxin (**1**) to its detoxification product **4** is highly variable between experiments, e.g. in the example chromatogram shown in (a) no **1** remains following addition of **1**.

To confirm the essential role of the GliP-T_3 domain, we heterologously expressed a GliP mutant protein in which the first and second T-domains were inactivated by substitution of serines 555 and 1582 for alanine (GliP-ΔT_1T_2). We then used synthetic L-Phe-L-Ser-*N*-acetylcysteamine (L-Phe-L-Ser-SNAC, **5**) to effect attachment of L-Phe-L-Ser to T_3 via transthiolation and monitored production of **2** (**Figures 4a** and **S8a**). Since thioester **5** could cyclize non-enzymatically or via catalysis by the C_T domain without first getting attached to T_3 (**Figure S8b**), we included control assays without GliP, without Sfp, or without coenzyme A (= no ppant functionalization on T_3), and using a GliP mutant protein in which all three T-domains were inactivated (GliP-$\Delta T_1T_2T_3$). Incubation of **5** under these conditions (**Figure 4a**) demonstrated that the presence of a functional T_3 domain significantly increases the cyclization activity of GliP.

Our results demonstrate that cyclization of tethered Phe-Ser dipeptide *en route* to **1** is not spontaneous, as proposed,[7-11] and rather requires two additional GliP domains, the second condensation-like domain (C_T domain) and the enigmatic terminal thiolation domain T_3 ("T_C" domain) to which the nascent dipeptide appears to be transferred prior to cyclization via C_T (**Figure 4b**). Significantly, mutation of the catalytic histidine in the C_T domain or the ppant attachment site within the T_3 domain

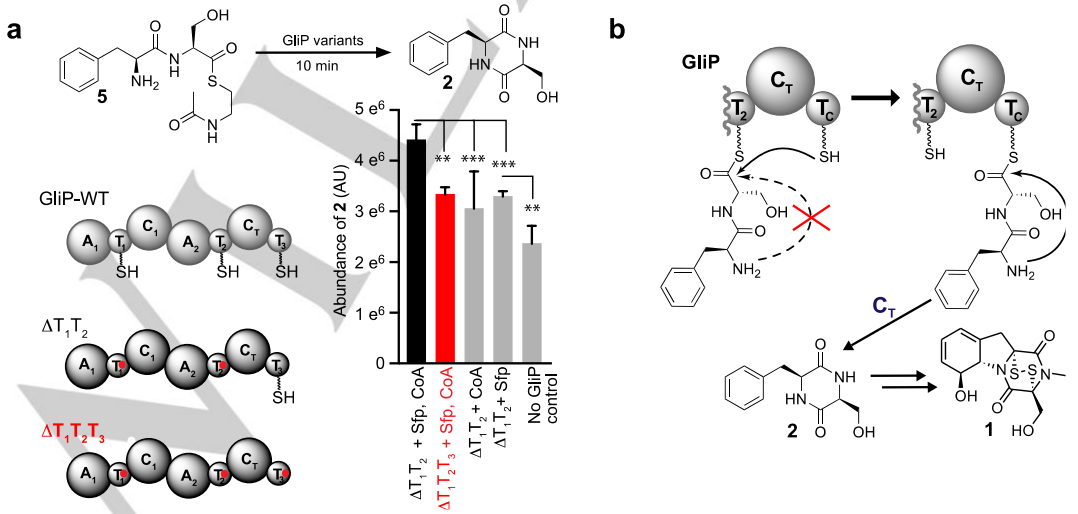

Figure 4.. (a) *In vitro* production of cyclo-Phe-Ser (**2**) using different GliP variants. Relative yields of **2** were measured via integration of the corresponding peak in LC-MS ion-chromatograms (n = 4). $^{**}p < 0.01$, $^{***}p < 0.001$. (b) Model for C_TT_C-catalyzed DKP formation in gliotoxin (**1**) biosynthesis.

is sufficient to almost completely abrogate gliotoxin biosynthesis. These observations highlight the importance of dedicated cyclization domains in fungal NRPSs.[25,26] Gliotoxin (**1**) is a representative member of a large class of NRPS-derived DKPs that seem likely to be produced via similar mechanisms. Analysis of available fungal genomes revealed 56 putative NRPSs that feature terminal domains homologous to the C_TT_3 tandem in GliP (see **Supporting Methods**, **Table S1**, and **Figure S9**), indicating a conserved biosynthetic strategy for DKP formation.[13,27]

Whereas the GliP C_T domain is homologous to the recently described C_T domains that catalyze cyclization of fungal tripeptides;[15] the requirement of an additional T_C domain for DKP formation is perhaps unexpected, given that dipeptide thioesters often cyclize non-enzymatically, as we showed in our *in vitro* studies. We note that T_C domains in DKP-producing NRPSs could serve as a tether for linear dipeptides during tailoring by other cluster enzymes. Although there is substantial evidence that, in the case of gliotoxin, many of the later steps of its biosynthesis proceed via untethered, cyclic intermediates, the structures of shunt metabolites in related DKP biosynthesis pathways may suggest tailoring of tethered dipeptides. For example, in the case of hexadehydroastechrome, which is derived from cyclo-Trp-Ala derivatives, abundant production of prenylated tryptophan in mutants defective in late-stage tailoring enzymes could be due to recycling of a tethered prenylated Trp-Ala dipeptide (**Figure S10**).[28]

In conclusion, our study suggests a general framework for fungal DKP biosynthesis, wherein the T_C domain serves as a tether for dipeptide cyclization by an adjacent C_T domain and potentially for tailoring by other cluster enzymes. Furthermore, our characterization of the biosynthetic roles of the C_T and T_C domains in DKP formation extends the functional repertoire of NRPS domains.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: gliotoxin • diketopiperazine • biosynthesis • cyclization • NRPS

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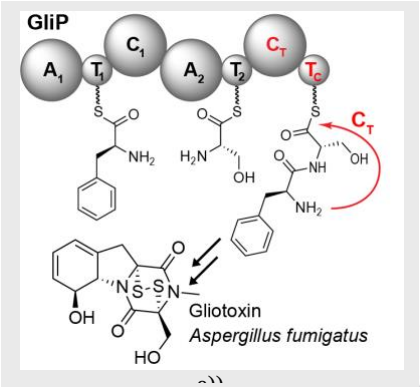
Seemingly extraneous: Diketopiperazines derived from non-ribosomal peptide synthetases, an important family of fungal virulence factors, do not form spontaneously, as presumed; instead cyclization relies on previously unannotated domains of the synthetase.



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