N-Heterocyclization in Gliotoxin Biosynthesis is Catalyzed by a Distinct Cytochrome P450 Monooxygenase

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Supporting Information
1. Experimental Section

General analytical procedures

Analytical HPLC was carried out on a Shimadzu LC-10Avp series HPLC system with a DAD (Nucleodur C18, 5 µm, 250 × 4 mm, flow rate 1 mL min⁻¹, gradient ACN/0.1% TFA-H₂O 0.5/95.5 1 min, then in 30 min to 98% ACN).

HRESI-MS was conducted on a Thermo Accela (LC) and Thermo Exactive (HRMS), an ESI source operating in the negative mode, and an orbitrap analyzer. 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.

NMR spectra were recorded on a Bruker Avance III 600 instrument in DMSO-d₆. Spectra were referenced to the residual solvent signals.

For open column chromatography, Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden) and Silica gel (Silica 60 M 0.04-0.063 mm from Macherey Nagel) were used.

Fungal strains, media and growth conditions

A. fumigatus strain ΔakuB[1] was used as wild-type strain and for generation of mutant strains. Strains were grown in Aspergillus minimal medium (AMM) as described previously.[2] AMM agar was prepared by addition of 1.6% (w/v) Select Agar (Invitrogen, Germany). For formation of conidia A. fumigatus wild-type and mutant strains were grown on AMM agar plates at 37 °C for five 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).

Isolation and manipulation of nucleic acids

Standard techniques for manipulation of DNA were carried out using standard procedures. Chromosomal DNA of A. fumigatus was prepared using the Master Pure Yeast DNA Purification Kit (Epicentre Biotechnologies). For Southern blot analysis, DNA fragments were separated on an agarose gel and blotted onto Hybond N+ nylon membranes (GE Healthcare Bio-Sciences). Labeling of DNA probes, hybridization and detection of DNA–DNA hybrids were performed as described previously.

Generation of A. fumigatus ΔgliF mutant strain

Partial deletion of gliF was done by using a PCR-based strategy. Upstream and downstream flanking regions of gene gliF (Afu6g09730) were amplified by PCR using primer pairs gliF5-for and gliF-ptrA-rev and gliF3-rev and gliF-ptrA-for, respectively. 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 gliF gene. The ptrA resistance cassette was amplified from plasmid pSK275 (kind gift from Sven Krappmann) 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 gliF5-for and gliF3-rev. All PCR reactions were performed with Phusion High-Fidelity DNA Polymerase (Finnzymes) according to the manufacturer's recommendations. The resulting 3.6 kb PCR product was purified and used for transformation of A. fumigatus wild type protoplasts as described previously. Pyrithiamine (1 mg mL⁻¹, Sigma-Aldrich) resistant transformants were analyzed for partial deletion of gliF by Southern blot analysis. One positive transformant, designated ΔgliF, was chosen for further analysis.

**Extraction and isolation of compound 7 from ΔgliF mutant.**

The ΔgliF mutant was cultivated in a 20 L fermenter for 6 days. The supernatant was extracted with ethyl acetate. The ethyl acetate extracts of the ΔgliF mutant was separated by open-column chromatography on silica gel using CHCl₃/MeOH mixtures of increasing polarity as eluents. The metabolite-containing fractions were further purified by size exclusion chromatography on Sephadex LH-20 (eluent MeOH) and preparative HPLC. Preparative HPLC was performed on a HPLC system consisting of a Shimadzu 20A prominence DAD and Shimadzu 8Avp pumps. YMC pack Pro C18 250 × 20 mm, flow rate 10 mL min⁻¹, gradient ACN/0.01% TFA 5:95 for 1 min, leading to 100% ACN in 35 min then keep 100% ACN for 1 min, decreasing to ACN/0.01% TFA 5:95 in 1 min. HRMS (ESI) calcd for C₁₂H₁₁N₂O₃S₂ (M–H): 295.0217, found: 295.0218

![Structure of compound 7](image)

**Figure S1.** Structure of compound 7 and key HMBC (-----) and COSY correlations (---).
Table S1. Physicochemical data of compound 7

| Compound 7 | | | |
|---|---|---|---|
| Position | H δ (ppm) J/ Hz | C δ (ppm) | HMBC |
| 1-NH | 9.55(s, 1H) | - | 2,3,5,6,7 |
| 2 | - | 165.2 | - |
| 3 | - | 72.5 | - |
| 4-NH | 9.70(s, 1H) | - | 2,3,5,6,8 |
| 5 | - | 165.4 | - |
| 6 | - | 73.3 | - |
| 6-OH | - | - | - |
| 7 | 4.01(12.12, d, 1H) 3.96(12.13, d, 1H) | 59.8 | 2, 3 |
| 7-OH | 5.54(s, 1H) | - | - |
| 8 | 3.57(14.14, d, 1H) 3.46(14.10, d, 1H) | 36.8 | 6, 8, 9, 10, 14, 5 |
| 9 | - | 134.5 | - |
| 10 | 7.51(7.57, d, 1H) | 130.9 | 8, 12, 14 |
| 11 | 7.29a(1H) | 127.9 | 9, 10, 13 |
| 12 | 7.24a(1H) | 127.2 | 10, 11, 13, 14 |
| 13 | 7.29a(1H) | 127.9 | 9, 10, 11 |
| 14 | 7.51(7.57, d, 1H) | 130.9 | 8, 10, 12 |

*a* adjacent signal overlapping

Construction of yeast expression plasmids for GliF.

For cloning of gliF in the yeast expression plasmid pYES2 (Life Technologies) the gene was amplified by PCR from plasmid DNA using the primer pairs pYES2gliF forward and pYES2gliF reverse. The PCR product was cut with HindIII/NotI and cloned into the HindIII/NotI of pYES2 to create pYES2GliF. The resulting plasmid was propagated in E. coli DH5α cells.
Preparation of S. cerevisiae microsomal fraction of GliF.

Preparation of microsomal fraction was done according to Watanabe et al. Preparation of microsomal fraction was done according to Watanabe et al. S. cerevisiae BY4741 (ura–) was transformed with pYES2GliF. Selected cells were grown in 2 mL of SC medium prepared using YNB with ammonium sulfate (Sigma-Aldrich) supplemented with amino acid solution without uracil at 30 °C for 48 h with 180 rpm. The culture was transferred into 100 mL of fresh SC medium without uracil and the culture was incubated at 30 °C for 48 h. YPD medium (500 mL) was inoculated with the culture (100 mL) and incubated at 30 °C for 15 h. Then, expression of each gene was induced with 2% of D-galactose at 30 °C, and incubation was continued for another 24 h. The cells were harvested by centrifugation at 2,500 × g. All subsequent procedures were performed at 4 °C or on ice. Harvested cells were resuspended in 200 mL of TEK buffer (0.1 M KCl, 50 mM Tris-HCl pH 7.5 and 1 mM EDTA). Cells were incubated at 4 °C or on ice and collected by centrifugation at 2,500 × g. Cells resuspended in 200 mL of TES buffer (0.6 M sorbitol, 50 mM Tris-HCl pH 7.5, 1 mM PMSF and 1 mM EDTA) were disrupted with high pressure homogenizer (Avestin), and the lysate was clarified by centrifugation at 10000 g. Then, the supernatant was fractionated by ultracentrifugation at 100,000 × g for 1 h. The pellet was resuspended in 2 mL of TEG buffer (20 % (v/v) glycerol, 50 mM Tris-HCl pH 7.5 and 1 mM EDTA) to yield a microsomal fraction.

Figure S2. Phylogenetic tree of cytochrome P450 isoform sequences including GliF and GliC and their homologues deduced from ETP biosynthesis gene clusters. Sequences were obtained from gene bank database based on previous studies. Proteins were aligned with ClustalW. Phylogenetic analysis was performed using maximum likelihood methods and bootstrap values were calculated using MEGA6. Clades were marked according to Figure 2.
**In vitro assays with GliF.**

The assay mixture (250 µL) containing 75 µL of microsomal fraction containing GliF, 1 mM of 7, 2 mM NADPH and 20 mM Tris-HCl pH 7.5, 150 mM NaCl was incubated at RT for 3 h. Heat-inactivated GliF was used in reactions as a negative control. The enzyme reaction was stopped by adding 500 µL of ethyl acetate. The organic phase was recovered and concentrated to dryness under a stream of nitrogen gas. The residue was dissolved in 200 µL methanol and analyzed by HPLC-HRMS (Exactive).

**Figure S3.** Mass spectra of 10, 11 and 12

**Table S2.** Oligonucleotides used in this study

| Oligonucleotide | Sequence 5’-3’ |
|-----------------|----------------|
| pYES2gliF forward | A CGT AAG CTT CAC ACA ATG GAC CAA GTC TAC CTC CCC CAG |
| pYES2gliF reverse | A CGT GC GGC CGC TTA TAC TCG TGG CTT CAA CTC CAC CTC |
| ptrA-for | GAA TTC GAT GGC CAC TCA GGC C |
| ptrA-rev | GCT TGA TGG CCT AGA TGG CCT C |
| GliF_5’_for | CAT TCG GAT GGA CCG AGT AG |
| GliF_3’_rev | GCT ACG ATG TCA GAA AGG C |
| GliF_ptrA_rev | GCC CTG AGT GCC CAT CGA ATT CGA TCC AGT GCG GGT AGA AG |
| GliF_ptrA_for | GAG GCC ATC TAG GCC ATC AAG CCT GCT CGC CCA TCA AGA AG |
2. NMR Spectra

**Figure S5.** $^1$H NMR spectrum of 7

**Figure S6.** $^{13}$C NMR spectrum of 7
Figure S7. DEPT135 spectrum of 7

Figure S8. H, H-COSY spectrum of 7
Figure S9. HSQC spectrum of 7

Figure S10. HMBC spectrum of 7
Figure S11 Model of radical-mediated enzymatic heterocyclization.

3. Supporting References

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