Published in final edited form as:

Bioorg Med Chem Lett. 2013 April 1; 23(7): 2155–2157. doi:10.1016/j.bmcl.2013.01.099.

Reconstitution of the early steps of gliotoxin biosynthesis in *Aspergillus nidulans* reveals the role of the monooxygenase GliC

Shu-Lin Chang^{a,d}, Yi-Ming Chiang^{b,d}, Hsu-Hua Yeh^{c,d}, Tung-Kung Wu^c, and Clay C. C. Wang^{d,e,*}

^a Department of Biotechnology, Chia Nan University of Pharmacy and Science, 60, Sec. 1, Erren Rd., Rende Dist., Tainan City 71710, Taiwan

^b Graduate Institute of Pharmaceutical Science, Chia Nan University of Pharmacy and Science, 60, Sec. 1, Erren Rd., Rende Dist., Tainan City 71710, Taiwan

^c Department of Biological Science and Technology, National Chiao Tung University, 1001, Daxue Rd., East Dist., Hsinchu City 30010, Taiwan

^d Department of Pharmacology and Pharmaceutical Sciences, University of Southern California, School of Pharmacy, 1985 Zonal Avenue, Los Angeles, California 90089, USA

^e Department of Chemistry, University of Southern California, Dornsife College of Letters, Arts, and Sciences, 1985 Zonal Avenue, Los Angeles, California 90089, USA

Abstract

The gliotoxin, a member of the epipolythiodioxopiperazine (ETP), has received considerable attention from the scientific community for its wide range of biological activity. Despite the identification of gliotoxin cluster, however, the sequence of steps in the gliotoxin biosynthesis has remained elusive. As an alternative to the gene knock out and biochemical approaches used so far, here we report using a heterologous expression approach to determine the sequence of the early steps of gliotoxin biosynthesis in *A. nidulans*. We identified the GliC, a monooxygenases that involved in the second step of gliotoxin biosynthesis pathway through the catalyzing the hydroxylation at the α -position of L-Phe.

Keywords

Biosynthesis; Gliotoxin; Heterologous expression

Gliotoxin belongs to a class of fungal secondary metabolites produced by nonribosomal peptide synthetase (NRPS) called epipolythiodioxopiperazine (ETP). ETPs are toxins that

© 2013 Elsevier Ltd. All rights reserved.

*Corresponding author. Tel.: + 323-442-1670; fax: 323-442-1390; clayw@usc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Supplementary Material

The primers and *A. nidulans* strains used in this study, nucleotide alignment analysis, experimental details, and characterization of compounds **4** (¹H NMR, ¹³C NMR and HRMS) are provided in the Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

are characterized by the presence of the transannular disulphide bridge which is the source of their biological activity. Extensive studies have revealed that this unique structure motif primarily imparts various properties of gliotoxin such as antiviral,^{1, 2} antibacterial³ and immunosuppressive^{4, 5} activities. The gliotoxin biosynthesis gene cluster has been identified in the human pathogen *Aspergillus fumigatus*, however, the exact sequence of steps in gliotoxin biosynthesis has remained unclear.⁶ Various pathways for gliotoxin biosynthesis have been proposed by different groups using either knock-out approaches in *A. fumigatus* targeting *gliP*,⁷ *gliT*,⁸ *gliZ*,⁹ *gliG*,^{10, 11} *gliH*,¹² *gliK*¹³ or *gliI*¹⁴ genes and by biochemical approaches. However to date, the role of other genes in gliotoxin biosynthesis remains unclear. As an alternative to the gene knock out and biochemical approaches used so far, we used a bottom up heterologous expression approach using *A. nidulans* an organism not known to produce gliotoxin as host to reconstitute the early steps of gliotoxin biosynthesis.

Works from the Walsh group have definitively demonstrated that the first step in gliotoxin biosynthesis is the formation of the cyclo-L-phenylalanyl-L-seryl by the NRPS, GliP.¹⁵ As the first step in our approach, we carried out the heterologous expression of the *A. fumigatus* *gliP* in *A. nidulans* under the control of the *alcA* promoter that can be induced strongly using cyclopentanone.¹⁶ We amplified the *gliP* gene and *pyroA* selective marker by PCR from *A. fumigatus* strain AF293. The *alcA* promoter and the two regions flanking the *yA* gene were amplified from the genomic DNA of *A. nidulans* strain LO2026.¹⁷ LO2026 is an *A. nidulans* strain where the FAS gene *stcJ* in the sterigmatocystin pathway has been deleted, thus will not produce major metabolite sterigmatocystin. This recipient strain also contains an *nkuA* mutation that greatly increases homologous recombination rate. Due to the large size of the *gliP* gene we split the gene into two incomplete fragments with 1 kb overlapping region. The first *gliP* fragment was fused to the upstream *yA*-flanking sequence-linked with the *alcA* promoter and the second *gliP* fragment was fused to the downstream *yA*-flanking sequence-linked with the *pyroA* marker. These constructs were generated by fusion PCR (Table S1) and characterized by DNA sequence analysis which is then subsequently used for *A. nidulans* transformation. The transformants with the correct insertion were further verified with diagnostic PCR (Fig. S1). In each case at least two transformants carrying the correct insertion were used for further study. We cultivated the *alcA-gliP A. nidulans* strain (CW2129, Table S2) in LMM medium (15 g/l lactose, 6 g/l NaNO₃, 0.52 g/l KCl, 0.52 g/l MgSO₄·7H₂O, 1.52 g/l KH₂PO₄, 1 ml/l trace element) supplemented with uracil (1 mg/ml) and uridine (10 mM) for 18 hours and then induced with cyclopentanone (10 mM) for another 48 hours. After 48 hours of incubation the medium was extracted with ethyl acetate followed by evaporating in *vacuo*. The residue was resuspended in 0.5 ml of 20% DMSO/MeOH and a portion (10 µl) was examined by HPLC-DAD-MS. We detected two UV active peaks with masses of 235 and 274 (*m/z*[M+H]⁺) (Fig. 1).

Characterization of the purified molecules from scaled-up 1 liter culture by NMR revealed that compound **1** is cyclo-(L-phenylalanyl-L-seryl) and compound **2** is cyclo-(L-tryptophanyl-L-seryl) (Fig. 1). The diketopiperazine **1** has been identified from a biochemical study using purified recombinant GliP and is an expected product from the pathway.¹⁵ The production of the diketopiperazine **2**¹⁸ however was unexpected and suggests that the substrate specificity of the first adenylation (A) domain in GliP is sufficiently relaxed to accommodate both L-Phe and L-Trp.

After the first step of gliotoxin biosynthesis by GliP, the subsequent steps are unclear. Intermediates isolated from a *gliG*, glutathione synthase knock out mutant and *gliZ* transcription factor knock out mutant of *A. fumigatus* revealed a series of intermediates oxidized at the α carbon of L-Phe suggesting that the next step in gliotoxin involves one of the two cytochrome P450 monooxygenases, GliC or GliF.^{9, 10} To identify which of the

monooxygenase is responsible for the oxidation at the α -carbon of L-Phe, we created an *alcA-gliP/alcA-gliC* and an *alcA-gliP/alcA-gliP/alcA-gliF* mutant strains using the same strategy we used to create *gliP*. We cultured both *alcA-gliP/alcA-gliC* (CW2142) and *alcA-gliP/alcA-gliF* (CW2221) *A. nidulans* mutant strains separately and examined the extracts from the two strains by HPLC-DAD-MS. Only in the *alcA-gliP/alcA-gliC* (CW2142) strain did we observe the disappearance of compound **1** and the emergence of a new peak (compound **3**) with a mass of 247 ($m/z[M-H]^-$) (Fig. 1). Compound **2** can still be detected in the *alcA-gliP/alcA-gliC* (CW2142) strain, suggesting that only cyclo-(L-phenylalanyl-L-seryl) is a substrate for GliC (Fig. 1). The *alcA-gliP/alcA-gliF* (CW2221) strain has similar metabolite profile as the single *alcA-gliP* strain (Fig. 1) suggesting that *gliF* is involved in the latter parts of the gliotoxin biosynthesis pathway if at all.

To characterize the new metabolite **3**, we scaled up the culture of the *alcA-gliP/alcA-gliC* (CW2142) strain and isolated the metabolite by column chromatography followed by preparative HPLC. Spectroscopic analysis of compound **3** (Table 1) revealed that compound **3** isolated from this study is structurally quite similar to the shunt product **4** isolated by Davis *et al* and Scharf *et al* from a *gliG* knockout mutant.^{10, 11} The two molecules differ only in the functional group attached to N1. In our molecule it is a hydroxy group while in the Davis *et al* molecule a methoxy group is attached to N1. The enzyme responsible for catalyzing the methoxy group is currently unknown although the gliotoxin cluster contains two methyltransferases GliM and GliN which are possible candidates. In our experiments, neither GliM nor GliN have been reconstituted in *A. nidulans* and could explain the lack of methylation in the substrate we isolated.

From the structure of compound **3**, we propose that the second step in the gliotoxin biosynthesis pathway is the hydroxylation at the α -position of L-Phe and this is catalyzed by GliC (Scheme 1). Our data is consistent with the work by Forseth *et al*. Where oxidation at the α -position is proposed to occur at an early state in the biosynthesis.⁹ The structure of compound **3** suggests that the molecule we isolated is probably a stable shunt metabolite that is formed because we have not reconstituted additional genes in the pathway.

From our approach, it is not clear the exact nature of the substrate of GliC. It is possible that the free standing diketopiperazine **1** is an intermediate although we can not exclude the other possibility that GliC recognizes the substrate still tethered to GliP. Additional studies will be necessary to clarify the two competing mechanisms.

In summary, using a heterologous expression technique we demonstrated that one of the two cytochrome P450 monooxygenase, GliC, is responsible for the oxidation of the α -carbon of L-Phe, and is the subsequent step after the condensation of L-Phe and L-Ser by GliP in gliotoxin biosynthesis. Further investigation of other components within the gliotoxin cluster will not only provide for intriguing mechanistic studies but also lay a foundation for the utilization of the gliotoxin pathway to generate new ETP molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The project described was supported by Grant Number PO1-GM084077 from the National Institute of General Medical Science to C.C.C.W.

References

1. Rightsel WA, Schneider HG, Sloan BJ, Graf PR, Miller FA, Bartz OR, Ehrlich J, Dixon GJ. *Nature*. 1964; 204:1333. [PubMed: 14254440]
2. Rodriguez PL, Carrasco L. *J. Virol.* 1992; 66:1971. [PubMed: 1372367]
3. Waring P, Beaver J. *Gen. Pharmacol.* 1996; 27:1311. [PubMed: 9304400]
4. Mullbacher A, Eichner RD. *Proc. Natl. Acad. Sci. U. S. A.* 1984; 81:3835. [PubMed: 6203127]
5. Mullbacher A, Waring P, Tiwari-Palni U, Eichner RD. *Mol. Immunol.* 1986; 23:231. [PubMed: 2422547]
6. Gardiner DM, Howlett BJ. *FEMS Microbiol. Lett.* 2005; 248:241. [PubMed: 15979823]
7. Cramer RA Jr, Gamcsik MP, Brooking RM, Najvar LK, Kirkpatrick WR, Patterson TF, Balibar CJ, Graybill JR, Perfect JR, Abraham SN, Steinbach WJ. *Eukaryot. Cell.* 2006; 5:972. [PubMed: 16757745]
8. Scharf DH, Remme N, Heinekamp T, Hortschansky P, Brakhage AA, Hertweck C. *J. Am. Chem. Soc.* 2010; 132:10136. [PubMed: 20593880]
9. Forseth RR, Fox EM, Chung D, Howlett BJ, Keller NP, Schroeder FC. *J. Am. Chem. Soc.* 2011; 133:9678. [PubMed: 21612254]
10. Davis C, Carberry S, Schrettl M, Singh I, Stephens JC, Barry SM, Kavanagh K, Challis GL, Brougham D, Doyle S. *Chem. Biol.* 2011; 18:542. [PubMed: 21513890]
11. Scharf DH, Remme N, Habel A, Chankhamjon P, Scherlach K, Heinekamp T, Hortschansky P, Brakhage AA, Hertweck C. *J. Am. Chem. Soc.* 2011; 133:12322. [PubMed: 21749092]
12. Schrettl M, Carberry S, Kavanagh K, Haas H, Jones GW, O'Brien J, Nolan A, Stephens J, Fenelon O, Doyle S. *PLoS Pathog.* 2010; 6:e1000952. [PubMed: 20548963]
13. Gallagher L, Owens RA, Dolan SK, O'Keefe G, Schrettl M, Kavanagh K, Jones GW, Doyle S. *Eukaryot. Cell.* 2012; 11:1226. [PubMed: 22903976]
14. Scharf DH, Chankhamjon P, Scherlach K, Heinekamp T, Roth M, Brakhage AA, Hertweck C. *Angew. Chem. Int. Ed. Engl.* 2012; 51:10064. [PubMed: 22936680]
15. Balibar CJ, Walsh CT. *Biochemistry.* 2006; 45:15029. [PubMed: 17154540]
16. Waring RB, May GS, Morris NR. *Gene.* 1989; 79:119. [PubMed: 2673931]
17. Chiang YM, Szewczyk E, Davidson AD, Keller N, Oakley BR, Wang CC. *J. Am. Chem. Soc.* 2009; 131:2965. [PubMed: 19199437]
18. Tullberg M, Luthman K, Grotli M. *J. Comb. Chem.* 2006; 8:915. [PubMed: 17096581]

Figure 1.

(A) HPLC-DAD-MS analysis of organic extracts from: recipient (*stcJ*Δ); GliP-expressing (*stcJ*Δ, *alcA-gliP*); GliP- and GliC-expressing (*stcJ*Δ, *alcA-gliP*, *alcA-gliC*); and GliP- and GliF-expressing (*stcJ*Δ, *alcA-gliP*, *alcA-gliF*) *A. nidulans* strains. (B) Structures of compound 1–4.

Scheme 1.

Proposed biosynthetic pathway for gliotoxin. Following the condensation of L-Phe and L-Ser by GliP, the oxidation of the α carbon of L-Phe was catalyzed by GliC. A series of subsequent reactions including a GliT-mediated tailoring reaction result in gliotoxin formation. Compound 3 was isolated as a stable shunt metabolite following co-expression of GliP and GliC. ^aCompound 5 is a hypothetical product which has not been detected in HPLC-DAD-MS.

NMR data comparison between compound **4** published from Davis *et al* and scharf *et al*. (600 and 150 MHz)^a and compound **3** isolated from this study (400 and 100 MHz)^b.

Table 1

4 (CDCl ₃)			3 (CD ₃ OD)		
position	δ _C	δ _H	δ _C	δ _H	
2	157.3	—	160.2	—	
3	132.2	—	N.O. ^c	—	
5	161.3	—	162.4	—	
6	82.4	—	84.0	—	
7	102.6	5.11, 5.57 (each 1H, s)	101.9	5.26, 5.34 (each 1H, brs)	
8	47.6	3.18, 3.33 (each 1H, d, J = 13.5 Hz)	47.1	2.94, 3.52 (each 1H, br d, J = 13.5 Hz)	
9	132.6	—	135.7	—	
10 and 14	130.7	7.19–7.20 (2H, m)	131.8	7.19 (5H, br s)	
11 and 13	128.8	7.24–7.27 (3H, m)	129.5		
12	128.0		128.3		
OCH ₃	62.0	3.71 (3H, s)	—	—	

^a published data obtained from Davis *et al* and Scharf *et al*.^{10,15}

^b Data obtained from this study.

^c Not observed.