Bioorganic & Medicinal Chemistry Letters 23 (2013) 2155–2157

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

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

ARTICLE INFO

Article history:
Received 19 December 2012
Revised 16 January 2013
Accepted 22 January 2013
Available online 4 February 2013

Keywords:
Biosynthesis
Gliotoxin
Heterologous expression

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 *Aspergillus 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.

© 2013 Elsevier Ltd. All rights reserved.

Gliotoxin belongs to a class of fungal secondary metabolites produced by nonribosomal peptide synthetase (NRPS) called epipolythiodioxopiperazine (ETP). ETPs are toxins that 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] antibacterial[3] and immunosuppresssive[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 $NaNO_3$, 0.52 g/l KCl, 0.52 g/l $MgSO_4{\cdot}7H_2O$, 1.52 g/l KH_2PO_4, 1 ml/l trace element) supplemented with uracil (1 mg/ml) and uridine (10 mM) for 18 h and then induced with cyclopentanone (10 mM) for another 48 h. After 48 h of incubation the medium was extracted with ethyl

* Corresponding author. Tel.: +1 323 442 1670; fax: +1 323 442 1390.
E-mail address: clayw@usc.edu (C.C.C. Wang).
† These authors contributed equally to this work.

0960-894X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmcl.2013.01.099

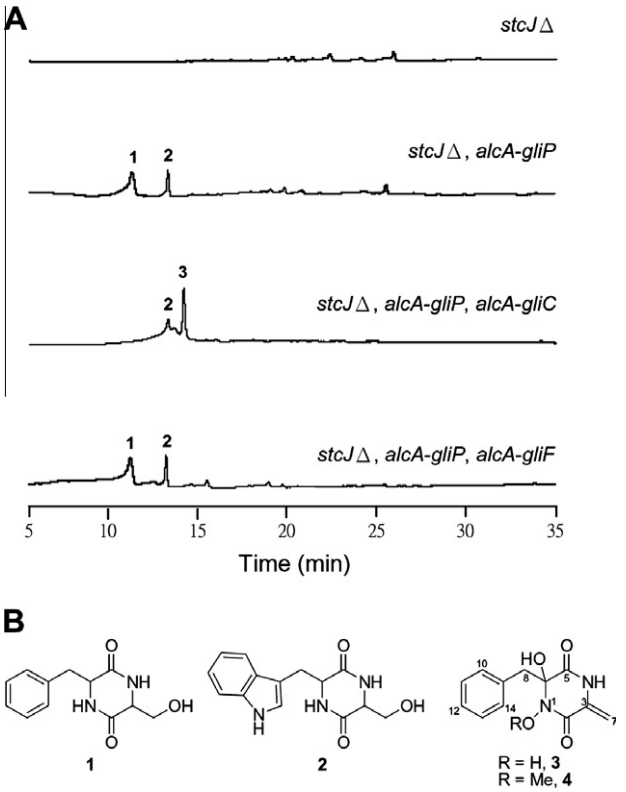


Figure 1. (A) HPLC–DAD-MS analysis of organic extracts from: recipient (*stcJ*Δ); GliP-expressing (*stcJ*Δ, *alcA-gliP*); GliP- and GliC-expressing (*stcJ*Δ, *alcA-gliP*, *alcA-gliC*); and GliP- and GliF-expressing (*stcJ*Δ, *alcA-gliP*, *alcA-gliF*) *A. nidulans* strains. (B) Structures of compound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

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).

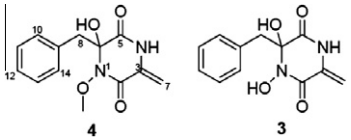
Characterization of the purified molecules from scaled-up 1 l culture by NMR revealed that compound **1** is cyclo-(L-phenylalanyl-L-seryl) and compound **2** is cyclo-(L-tryptophanyl-L-seryl) (Fig. 1). The diketopiperazine **1** has been identified from a biochemical study using purified recombinant 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-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* knock-out 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.

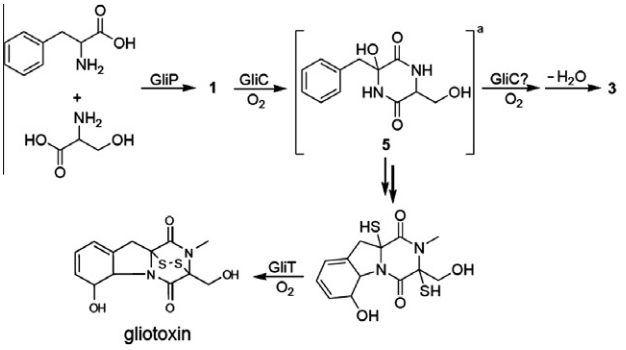
From the structure of compound **3,** we propose that the second step in the gliotoxin biosynthesis pathway is the hydroxylation at

Table 1
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]



	4 ($CDCl_3$)		**3** (CD_3OD)	
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, br s)
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 (2H, br s)
11 and 13	128.8	7.24–7.27 (2H, m)	129.5	7.19 (2H, br s)
12	128.0	7.24–7.27 (1H, m)	128.3	7.19 (1H, br s)
OCH_3	62.0	3.71 (3H, s)	—	—

[a] Published data obtained from Davis et al. and Scharf et al.[10,11]
[b] Data obtained from this study.
[c] Not observed.



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. [a]Compound **5** is a hypothetic product which has not been detected in HPLC–DAD-MS.

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.

Acknowledgement

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

Supplementary data

Supplementary data (the primers and *A. nidulans* strains used in this study, nucleotide alignment analysis, experimental details, and characterization of compound **3** (^{1}H NMR, ^{13}C NMR and HRMS) are provided) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.01.099.

References and notes

1. Rightsel, W. A.; Schneider, H. G.; Sloan, B. J.; Graf, P. R.; Miller, F. A.; Bartz, O. R.; Ehrlich, J.; Dixon, G. J. *Nature* **1964**, *204*, 1333.
2. Rodriguez, P. L.; Carrasco, L. *J. Virol.* **1971**, *1992*, 66.
3. Waring, P.; Beaver, J. *Gen. Pharmacol.* **1996**, *27*, 1311.
4. Mullbacher, A.; Eichner, R. D. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3835.
5. Mullbacher, A.; Waring, P.; Tiwari-Palni, U.; Eichner, R. D. *Mol. Immunol.* **1986**, *23*, 231.
6. Gardiner, D. M.; Howlett, B. J. *FEMS Microbiol. Lett.* **2005**, *248*, 241.
7. 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.
8. Scharf, D. H.; Remme, N.; Heinekamp, T.; Hortschansky, P.; Brakhage, A. A.; Hertweck, C. *J. Am. Chem. Soc.* **2010**, *132*, 10136.
9. Forseth, R. R.; Fox, E. M.; Chung, D.; Howlett, B. J.; Keller, N. P.; Schroeder, F. C. *J. Am. Chem. Soc.* **2011**, *133*, 9678.
10. 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.
11. 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.
12. Schrettl, M.; Carberry, S.; Kavanagh, K.; Haas, H.; Jones, G. W.; O'Brien, J.; Nolan, A.; Stephens, J.; Fenelon, O.; Doyle, S. *PLoS Pathog.* **2010**, *6*, e1000952.
13. Gallagher, L.; Owens, R. A.; Dolan, S. K.; O'Keeffe, G.; Schrettl, M.; Kavanagh, K.; Jones, G. W.; Doyle, S. *Eukaryot. Cell* **2012**, *11*, 1226.
14. Scharf, D. H.; Chankhamjon, P.; Scherlach, K.; Heinekamp, T.; Roth, M.; Brakhage, A. A.; Hertweck, C. *Angew. Chem., Int. Ed.* **2012**, *51*, 10064.
15. Balibar, C. J.; Walsh, C. T. *Biochemistry* **2006**, *45*, 15029.
16. Waring, R. B.; May, G. S.; Morris, N. R. *Gene* **1989**, *79*, 119.
17. Chiang, Y. M.; Szewczyk, E.; Davidson, A. D.; Keller, N.; Oakley, B. R.; Wang, C. C. *J. Am. Chem. Soc.* **2009**, *131*, 2965.
18. Tullberg, M.; Luthman, K.; Grotli, M. *J. Comb. Chem.* **2006**, *8*, 915.