

Biosynthesis of epipolythiodioxopiperazine toxins in fungi

Collaborations between fungal molecular biologists and chemists are the key to understanding the complex biosynthetic pathways of epipolythiodioxopiperazines, a class of secondary metabolite toxins. Barbara Howlett explains.

Filamentous fungi produce a range of natural products. Nowadays, with the rapidly increasing number of sequenced fungal genomes and advanced molecular tools, it is relatively easy to identify the biosynthetic genes for a toxin, but often much harder to deduce roles of individual genes. Such is the case for the epipolythiodioxopiperazines (ETPs), a class of secondary metabolite toxins that includes molecules contributing to virulence in plants and animals.

Structure and distribution of epipolythiodioxopiperazines

Toxicity of ETPs is due to the presence of a disulfide bridge across the diketopiperazine ring (Fig. 1A), which can inactivate proteins via reaction with thiol groups, or by generation of reactive oxygen species in redox cycling. At least 14 different ETPs are known; the best characterised one, gliotoxin (Fig. 1B), is made by several fungi including *Aspergillus fumigatus*, which infects immunocompromised humans. Gliotoxin is immunosuppressive and contributes to virulence associated with aspergillosis, a fatal disease. Another ETP, sporidesmin, is implicated in facial eczema, a disease that causes losses of more than \$100 million annually to the New Zealand sheep industry.

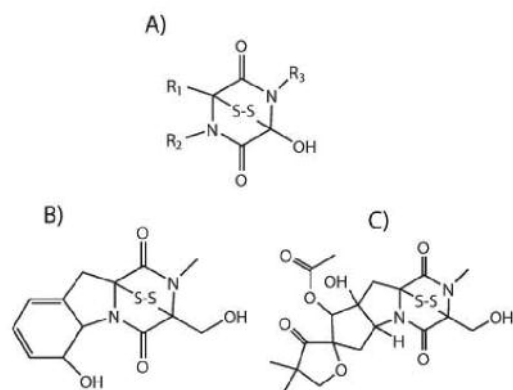


Figure 1. (A) Generic structure of an epipolythiodioxopiperazine: the most common form of ETPs. R is any atom or group; (B) gliotoxin; (C) sirodesmin PL.

Sporidesmin is produced by a fungus that grows on dead litter at the base of pasture grasses. Sirodesmin PL (Fig. 1C) produced by the blackleg fungus, *Leptosphaeria maculans*, contributes to virulence in canola. Sirodesmin and gliotoxin are the focus of this article, which is based on two recent reviews.^{1,2}

Biosynthesis of epipolythiodioxopiperazines

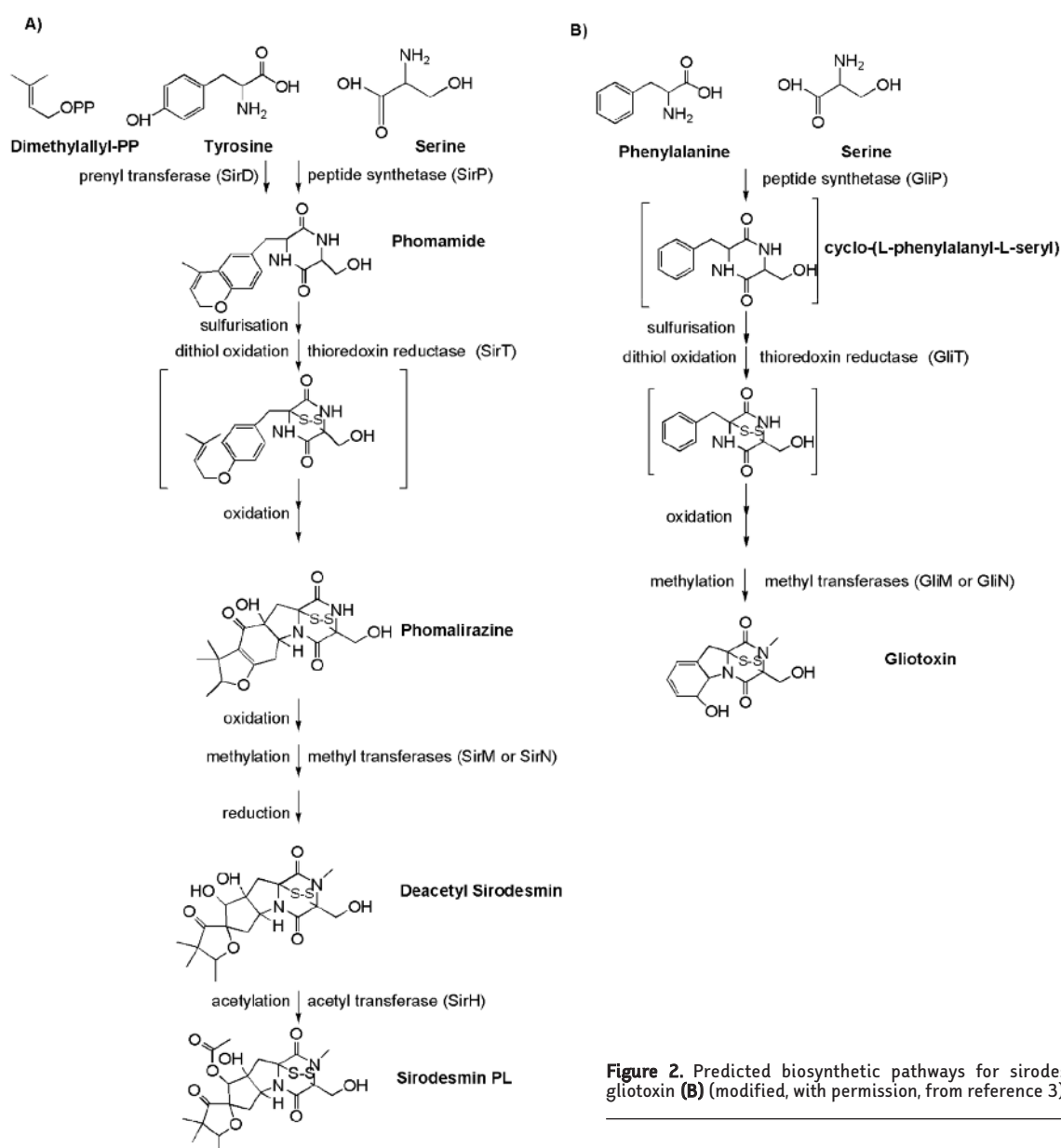
In spite of important biological effects of ETPs, little is known about their biosynthesis. Usually, the most direct way to determine a biosynthetic pathway is to feed an organism putative intermediates or precursors that have been radio-labelled.

Epipolythiodioxopiperazines are derived from amino acids, and labelling experiments have implicated cyclic dipeptides as intermediates. For sirodesmin PL biosynthesis, three intermediates have been identified: phomamide, a prenylated cyclic dipeptide; and two ETPs, phomarilazine and deacetyl sirodesmin (Fig. 2A). Hence the diketopiperazine ring is formed at an early stage in biosynthesis. Introduction of the sulfur atoms is poorly understood. Labelling experiments have shown that methionine, cysteine and sodium sulfate can all act as sources of sulfur, although cysteine is thought to be the direct donor. Figure 2 summarises the predicted pathways for the biosynthesis of sirodesmin PL and gliotoxin.

The bracketed compounds in Figure 2 are predicted and have not been isolated. Enzymes predicted to catalyse particular steps are denoted by a *Gli* or *Sir* prefix with an uppercase letter (e.g. P, T, N, M) as described below.

Identifying biosynthetic genes for epipolythiodioxopiperazines

The genes that encode enzymes for biosynthesis of secondary metabolites are usually clustered in fungal genomes. The discovery of the first set of biosynthetic genes for an ETP, sirodesmin PL, was serendipitous and arose from a project where 500 random genes in the blackleg fungus, *L. maculans*, were sequenced and analysed. One of these genes had high sequence similarity to a prenyl transferase in



another fungus. Such an enzyme is predicted to catalyse an early step in sirodesmin PL biosynthesis. Sequencing of flanking genes on the chromosome of *L. maculans* revealed the presence of a bi-modular, non-ribosomal peptide synthetase, which would catalyse condensation of two amino acids, serine and tyrosine. Furthermore, other genes with predicted roles in sirodesmin PL production were identified amongst a cluster of 18 genes (Fig. 3A). All these genes were activated concurrently during sirodesmin production, whilst genes at the extremes of the cluster were not.

This result provided strong evidence for the cluster being responsible for sirodesmin PL production. This hypothesis was confirmed by the finding that a mutant in the non-ribosomal peptide synthetase could not make sirodesmin PL.³ The mutant caused smaller stem lesions on canola than

the wild type, thus the toxin is important in the colonisation of canola stems fungus.⁴ Examination of the genome sequences of *A. fumigatus* revealed a similar set of genes, which were subsequently identified as the gliotoxin biosynthetic cluster (Fig. 3B).

Ten genes were common to the sirodesmin and gliotoxin clusters. These had high sequence similarity (best matches) to non-ribosomal peptide synthetase (P), thioredoxin reductase (T), methyl transferases (M and N), glutathione S-transferase (G), cytochrome P450 mono-oxygenase (C), aminotransferase or cystine lyase (I), dipeptidase (J), as well as a transcriptional regulator (Z) and a membrane transporter (A), which is predicted to pump the toxin from the fungal cell, thus protecting the fungus from the toxic effects of ETPs (Fig. 3). All these genes are proposed to be

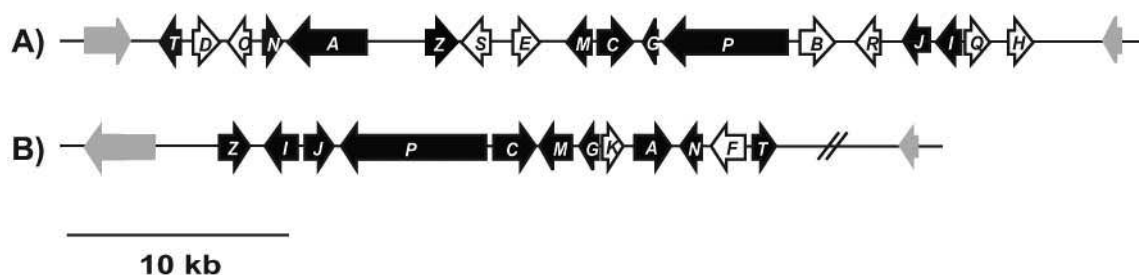


Figure 3. The sirodesmin (A) and gliotoxin (B) biosynthetic gene clusters from *Leptosphaeria maculans* and *Aspergillus fumigatus*, respectively. Common ETP moiety genes (white text on black background). Genes (black text on white background) predicted to be involved in modification of the side chains of the core ETP moiety. Genes (grey) encode proteins with best matches to proteins with no roles in ETP biosynthesis; such genes do not appear to be part of the cluster. The forward slash marks represent a 17 kb region of repetitive DNA.

responsible for the core ETP structure (Fig. 1A). Genes unique to either cluster included those with best matches to cytochrome P450 mono-oxygenases (F, B and E), prenyl transferase (D), acetyl transferase (H), epimerases (Q, S and R) and oxidoreductase (O).

By considering the best match of the gene sequence and predicted reactions in the pathway, the role of particular genes can be proposed as shown in Figure 2. For example, in the sirodesmin PL biosynthetic gene cluster, the prenyl transferase (D) and the acetyl transferase (H) are most likely involved in prenylation of the dipeptide and acetylation, respectively. Gliotoxin does not have these modifications and, as expected, these genes are absent from the gliotoxin gene cluster. As mentioned above, introduction of the sulfur atoms is poorly understood. The glutathione S-transferase (G) and a gene with close matches to enzymes such as aminotransferase or cystine lyase (I), that use pyridoxal 5-phosphate (PLP) as a cofactor, may be involved. These genes are predicted to encode enzymes that form/break bonds between sulfur and carbon. The thioredoxin reductase (T) may oxidise the dithiols to form the S-S bond. However, these assignments of function are purely speculative at this stage.

Conclusions and future prospects

As more fungal genome sequences become available, comparative analyses will enable the role of particular genes in ETP biosynthetic pathways to be proposed. For example, analysis of biosynthetic gene clusters for ETPs (e.g. arantoin, epicorazine) that lack methyl groups may reveal which of the putative methyl transferases (M or N) is responsible for N-methylation of ETPs such as gliotoxin or sirodesmin. Recently many fungal genome sequences have been released publicly. Examination of 30 such genome sequences revealed the presence of putative ETP biosynthetic gene clusters in 14 of them.⁵ In many cases it is not known whether an ETP is produced by these fungi. These putative ETP gene clusters appear to have arisen by 'horizontal gene transfer', similar to the way that antibiotic resistance can be transferred between

bacteria, rather than by evolution.⁵ The ability of fungi to transfer gene clusters that encode toxins or virulence factors presents important implications, particularly for fungi that are plant and animal pathogens.

Although biosynthetic genes for ETPs can be deduced by comparative genome analysis, this is an *in silico* bioinformatics approach and it is often difficult to determine the role of such genes in biosynthesis. The ability to generate mutations in particular genes, coupled with analytical techniques such as HPLC and mass spectrometry, allows discovery of pathway intermediates. Collaborations between fungal molecular biologists and chemists are essential for these pathways to be elucidated. This is a rewarding pursuit, and not just intellectually; knowledge of the actions of genes in these pathways may allow the design of inhibitors of enzymes involved in ETP biosynthesis. Such inhibitors may alleviate symptoms of secondary fungal toxicoses associated with important medical complaints, such as organ transplants and HIV AIDS. This is a fertile field for future research.

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REFERENCES

- 1 Fox E.M., Howlett B.J. *Mycol. Res.* 2008, **112**, 162-9.
- 2 Gardiner D.M., Waring P., Howlett B.J. *Microbiol.* 2005, **151**, 1021-32.
- 3 Gardiner D.M., Cozijnsen A.J., Wilson L.M., Pedras M.S.C., Howlett B.J. *Mol. Microbiol.* 2004, **53**, 1307-18.
- 4 Elliott C.E., Gardiner D.M., Thomas G., Cozijnsen A.J., Van De Wouw A., Howlett B.J. *Mol. Plant Path.* 2007, **8**, 791-802.
- 5 Patron N.J., Waller R.F., Cozijnsen A.J., Straney D.C., Gardiner D.M., Nierman W.C., Howlett B.J. *BMC Evol. Biol.* 2007, **7**, 174.

Barbara J. Howlett, School of Botany, University of Melbourne