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Review

The epipolythiodioxopiperazine (ETP) class of fungal toxins: distribution, mode of action, functions and biosynthesis

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Epipolythiodioxopiperazines (ETPs) are toxic secondary metabolites made only by fungi. The best-known ETP is gliotoxin, which appears to be a virulence factor associated with invasive aspergillosis of immunocompromised patients. The toxicity of ETPs is due to the presence of a disulphide bridge, which can inactivate proteins via reaction with thiol groups, and to the generation of reactive oxygen species by redox cycling. With the availability of complete fungal genome sequences and efficient gene-disruption techniques for fungi, approaches are now feasible to delineate biosynthetic pathways for ETPs and to gain insights into the evolution of such gene clusters.

Introduction

Most fungal toxins are secondary metabolites – low-molecular-mass compounds that are dispensable, but may provide selective advantage under particular conditions. Well-characterized classes of toxins include polyketides (e.g. aflatoxins), cyclic peptides, alkaloids and sesquiterpenoids (e.g. trichothecenes). Another class, the epipolythiodioxopiperazines (ETPs), is characterized by the presence of an internal disulphide bridge (Fig. 1). The diketopiperazine ring is derived from a cyclic dipeptide and its sulphur bridge imparts all known toxicity of these molecules (Mullbacher *et al.*, 1986).

Gliotoxin was the first ETP reported and is the best-characterized. Its name is derived from its identification as a metabolite of *Gliocladium fimbriatum* (possibly a *Trichoderma* sp.) (Weindling & Emerson, 1936; Weindling, 1941). Gliotoxin is implicated in animal mycoses. It is immunosuppressive and causes apoptotic and necrotic cell death *in vitro*. The toxicity of ETPs has made them attractive as potential therapeutic agents for diseases such as cancer (Vigushi *et al.*, 2004). In spite of their important biological effects, little is known about their biosynthesis or even their primary role in the biology of the organisms that produce them. Furthermore these aspects, as well as the discontinuous taxonomic distribution of ETPs amongst fungi, and their structural relatedness, have never been reviewed. Such a review is timely as tools are now available to analyse their biosynthetic pathways and to determine unequivocally their role in disease. These tools include complete fungal genome sequences and efficient gene-disruption techniques for fungi. Recently these approaches have been applied to identify a biosynthetic gene cluster for an ETP, sirodesmin PL, in *Leptosphaeria maculans*, and to predict genes in the gliotoxin biosynthetic pathway in *Aspergillus fumigatus* (Gardiner *et al.*, 2004). In this review we describe the range of fungi that produce ETPs, and the proposed modes of ETP activity, biosynthesis and transport. We also discuss current opinion about the evolution of such biosynthetic gene clusters.

Diversity of ETP toxins and their discontinuous distribution

At least 14 different ETPs (excluding those with minor modifications) are known (Table 1). The diversity of structures stems from the amino acids of the core ETP moiety, as well as the modifications of these amino acids. All natural ETPs isolated to date contain at least one aromatic amino acid. A diverse range of filamentous ascomycetes

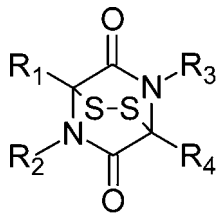

Fig. 1. Generic structure of an epidithiodioxopiperazine, the most common form of ETP. In some circumstances the sulphur bridge contains one, three or four sulphur atoms; these compounds are usually co-produced with those containing the disulphide. R = any atom or group.

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Table 1. ETP molecules from various fungal species

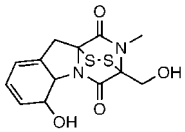
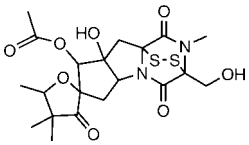
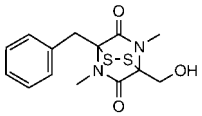
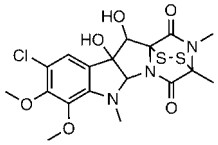
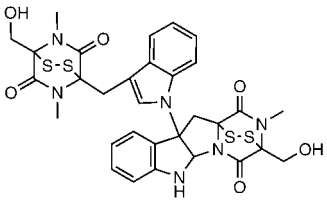
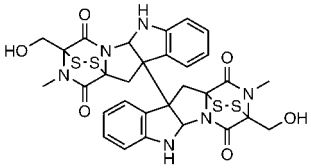
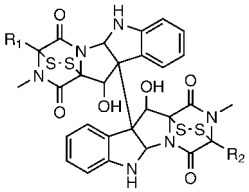
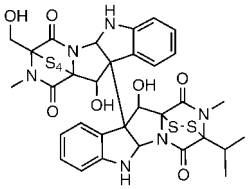
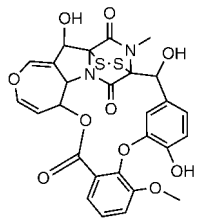
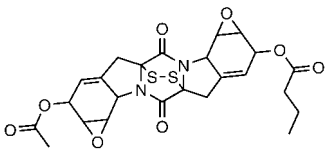
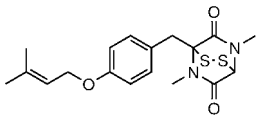
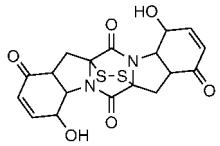
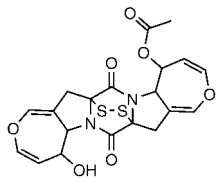
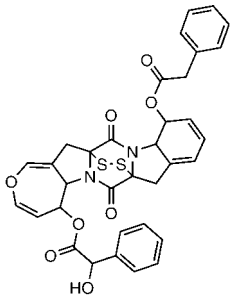
Compound	Structure*	Producing organism(s)	Predicted amino acids†	References
Gliotoxin		*Aspergillus fumigatus*, *Trichoderma virens*, *Penicillium* spp., *Candida albicans*	Phe, Ser	Kirby & Robins (1980); Shah & Larsen (1991)
Sirodesmin		*Leptosphaeria maculans*, *Sirodesmium diversum*	Tyr, Ser	Curtis *et al.* (1977); Ferezou *et al.* (1977)
Hyalodendrin		*Hyalodendron* sp.	Phe, Ser	Stillwell *et al.* (1974)
Sporidesmin A		*Pithomyces chartarum*	Trp, Ala	Kirby & Robins (1980)
Chaetomin		*Chaetomium globosum*	Trp, Ser	Sekita *et al.* (1981)
Chaetocin		*Chaetomium* spp.	Trp, Ser	Sekita *et al.* (1981)
Verticillins		*Verticillium* spp. (A and B), *Penicillium* sp. (A), *Gliocladium catenulatum* (D)	Trp and A: $R_1 = R_2 = CH_3$ (Ala) B: $R_1 = CH_3$ (Ala), $R_2 = CH_2OH$ (Ser) D: $R_1 = R_2 =$ $CH_2(OH)CH_3$ (Thr)	Byeng *et al.* (1999); Joshi *et al.* (1999); Kirby & Robins (1980); Sekita *et al.* (1981)
Leptosin		*Leptosphaeria* sp. OUPS-4	Trp, Ser, Val	Takahashi *et al.* (1994)
Emestrin		*Aspergillus* spp.	Phe, Tyr	Refs in Seya *et al.* (1986)

Table 1. cont.

Compound	Structure*	Producing organism(s)	Predicted amino acids†	References
Scabrosin (ester 2)		*Xanthoparmelia scabrosa*	Phe	Ernst-Russell *et al.* (1999); Moerman *et al.* (2003)
Dithiosilvatin		*Aspergillus silvaticus*	Tyr, Gly	Kawahara *et al.* (1987)
Epicorazine		*Stereum hirsutum* (epicorazine C), *Epicoccum purpurascens* (epicorazine B), *Epicoccum nigrum* (epicorazine A and B)	Phe	Deffieux *et al.* (1977); Kleinwachter *et al.* (2001)
Aranotin		*Arachniotus aureus*, *Aspergillus terreus*	Phe	Neuss *et al.* (1968)
Emethallicin		*Aspergillus heterothallicus*	Phe	Kawahara *et al.* (1989)

*Many species produce multiple related ETPs, which often differ in either the number of sulphurs in the bridged piperazine, or side groups. In most cases the most abundant ETP produced by the fungus is shown. ETPs with a disulphide-bridged piperazine are the most common and stable.

†Constituent amino acids of the core ETP moiety in many cases are predicted from the final structure and by comparison to other ETPs. Where only one amino acid is listed, it is assumed that the same amino acid is used for both halves of the ETP.

produce ETPs. Five classes representing 14 genera are shown in Table 1: Dothideomycetes, Eurotiomycetes, Lecanoromycetes, Saccharomycetes and Sordariomycetes. At least two basidiomycetes, *Stereum hirsutum* and a *Hyalodendron* sp., produce ETPs: epicorazine and hyalodendrin, respectively (Kleinwachter *et al.*, 2001; Stillwell *et al.*, 1974). Lichens (presumably the fungal partner) produce the scabrosin ester ETPs (Ernst-Russell *et al.*, 1999; Moerman *et al.*, 2003).

Production of ETPs amongst fungal species is discontinuous. For example, the eurotiomycete *Aspergillus fumigatus* produces gliotoxin, but other *Aspergillus* spp. do not. The dothideomycete *L. maculans* produces sirodesmin PL, but the very closely related *Leptosphaeria biglobosa* (until recently classified as a member of the *L. maculans* species complex) does not. Another dothideomycete, *Sirodesmium diversum*, synthesizes sirodesmin, but the major chiral isomer is different from that produced by *L. maculans*.

Also, distantly related fungi can produce the same molecule. For instance gliotoxin is produced by unrelated fungi, the opportunistic animal pathogens *A. fumigatus*, *Penicillium* spp., *Candida* spp. and mycoparasitic *Trichoderma* spp. (Macdonald & Slater, 1975; Shah & Larsen, 1991; Weindling & Emerson, 1936). The verticillins are produced by sordariomycetes and eurotiomycetes, and the closely related molecules chaetocin, chaetomin and leptosin are produced by sordariomycetes and dothideomycetes (Table 1).

Mechanisms of toxicity

The effect of gliotoxin on animal cell cultures has been studied extensively, and most of the research described in this section pertains to this ETP. Interest in gliotoxin as a therapeutic agent was initiated when it was shown to prevent viral RNA replication, via inhibition of reverse transcriptase (De Clercq *et al.*, 1978; Rightsel *et al.*, 1964). The bridged disulphide ring is essential for this activity, and removal of the sulphur atoms or addition of the reducing agent dithiothreitol completely abrogates this inhibition (Rodriguez & Carrasco, 1992; Trown & Bilello, 1972). Toxicity is thought to be mediated in at least two ways: (1) conjugation to proteins with susceptible thiol residues and subsequent inactivation; and (2) generation of reactive oxygen species via redox cycling. However, as discussed below, the role of these processes in toxicity is equivocal and many findings appear to be contradictory.

ETPs do not have exclusive protein targets. For some proteins, the cysteine residues that form mixed disulphide bonds with ETPs have been identified. For instance, gliotoxin forms a 1 : 1 covalent complex with alcohol dehydrogenase via cysteine residue 281 or 282 (Waring *et al.*, 1995b). Gliotoxin catalyses formation of an internal disulphide bond between the physically close cysteines 282 and 73 in creatine kinase, which can be reversed by reducing agents (Hurne *et al.*, 2000). Although *in vitro* studies show mixed disulphide formation with proteins, the role of mixed disulphides in the cellular toxicity of ETPs has not been established unequivocally. One target is the transcription factor NF-κB, which is inhibited in cells by gliotoxin, probably via interaction with an essential thiol residue (Pahl *et al.*, 1996). Since this factor is an integral part of the inflammatory immune response and controls expression of some cytokines, its inhibition may account for the immunosuppressive properties of ETPs. Another cellular target is the mitochondrion. Mitochondrial function in intact cells is inhibited by the scabrosin ester ETP (Moerman *et al.*, 2003). Initially mitochondrial ATP synthase is inhibited, then the mitochondrial membrane becomes hyperpolarized, and finally apoptotic cell death occurs. Effects of gliotoxin on isolated mitochondria include release of both calcium and magnesium (Salvi *et al.*, 2004; Schweizer & Richter, 1994). It also causes a redox-dependent change in electrophoretic mobility of a component of the mitochondrial permeability transition pore, the adenine nucleotide transporter (ANT). This transition pore is an essential mediator of apoptosis modulated by mitochondria (Orr *et al.*, 2004). The ANT is sensitive to oxidizing agents and has two cysteine residues which may be targets of gliotoxin (McStay *et al.*, 2002).

Gliotoxin and other ETP toxins can redox cycle, whereby the reduced ETP auto-oxidizes back to the disulphide form and produces deleterious reactive oxygen species (ROS) such as superoxide or hydrogen peroxide (Fig. 2). The toxic effects of sporidesmin on erythrocytes appear to be modulated by ROS (Munday, 1982). The recent finding that gliotoxin is almost completely reduced to the dithiol form in P388D1 cells suggests that redox cycling may be important for its toxicity (Bernardo *et al.*, 2003). However, this cannot account for all the activities of ETPs. For instance, it cannot fully explain how gliotoxin causes apoptotic and necrotic death of animal cells. At gliotoxin concentrations above 10 μM, the nature of cell death switches from apoptosis to necrosis, with almost complete necrosis above 50 μM (Beaver & Waring, 1994). Apoptosis is not inhibited in gliotoxin-treated cells by a range of scavengers of ROS, which implies that redox cycling is not involved in the induction of apoptosis (Waring *et al.*, 1995a). Furthermore, the nanomolar concentrations of gliotoxin that induce apoptosis would not be expected to induce oxidative stress.

Calcium influx has been indirectly implicated in the toxicity of gliotoxin. Necrosis of thymocytes caused by gliotoxin (>50 μM) has been attributed to increased cellular calcium levels. This increase is induced by interaction of gliotoxin with a thiol residue in the plasma membrane calcium channel, which is redox sensitive (Hurne *et al.*, 2002). Increased calcium flux may then cause subsequent oxidative damage. Significantly, addition of either dithiothreitol or glutathione prevents calcium influx since gliotoxin in the reduced form cannot oxidatively modify thiol residues. This argues for mixed disulphide formation, rather than redox cycling, as an explanation of these necrotic effects of gliotoxin.

Recently a redox-uptake mechanism for gliotoxin has

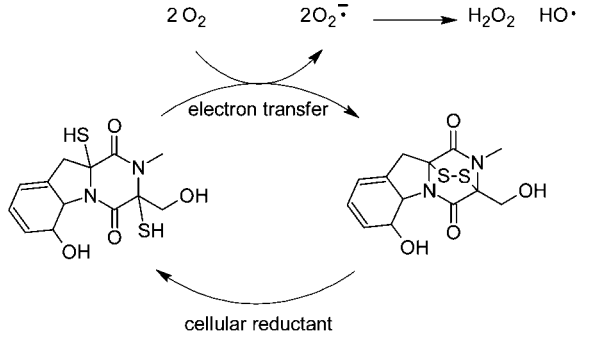


Fig. 2. Redox cycling between the reduced (dithiol) and oxidized (disulphide) forms of gliotoxin. The oxidation of gliotoxin and presumably other ETPs generates reactive oxygen species.

been described (Bernardo *et al.*, 2003). The concentration within a cell (animal cells and cell lines) can be several orders of magnitude greater (up to 1500-fold) than the applied concentration. This accumulation appears to enhance toxicity of ETPs. The reduced form of the toxin is trapped in the cell and appears to be incapable of traversing the plasma membrane. Reduction occurs rapidly by cellular glutathione and is reversible. Transient oxidation of glutathione in cells loaded with gliotoxin results in rapid efflux of the oxidized form of gliotoxin. Furthermore, treatment of the cells to deplete glutathione, or extracellular reduction of the toxin by addition of dithiothreitol, abrogates both uptake and biological activity of gliotoxin.

This redox uptake mechanism can explain the effect of cell density on efficacy of killing by ETP toxins, whereby cytotoxicity of gliotoxin increases as cell density decreases (Jordan & Pedersen, 1986). As cell density decreases, more toxin per cell is at equilibrium between reduced and oxidized forms. The equilibrium levels of accumulated gliotoxin in animal cells is independent of the mode by which the toxin traverses the plasma membrane, although gliotoxin is able to diffuse through a simple lipid bilayer (Green *et al.*, 2000). However, the initial rate of uptake into cells is saturable, suggesting that facilitated diffusion occurs.

Putative functions of ETPs: virulence and/or defence

Many secondary metabolites are assumed to be part of the chemical defence system of micro-organisms, and beneficial or detrimental effects on humans are presumed to be accidental. A number of ETPs have also been specifically associated with mammalian or plant disease. Gliotoxin is immunosuppressive, with selective effects on mature lymphocytes and macrophages (Mullbacher *et al.*, 1985, 1988). Gliotoxin production by *A. fumigatus* is thought to contribute to invasive aspergillosis of mammals (Sutton *et al.*, 1994). Its role as a virulence factor is deduced from a range of studies (Hope & Denning, 2004). Its production by *A. fumigatus* at zones of cellular detachment of A549 human cells grown in agar with the fungus indicates a role in tissue colonization (Daly & Kavanagh, 2002). Levels of gliotoxin secreted by different *A. fumigatus* strains correlate with virulence on larvae of the insect *Galleria mellonella*. This insect system is being trialled as a model for *in vivo* testing of virulence of *Aspergillus* spp. (Reeves *et al.*, 2004). Gliotoxin can also disrupt cellular responses to fungal infections involving the NADPH oxidase enzyme complex in human polymorphonuclear leukocytes. Its mode of action is via inhibition of assembly and function of this enzyme complex (Nishida *et al.*, 2005; Tsunawaki *et al.*, 2004).

Other ETPs with suggested roles in animal diseases include sporidesmins, which are produced by *Pithomyces chartarum* on infected grasses and cause facial eczema and liver diseases of grazing animals (Cheeke, 1995). Chaetomin is an ETP produced by *Chaetomium globosum*, some isolates of which infect skin and nails of humans. Also *C. globosum* is a deadly systemic pathogen of immunocompromised humans (Serena *et al.*, 2003). Other *C. globosum* isolates are antagonists of soil-borne pathogens, including the fungus *Cochliobolus sativum*, which causes spot blotch of wheat (Aggarwall *et al.*, 2004). The role of chaetomin in these activities is circumstantial (like that of gliotoxin in aspergillosis).

Sirodesmin PL is the major secreted metabolite of *L. maculans*. Its phytotoxic activities suggest a role for it in blackleg disease of *Brassica napus* (canola, oilseed rape) (Rouxel *et al.*, 1988). The lifestyle of this pathogen *in planta* is complicated. Initially *L. maculans* is biotrophic during invasion of leaves, causing little damage to the plant. Later as it grows down the stem, it becomes necrotrophic, causing blackened cankers at the base of the stem. After harvest *L. maculans* survives on stubble as a saprophyte and undergoes sexual crossing, producing windborne ascospores that infect canola crops sown the following year (Howlett, 2004). Sirodesmin could be involved either in the necrotrophic phase of infection or in competition with other micro-organisms during saprophytic growth. Analysis of a sirodesmin non-producing mutant (created via disruption of a non-ribosomal peptide synthetase gene) has shown that sirodesmin is not essential for lesion formation on leaves (Gardiner *et al.*, 2004). These results are consistent with those obtained by Sock & Hoppe (1999) with UV-induced mutants of *L. maculans* that were unable to produce sirodesmin: these mutants caused similar-sized leaf lesions to those caused by the wild-type isolates. The latter authors showed that UV mutants were less virulent on stems of *B. napus*. Measurements of fungal biomass (determined by quantitative PCR) in *B. napus* stems infected by the peptide synthetase mutant are consistent with this finding (D. M. Gardiner, A. J. Cozijnsen and B. J. Howlett, unpublished data).

While the focus of much mycotoxin research, including that on ETPs, is on the role of such compounds in disease, their potential function as defence molecules should also be considered. For *A. fumigatus*, which is primarily saprophytic and is only pathogenic towards immunocompromised individuals, it is difficult to conceive that the evolutionary force that led to and maintained its ability to produce gliotoxin was enhancement of virulence towards animals. Another possibility is that gliotoxin (and other ETPs) plays a role in allowing the fungi that produce it to successfully compete with other micro-organisms in its ecological niche. To our knowledge, only one study describing competition between ETP-producing and non-ETP-producing fungi has been reported. However, in that study, strains of *P. chartarum* unable to produce sporidesmin out-competed toxigenic strains in grass pastures (Fitzgerald *et al.*, 1998). Thus, this argues against the role of ETPs proposed above. Clearly the primary role of ETPs is unknown. Nonetheless, it is intriguing that for

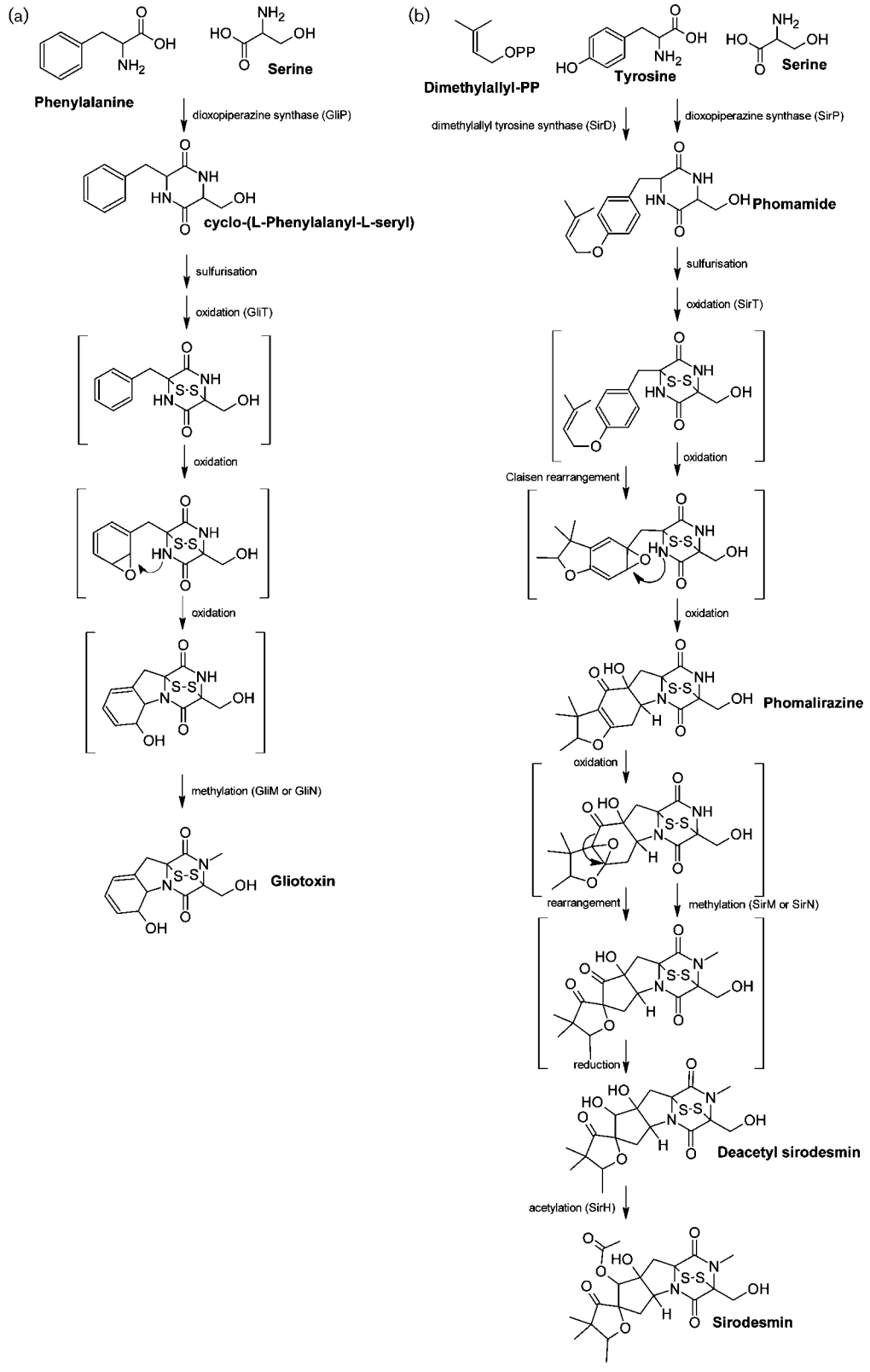


Fig. 3. Predicted biosynthetic pathways for (a) gliotoxin and (b) sirodesmin. The bracketed compounds are predicted and have not been isolated. Reactions predicted to be unique are shown to the left of the sirodesmin biosynthesis pathway, whilst those in common between gliotoxin and sirodesmin are shown to the right in the sirodesmin pathway. Enzymes predicted to catalyse particular steps are denoted by a Gli or Sir prefix, consistent with the nomenclature used by Gardiner *et al.* (2004).

several fungi there are associations between disease and ability to produce ETPs.

ETP biosynthesis

Core ETP moiety

In spite of almost 50 years of research on ETPs since the structure of gliotoxin was first described (Bell *et al.*, 1958), very little is known about ETP biosynthesis. Labelling experiments demonstrate that amino acids are precursors for ETPs (Suhadolnik & Chenoweth, 1958; Winstead & Suhadolnik, 1960). Feeding experiments with cyclic dipeptides and the identification of these molecules in culture filtrates of ETP-producing organisms has suggested them as intermediates (Ferezou *et al.*, 1980; Kirby *et al.*, 1978). For sirodesmin biosynthesis, in addition to the (prenylated) cyclic dipeptide phomamide, two other intermediates have been identified, which are themselves ETPs; phomarilazine and deacetyl sirodesmin (Pedras *et al.*, 1990). Hence the core ETP structure is formed at an early stage in the pathway. Fig. 3 summarizes predicted pathways for the biosynthesis of gliotoxin and sirodesmin.

Fungal secondary metabolites that incorporate more than one amino acid are typically synthesized via non-ribosomal peptide synthetases (NRPSs), modular enzymes that contain one module per amino acid added to the peptide (Mootz & Marahiel, 1997). Recently a two-module NRPS (presumed to be enzymically a dioxopiperazine synthase) was shown to be involved in the biosynthesis of sirodesmin in *L. maculans*. Targeted disruption of the gene (*sirP*) encoding this enzyme resulted in a mutant unable to synthesize sirodesmin or the easily detectable precursors phomamide and deacetyl sirodesmin (Gardiner *et al.*, 2004).

The genes that encode enzymes for biosynthesis of fungal secondary metabolites are usually clustered in the genome. Biosynthetic gene clusters have been identified for compounds such as penicillin, aflatoxin, trichothecenes and ergot alkaloids (Brown *et al.*, 2003; Smith *et al.*, 1990; Tudzynski *et al.*, 1999; Yu *et al.*, 2004). The *sirP* gene is in a cluster of 18 genes in *L. maculans* (Fig. 4), for which many of the products can be assigned roles in sirodesmin biosynthesis. For example, prenyl transferase (SirD), thioredoxin reductase (SirT) and acetyl transferase (SirH) are assigned the roles shown in Fig. 3 (Gardiner *et al.*, 2004). Generation of mutations in these (and other) cluster genes and analysis of secondary metabolite profiles of the resultant mutants will allow identification of intermediates in the biosynthetic pathway for sirodesmin PL.

The recent availability of complete fungal genomes has allowed identification of putative ETP clusters in other fungi. Six genomes were examined (Gardiner *et al.*, 2004). The yeasts *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Candida albicans* did not contain such a cluster. *Sacch. cerevisiae* and *Schiz. pombe* are not reported to produce ETPs, but gliotoxin production by *C. albicans* has been reported in some isolates cultured from infected humans (Shah & Larsen, 1991). A putative ETP cluster was identified in the genome of *A. fumigatus* (Fig. 4), but not in *Aspergillus nidulans*, which does not produce gliotoxin. Expressed sequence tags (ESTs) were identified from *Chaetomium globosum*, which produces chaetomin (Table 1). ETP-like gene clusters are present in other organisms, such as the wheat head scab fungus *Fusarium graminearum* and the rice-blast fungus *Magnaporthe grisea* (Gardiner *et al.*, 2004), but the presence of ETP-like molecules has not been described for these species. However, the composition of culture filtrates of these fungi, particularly *M. grisea*, has not been studied in much detail.

The identification of these putative ETP biosynthetic gene clusters is a major step forward in understanding how these molecules are made. The number of genes can be defined by the physical limits of the cluster and co-regulation of the gene transcripts with ETP production. This has been done for the sirodesmin and putative gliotoxin gene clusters (Gardiner *et al.*, 2004, D. M. Gardiner, unpublished

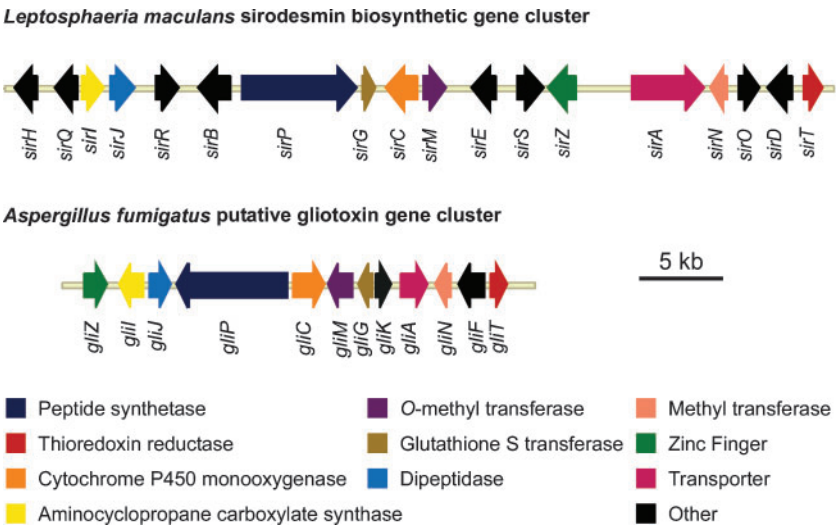


Fig. 4. Comparison of the sirodesmin and gliotoxin biosynthetic gene clusters from *Leptosphaeria maculans* and *Aspergillus fumigatus*, respectively. Genes with obvious homologues in the clusters are coloured. The 'other' category contains genes encoding cytochrome P450 monooxygenases (GliF, SirB, SirE), a prenyl transferase (SirD), an acetyl transferase (SirH), epimerases (SirQ, SirR, SirS), an oxidoreductase (SirO) and a hypothetical protein (GliK).

results). Targeted gene disruption of predicted genes in the *A. fumigatus* cluster is required to confirm that this cluster encodes enzymes involved in gliotoxin biosynthesis.

Comparisons of the gene clusters in *L. maculans* and *A. fumigatus*, and ESTs of *C. globosum*, identified ten genes with predicted homologous functions (Gardiner *et al.*, 2004). Eight of these genes are likely to encode biosynthetic enzymes for the core ETP moiety (Fig. 1) and are described in Table 2. The other two genes encode a zinc finger transcriptional regulator (GliZ for gliotoxin and SirZ for sirodesmin), presumably required for regulation of genes in the clusters, and transport proteins (GliA and SirA) commonly found in secondary metabolite gene clusters. These transport proteins are discussed in more detail later in this review.

As more fungal genome sequences become available, comparative analyses will be extremely powerful in identifying ETP biosynthetic pathways, and in particular the core ETP biosynthetic genes (Table 2). For example, the analysis of biosynthetic clusters for ETPs (e.g. aranotin, emethacillin, epicorazine and scabrosin ester) which are derived from two aromatic amino acids and lack methyl groups may reveal which of the several potential methyl transferases (GliM or GliN) is responsible for *N*-methylation of ETPs such as gliotoxin that contain only one aromatic amino acid. Similarly, analysis of genes responsible for biosynthesis of the ETPs such as dithiosilvatin and hyalodendrin that do not contain a pyrrolidine ring (five membered ring with one nitrogen) may indicate enzymes in the gliotoxin and sirodesmin clusters involved in ring cyclization. Directly determining the enzymes responsible for this ring formation will be difficult by chemical analyses as this reaction is thought to proceed via an unstable epoxide intermediate (second bracketed compound in pathways in Fig. 3).

Introduction of the sulphur atoms into the core ETP moiety is poorly understood. Labelling experiments have shown that methionine, cysteine and sodium sulphate can all act as sources of sulphur, although cysteine is thought to be the direct donor (Kirby & Robins, 1980). However, the mechanism by which the sulphurs are introduced is unknown and has no precedent in fungal secondary metabolism. The *gliG*/*sirG* and *gliI*/*sirI* gene products are possible candidates for such reactions, as these are predicted to encode enzymes that form/break bonds between sulphur and carbon; the latter genes have not been previously described in fungal secondary metabolite clusters.

Modifications of side chains of ETPs

Side chain modifications that are predicted in ETP biosyntheses include methylations (e.g. hyalodendrin), esterifications (e.g. scabrosin ester), chlorinations (e.g. sporidesmin), oxidations (e.g. sporidesmin), rearrangements (e.g. sirodesmin) and dimerizations (e.g. chaetocin). The nature of side groups of ETPs does not appear to affect toxicity. Indeed the synthetic ETP 1,4-dimethyl-3,6-epidithio-2,5-dioxopiperazine has similar toxicity to that of natural ETPs (Mullbacher *et al.*, 1986). However, modifications may have evolved to mask the core ETP moiety and prevent degradation by target organisms. Comparative genomics will allow the prediction of genes responsible for side group modifications. For example, in the sirodesmin biosynthetic gene cluster the *sirD* and *sirH* gene products are most likely involved in side group modification as they are absent from the gliotoxin cluster and they have predictable functions required for sirodesmin biosynthesis as shown in Fig. 3 (Gardiner *et al.*, 2004).

Aspects of sirodesmin biosynthesis are unique amongst fungal secondary metabolism. Labelling experiments confirm that a Claisen rearrangement, i.e. a stereoselective [3,3]-rearrangement of allyl vinyl or allyl aryl ethers to yield unsaturated carbonyl compounds or *O*-allyl substituted phenols, is involved in the modifications of the side chain of sirodesmin (Bu'Lock & Clough, 1992; Ferezou *et al.*, 1980). Claisen rearrangements are a mechanistically diverse group of chemical reactions. However, only one enzyme, chorismate mutase, is known to catalyse such a reaction. This enzyme is involved in biosynthesis of phenylalanine and tyrosine and was identified over 30 years ago (Martin Castro, 2004). The enzymes responsible for this

Table 2. Predicted core biosynthetic enzymes for ETPs that contain an *N*-methylated pyrrolidine ring

Enzyme (gliotoxin/sirodesmin)	Comments and proposed function
GliP/SirP	Two-module non-ribosomal peptide synthetase (dioxopiperazine synthase) involved in synthesis of the dipeptide
GliT/SirT	Thioredoxin reductase (dithiolpiperazine oxidase); formation of disulphide bond
GliC/SirC	Cytochrome P450 monooxygenase
GliI/SirI	High similarity to amino cyclopropane carboxylate synthases; unknown function, encodes enzyme that makes/breaks C–S bonds
GliJ/SirJ	Dipeptidase domain; unknown function
GliM/SirM	*O*-Methyl transferase domain; unknown function
GliN/SirN	Methyl transferase domain; unknown function
GliG/SirG	Glutathione *S*-transferase domain; unknown function, encodes enzyme that makes/breaks C–S bonds

reaction in sirodesmin biosynthesis are presumably encoded by one or more of the genes that do not have homologues in the gliotoxin gene cluster of *A. fumigatus*.

Efflux of ETPs from fungi

ETPs are fungitoxic. In *Sacch. cerevisiae*, resistance to sporidesmin is provided by the ATP-binding cassette (ABC) transporter PDR5 (Bissinger & Kuchler, 1994), which forms part of the multidrug resistance network (Balzi & Goffeau, 1995). Although uptake mechanisms by mammalian target cells are well described, the manner in which these compounds are exported by such cells or from the fungi that produce them has not been reported.

Transport proteins are commonly found in fungal secondary metabolite gene clusters (del Sorbo *et al.*, 2000). Despite the significant similarity between the gliotoxin and sirodesmin biosynthetic gene clusters, these clusters contain transport proteins from different classes. The putative gliotoxin cluster from *A. fumigatus* encodes a major facilitator superfamily member (GliA), whereas the sirodesmin cluster contains an ABC transporter (SirA) of the $(TMD_6\text{-}NBF)_2$ topology (transmembrane domain, nucleotide binding fold). Disruption of *sirA* demonstrated that this transporter was not necessary for sirodesmin production (Gardiner *et al.*, 2005). This gene, *sirA*, imparts resistance to both sirodesmin and gliotoxin. The *gliA* gene also provides resistance to gliotoxin when expressed in *L. maculans*, but does not alter sensitivity of *L. maculans* to sirodesmin (Gardiner *et al.*, 2005). These results indicate a difference between the mechanisms of primary toxin export and self-protection in *L. maculans*.

Evolution of the ETP gene clusters

Genes for secondary metabolism have been adapted by various fungi for production of a variety of compounds. For example prenyl transferase, SirD, and its homologues from *Claviceps purpurea* and *Penicillium paxilli* are involved in biosynthesis of three very different metabolites, despite their high protein sequence similarity (Gardiner *et al.*, 2004; Tudzynski *et al.*, 1999; Young *et al.*, 2001). Horizontal transmission of gene clusters has been proposed to account for evolution of fungal secondary metabolite gene clusters (Walton, 2000). However, it is difficult to differentiate between horizontal transfer versus vertical transmission followed by selective loss during evolution of filamentous fungi (Kroken *et al.*, 2003). Given the discontinuous distribution of ETP production by fungi, horizontal transfer of entire gene clusters is *prima facie* an attractive explanation for evolution of the ability to produce an ETP.

Since gliotoxin is made by a range of fungi, including *A. fumigatus*, *Penicillium* spp. and *Trichoderma* spp., comparison of gene order, promoter regions and flanking genes in the various gene clusters may give insights into the origin of this gene cluster. Genes involved in the production of secondary metabolites have been analysed at a number of phylogenetic levels. For instance, trichothecene biosynthetic gene clusters within the *Fusarium graminearum* species complex have been analysed (Ward *et al.*, 2002). The β-lactam antibiotics have been analysed with respect to inter-genus and inter-kingdom relatedness of the NRPS gene, and horizontal transfer from bacteria to fungi has been suggested to account for evolution of β-lactam gene clusters (Buades & Moya, 1996). Other than the β-lactams, the evolution of mycotoxin biosynthetic gene clusters has not been investigated across such a broad spectrum of organisms. The ETPs are molecules whose genes for biosynthetic pathways can be analysed across a large number of fungal classes. For instance, distantly related ascomycetes (classes Sordariomycetes and Eurotiomycetes) produce verticillins, whose only difference is the identity of the non-aromatic amino acid in the core ETP moiety. This specificity is most likely provided by the amino acid binding pocket of the adenylation domain of the peptide synthase module responsible for this amino acid (Challis *et al.*, 2000). Individual or a few variations in amino acids in this pocket would confer these specificities. Thus despite the evolutionary distance between such fungi, their biosynthetic genes for verticillins may be very similar. Clues to whether the biosynthetic gene cluster for verticillins have arisen by horizontal gene transfer or co-evolution can be obtained via sequence analysis of their biosynthetic genes, when they become available.

Conclusions and future prospects

ETPs are an interesting class of fungal toxins, whose primary role is as yet unknown. Currently the biosynthetic gene cluster for only one ETP, sirodesmin, has been unequivocally determined. Genes in the gliotoxin biosynthetic gene cluster have been predicted, but disruption of one or more of these genes is required to confirm this unambiguously. The acquisition of such mutants will allow the role of gliotoxin in diseases such as aspergillosis to be determined. As genome sequences for other fungi become available, comparative analyses will enable biosynthetic pathways for other ETPs to be delineated. For instance a draft sequence for *C. globosum* was released in January 2005 (http://www.broad.mit.edu/annotation/fungi/chaetomium_globosum/). Knowledge of the actions of genes in these pathways may lead to the ability to engineer fungal secondary metabolites with altered specificity. Additionally this could lead to the design of drugs that inhibit enzymes involved in ETP biosynthesis; such inhibitors may alleviate symptoms of secondary fungal toxicoses associated with leukaemia, organ transplants and HIV AIDS. Recent studies highlight the potential of gliotoxin as an anticancer agent. Gliotoxin inhibits farnesyl transferase, an enzyme required for normal functioning of the *ras* oncogene (Van der Pyl *et al.*, 1992). Compounds with such properties have been explored as putative anti-cancer agents, and gliotoxin has pronounced *in vivo* activity against rat mammary carcinomas without detectable toxicity (Vigushi *et al.*, 2004). This is the first demonstration that an ETP toxin has

anti-cancer properties in a whole animal and may herald renewed interest in this class of metabolites. Gliotoxin also can selectively kill activated hepatic stellate cells in rats, which is a model system for liver fibrosis (Dekel *et al.*, 2003; Wright *et al.*, 2001). This morbidity is caused by selective induction of apoptotic cell death in stellate cells through redox-dependent effects on the adenine nucleotide transporter (Orr *et al.*, 2004). However, the usefulness of ETPs such as gliotoxin as antimicrobial agents, and as clinical immunosuppressive agents, will be limited by their toxicity. Nonetheless, the unique nature of the internal disulphide bond, mode of action and uptake into cells make ETPs interesting molecules for design of future chemotherapeutic agents. In particular, their ability to efficiently kill cells with raised glutathione levels may suggest ways to address tumour cell resistance caused by elevated levels of glutathione and/or glutathione-detoxifying enzymes. Their targeting of mitochondrial function is also significant, given the importance of this organelle in triggering cell death and renewed interest in mitochondria as cellular targets in cancer therapy (Debatin *et al.*, 2002).

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