Chemistry of endophytes: patterns and diversity

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Abstract

Neotyphodium spp. fungi in pasture grasses synthesise a diverse range of metabolites. In this report, recent progress in extending knowledge of the diversity of endophyte metabolites is reviewed, in particular the elucidation of indole-diterpenes and ergot alkaloids. Some 20 indole-diterpenes have been identified from *N. lolii*-infected perennial ryegrass, and several ergot alkaloids additional to ergovaline have been identified in perennial ryegrass and other grasses infected with *Neotyphodium spp.* endophytes. While lolitrem B, ergovaline, and peramine remain significant factors in understanding the biological activity of *N. lolii*-infected perennial ryegrass, a more complex and complete view of endophyte chemical ecology must now be developed.

Keywords: chanoclavine-I, dehydroergovaline, endophyte, ergine, ergot alkaloid, indole-diterpene, *Neotyphodium spp*., perennial ryegrass, tall fescue

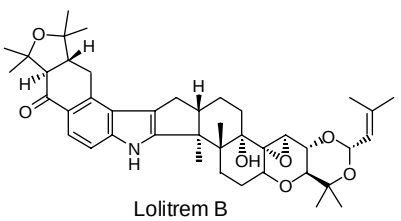
Introduction

When humans intervene with chemical agents to manage plant production, we typically employ a rather modest range of biologically active chemicals as pesticides, fungicides or herbicides, each preferably with a well-defined spectrum of activity. It would be convenient for research into natural chemical defence systems in agricultural ecosystems if nature followed the same parsimonious route, and each mode of biological activity observed could be ascribed to a single chemical agent. However the natural chemical defence systems generated by evolutionary processes are generally very complex, involving diverse mixtures of biologically active chemicals, each often with a broad and distinctive spectrum of activity.

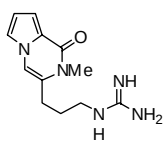
Since the biologically potent role of endophytic *Neotyphodium* fungi infecting perennial ryegrass in New Zealand pastures was first recognised (Fletcher & Harvey 1981) a simple model of the chemical armoury of the *N. lolii–L. perenne* symbiosis has been developed to account for its major features (Figure 1). Ryegrass staggers has been linked to the accumulation of the powerful and long-lasting tremorgen lolitrem B (Gallagher *et al.* 1984; Gallagher *et al.* 1981). The insect feeding deterrent peramine (Rowan & Gaynor 1986) has been implicated in resistance to Argentine stem weevil. More recently, the occurrence of heat stress in stock grazing endophyte-infected perennial ryegrass has been attributed to the accumulation of the ergot alkaloid, ergovaline (Easton *et al.* 1996; Rowan & Shaw 1987).

The simple model based on lolitrem B, ergovaline and peramine has proved very useful in screening for safe endophyte-grass associations (Tapper & Latch 1999). However, continuing research is revealing greater complexity to the chemical defenses which endophytic *Neotyphodium* fungi provide to their host grass. We are now aware of an expanded range of compounds related to lolitrem B or to ergovaline which are synthesised by the fungus.

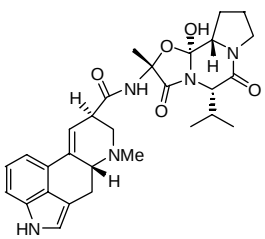
Figure 1 Alkaloids of *Neotyphodium lolii–Lolium perenne* implicated in biological activity of endophyte in agriculture in New Zealand.



Lolitrem B



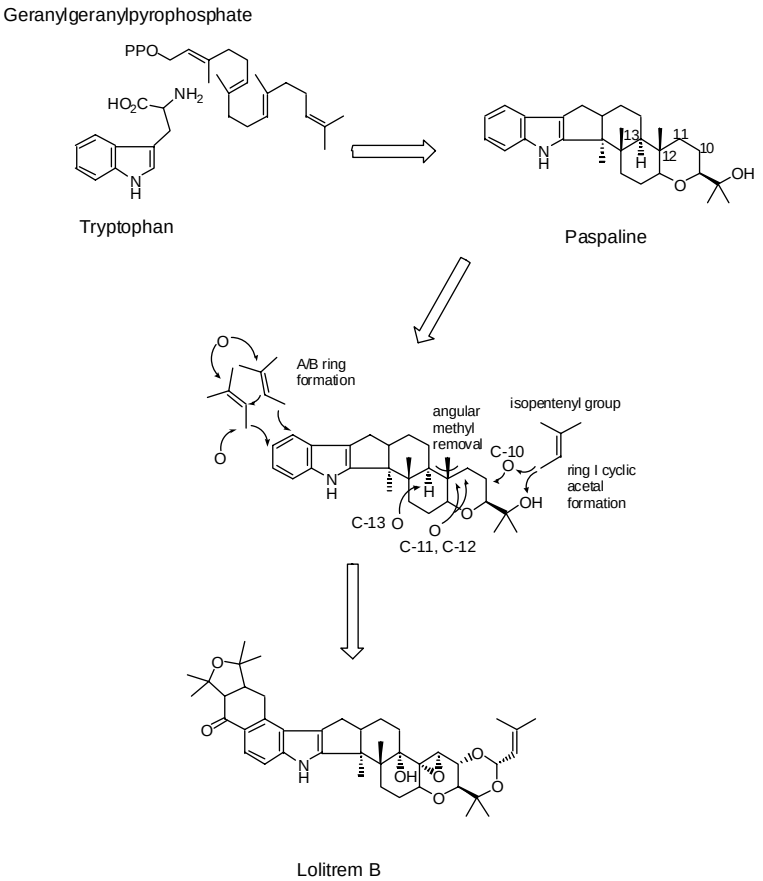
Peramine



Ergovaline

In this report, recent progress in extending knowledge of the diversity of endophyte metabolites is reviewed, in particular the elucidation of additional indole-diterpene compounds structurally-related to lolitrem B, and of further ergot alkaloids related to ergovaline. The agricultural implications of this diverse biosynthetic capability are as yet unclear, and a more complete and complex model of the chemical ecology of *N. lolii–L. perenne* association awaits development. Some of the resulting issues and opportunities are outlined in the discussion.

Figure 2 Biosynthesis of lolitrem B; schematic diagram showing precursors and transformation steps.



Endophyte metabolites

Although much of the scientific discussion of the biological activity of endophyte-infected ryegrass pastures has been delineated in terms of the three metabolites shown in Figure 1, for most classes of endophyte secondary metabolites, complexity and diversity is the rule. Peramine stands alone as the unique representative of a structural class of secondary metabolites characteristic of *Neotyphodium* fungi. Further studies may remove this unique status, as antibody binding measurements using ELISA (enzyme-linked immuno-sorbent assay) suggest the presence of an unidentified structurally related compound in *Achnatherum inebrians* (Miles *et al.* 1996). For other classes of compounds there is mounting evidence of diversity. This evidence is most dramatic in the realm of indole-diterpenes, but additional endophyte ergot alkaloids have also been identified. Developments for other classes of metabolites have been more limited.

Indole-diterpenes

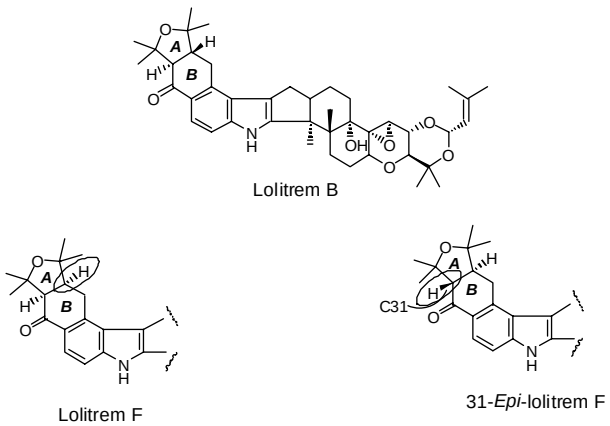
The initial report of the identification of lolitrem B indicated that at least one other related compound was present in *Neotyphodium lolii*-infected ryegrass seed (Gallagher *et al.* 1981). Detailed investigations by the AgResearch group at Ruakura have now established that a diverse array of indole-diterpene compounds is synthesised by this fungus. Lolitrem B, the first tremorgen characterised from *N. lolii*-infected ryegrass (Gallagher *et al.* 1984), remains the prime candidate as the major causative agent of ryegrass staggers. Lolitrem B is an indole-diterpene, one of a class of complex secondary metabolites derived from tryptophan and geranylgeranylpyrophosphate (Figure 2). Paspaline is the base structure and a probable key intermediate in the biosynthesis of many complex indole-diterpenes such as lolitrem B (Munday-Finch *et al.* 1996a). Indole-diterpenes have been found in a range of fungi, with a variety of substitution patterns and variants of elaboration of the carbon skeleton, and many have tremorgenic and anti-insect activity (Munday-Finch 1997; Munday-Finch *et al.* 1996a; Steyn & Vleggaar 1985).

The process of isolation of lolitrem B from *N. lolii*-infected ryegrass seed for studies of its tremorgenic activity has provided a source of material for detailed investigation of the indole-diterpene content. Some 20 indole-diterpenes have now been reported (Gatenby *et*

al. 1999, and references cited; Munday-Finch 1997), and a number of these are shown in Figures 3 and 4. Many of these compounds have the fused ring A/B system of lolitrem B, and this structural feature appears to be unique to the indole-diterpenes of *Neotyphodium spp.* fungi.

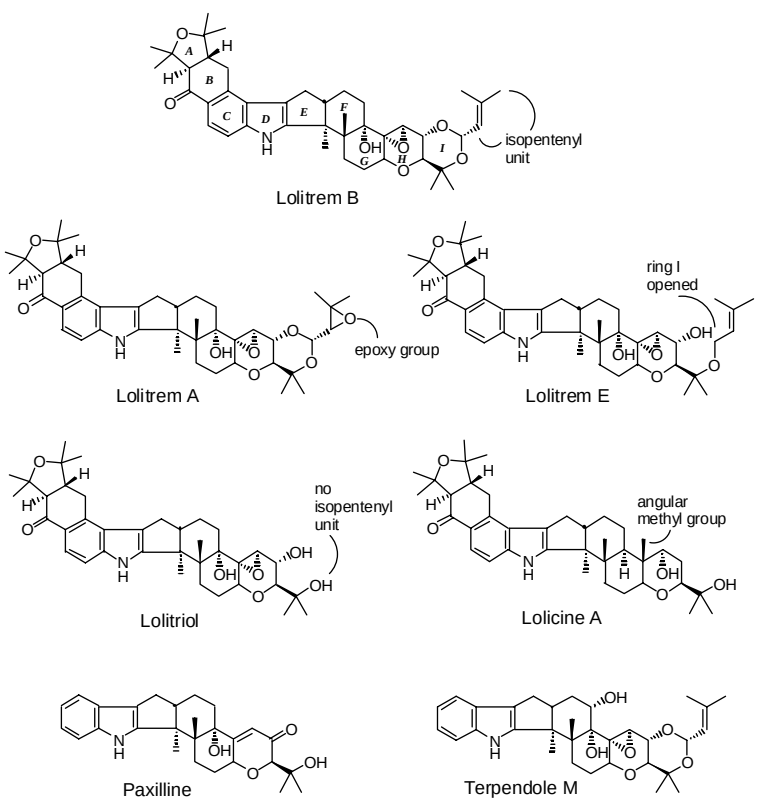
Some of these compounds differ only in stereochemistry around the ring A/B junction. Lolitrem F (Munday-Finch *et al.* 1996b) and 31-*epi*lolitrem F (Munday-Finch *et al.* 1998) are identical to lolitrem B but for the different spatial arrangement of the hydrogens attached to the ring A/B junction (Figure 3; the dotted wedged line indicates a hydrogen behind the plane, and a bold wedged line, a hydrogen in front of the plane). As a result, they differ rather subtly in molecular shape from lolitrem B. Both these compounds are also tremorgenic.

Figure 3 Lolitrem B and naturally-occurring ring A/B stereoisomers from *N. lolii–Lolium perenne*. (Wavy lines indicate remainder of structure identical to lolitrem B.)



Other compounds vary in substitution around rings F-I and the attached isopentenyl unit of lolitrem B and a representative selection of structures is shown in Figure 4. Lolitrem A (Munday-Finch *et al.* 1995) is a homologue of lolitrem B with an additional epoxy group on the isopentenyl unit. Lolitrem E is a variant with ring I opened (Miles *et al.* 1994), and lolitriol (Miles *et al.* 1992) lacks the attached isopentenyl unit of lolitrem E. Lolicine A (Munday-Finch *et al.* 1998) is less oxygenated than lolitriol, and has an additional angular methyl group. Some seventeen compounds with the distinctive ring A/B system have been reported to date. A range of substitution patterns around rings F-I has been found, many as mixtures of ring A/B stereoisomers (references cited above and Munday-Finch 1997; Munday-Finch *et al.* 1997).

Figure 4 Lolitrem B and selected homologues from *N. lolii–Lolium perenne* varying in ring substitution.



Indole-diterpenes lacking the distinctive lolitrem ring A/B system have also been found from *N. lolii*. Paxilline (Figure 4) was identified by Weedon and Mantle (1987) in *N. lolii* grown in culture. Recently further examples, including paspaline (the base structure for these complex indole-diterpenes, Figure 2) and terpendole M (Figure 4) (Gatenby *et al.* 1999) have been isolated from *N. lolii*-infected perennial ryegrass seed.

This plethora of variants of complex molecular structures, and the associated alphabet soup of

nomenclature, is rather daunting. No doubt yet more structural diversity remains to be revealed. However, there are patterns underlying this diversity. The indole-diterpenes of *N. lolii* can all be considered as products of the modification and elaboration of paspaline (Gatenby *et al.* 1999). Conversion to lolitrem B involves a number of steps (shown schematically in Figure 2): construction of the ring A/B system fused to the indole ring; removal of an angular methyl group at C-12; oxygenation at C-10, C-11, C-12, and C-13; and formation of ring I, a cyclic acetal with a pendant isopentenyl unit.

Many of these steps involve complex multi-stage chemical transformations, and details of the biosynthesis are not yet known. However, the diversity of products can be accounted for if this ensemble of transformations can occur in a variety of sequences. A matrix of inter-conversions can be envisaged, with multiple possible paths to any product (Gatenby *et al.* 1999). The compounds so far identified can be envisaged as intermediates, end products, or side products of this grid of transformations.

The biosynthetic pathways are largely hypothetical at this stage. The diversity of products may derive from transformations by enzyme systems of broad specificity accepting a range of substrates. Alternatively there may be a diversity of specific and selective biosynthetic genes and enzymes. However, the fungus appears to be able to biosynthesise a very wide range of indole-diterpene structures out of standard building blocks and transformations steps, effectively a biosynthetic kitset.

The modular mode of indole-diterpene synthesis is not unique to *Neotyphodium spp.* While the lolitrem ring A/B system is unique, most of the other conversion steps are to be found in the repertoire of other fungi, from several different fungal genera, which also synthesise indole-diterpenes. A variety of carbon structures attached to the benzenoid ring of the indole nucleus (the "left-hand side" of the structure), differing from the lolitrem ring A/B system, have been found in other fungi. However, the patterns of oxygenation and substitution of rings F-I are typical of fungal indole-diterpenes (Munday-Finch 1997; Steyn & Vleggaar 1985).

Although many of these compounds have been as yet reported only from the *N. lolii–L. perenne* association, this diverse biosynthetic capacity is unlikely to be unique to this single species of endophytic fungus. Lolitrem B has been reported in several other *Neotyphodium spp.* fungi *in planta* (reviewed in Lane *et al.* in press), and paxilline (or its analogues) has been found in a variety of *Neotyphodium spp.* grown in culture (Penn *et al.* 1993). In addition, ELISA evidence has been reported indicating unidentified indole-diterpenes are present in endophyte-infected *Echinopogon ovatus*, *Melica decumbens*, and *Poa huecu* (Miles *et al.* 1998 and references cited), each of which is associated with staggers syndromes.

Investigations of the biological role of *Neotyphodium* indole-diterpenes other than lolitrem B are very limited. Some patterns to the structural properties required for tremorgenicity are emerging (Gatenby *et al.* 1999; Munday-Finch *et al.* 1998) and are addressed elsewhere in this volume (Munday-Finch & Garthwaite 1999). Evidence of insect toxicity or feeding deterrence to pasture insects has been reported for a few of the compounds (Ball *et al.* 1997; Prestidge & Ball 1993). Several indole-diterpenes of other fungi have been isolated as insect-active factors (e.g., Belofsky *et al.* 1995). However, evidence of their significance as biologically active agents in grazed pasture awaits investigation.

Ergot alkaloids

While ergovaline retains its significance as a major factor in the biological activity of *Neotyphodium*-infected perennial ryegrass (and tall fescue), there is recent evidence of other compounds of the ergot alkaloid class which are possible precursors or products of ergovaline in these associations. Investigations in the USA of fescue toxicosis led to the discovery of ergot alkaloids, in particular the ergopeptide ergovaline, in the tall fescue endophyte, *Neotyphodium coenophialum* in culture (Porter *et al.* 1981) and *in planta* (Lyons *et al.* 1986; Yates *et al.* 1985). Subsequently Rowan and Shaw (1987) showed ergovaline, along with other ergo-peptides, was present in *N. lolii*-infected ryegrass. The probable role of ergovaline in heat stress of livestock in New Zealand has been noted (Easton *et al.* 1996). It was observed in this latter study that symptoms of heat stress in cattle grazing *N. lolii*-infected ryegrass in New Zealand are generally much less marked than for cattle grazing *N. coenophialum*-infected tall fescue in the USA, with similar or lower estimated concentrations of ergovaline.

Ergovaline undergoes ready isomerisation with its 8-epimer, ergovalinine, which differs only in the stereochemistry of attachment to the ergoline ring system (Figure 5). Ergovaline is thought to be the biologically active form of the compound, but isomerisation can take place variably in the plant, in stored samples, and during analysis. The practice in investigations in New Zealand has therefore been to measure ergovaline as the sum of both isomers (e.g., Easton *et al.* 1996). Thus the ergovaline values reported in the New Zealand literature may be 30–40% higher than in studies in the USA in which ergovaline alone has been measured. However

neither this difference of procedure, nor climatic differences (Easton *et al.* 1996), appear to account entirely for the difference in the impact on livestock, and there appear to be some underlying chemical differences.

Before its discovery in grasses infected with *Neotyphodium* species, the ergot alkaloid ergovaline was already known from the ergot fungus, *Claviceps purpurea* (Brunner *et al.* 1979). An extensive and diverse array of ergot alkaloids has been isolated from *Claviceps spp.* fungi (Flieger *et al.* 1997). These include clavine alkaloids, tricyclic or tetracyclic diamino compounds, such as chanoclavine-I; tetracyclic lysergic acid amides, such as ergine; in addition to complex ergopeptides such as ergovaline. They are all derived from the amino acid tryptophan and dimethylallyl pyrophosphate, and the biosynthesis of lysergic acid amides and ergopeptides takes place via the clavine alkaloid, chanoclavine-I (Gröger & Floss 1998) (Figure 6). Ergot alkaloids are known for their potent and diverse biological activity. Symptoms of ergot poisoning such as gangrene, and disturbances of the central nervous system, derive from the capacity of these compounds to interact with a wide spectrum of important receptors (e.g., Pertz & Eckart 1999). In addition, some ergot alkaloids show antimicrobial, cytostatic (Eckart & Pertz 1999) and immunomodulatory activity (Fiserova & Pospisil 1999).

There has been continuing uncertainty among US researchers about how significant ergot alkaloids other than ergovaline might be in fescue toxicosis (see e.g., Piper *et al.* 1997). Recently, Shelby and Flieger and co-workers (1997) have reinvestigated the ergot alkaloids of *N. coenophialum*-infected tall fescue in the U.S.A. by the powerful technique of liquid chromatography–mass spectrometry and made some interesting discoveries. In addition to ergovaline, and the known ergopeptides ergosine and ergonine (as minor components), Shelby *et al.* (1997) reported two unusual modified ergopeptides, dehydroergovaline and *aci*-ergovaline (Figure 5). While *aci*-ergovaline was already known as a product of isomerisation of ergovaline in the laboratory, dehydroergovaline was a novel compound, and some ambiguity remains about the details of its structure. Both these compounds retain the ergoline moiety of ergovaline unaltered, but differ in the tri-peptide structure. *Aci*-ergovaline differs from ergovaline only in the stereochemistry at C-2'. Dehydroergovaline is thought to have an additional carbon–carbon double bond either in the side-chain, or in the proline ring (Shelby *et al.* 1997) (see Figure 5). (Note: The trivial

Figure 5 Ergovaline, its C-8-stereoisomer ergovalinine, and closely-related compounds from *N. coenophialum–Festuca arundinaceae*, the C-2'-stereoisomer *aci*-ergovalinine and dehydroergovaline. (Wavy lines indicate remainder of structure identical to ergovaline.)

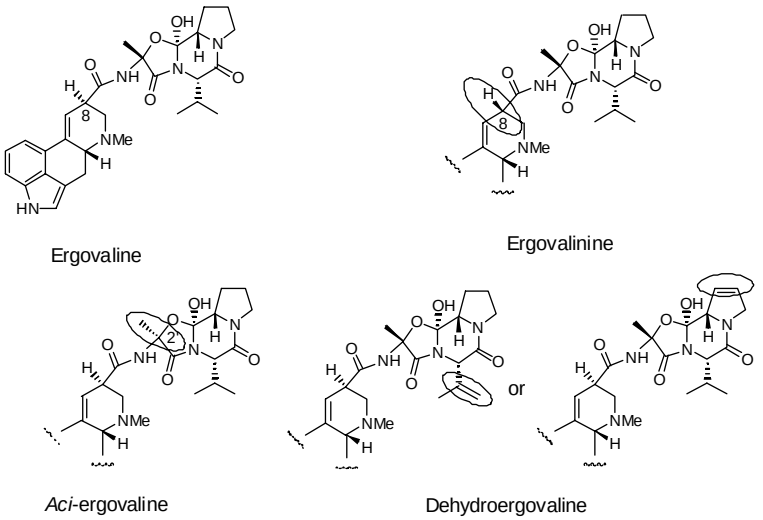
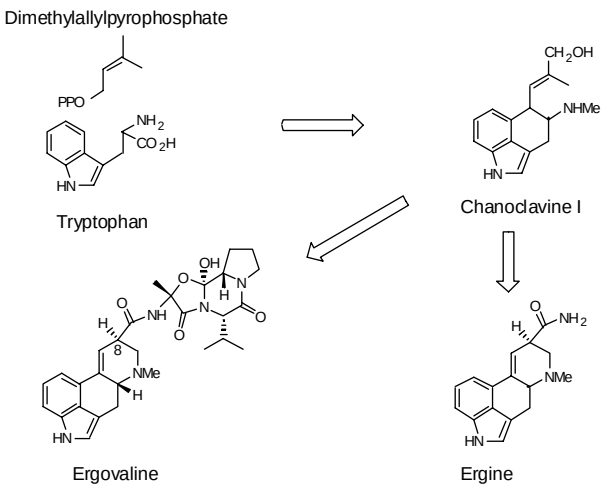


Figure 6 Biosynthesis of chanoclavine-I, ergine and ergovaline in *Claviceps spp.* fungi; schematic diagram showing precursors.



name dehydroergovaline is used here rather than didehydroergovaline as used by Shelby *et al.* (1997), as more appropriate for the compound corresponding to ergovaline–H_2).

Shelby *et al.* (1997) suggested that rather than being direct products of the endophyte itself, dehydroergovaline and *aci*-ergovaline may derive from modification of ergovaline by plant enzymes. They also suggested that other ergopeptides, such as ergotamine, previously reported from endophyte-infected grasses (Rowan & Shaw 1987; Yates *et al.* 1985), but typical of *C. purpurea*, are likely to have derived from low levels of contamination of samples by ergots.

We have followed up on these investigations, with the view that discoveries in the *N. coenophialum*–tall fescue association might be pertinent to the *N. lolii*–ryegrass association. We have also had the impetus of local scientific history. Cunningham (1949), on the basis of feeding experiments with tall fescue herbage at Wallaceville, first recognised that "fescue foot" of cattle grazing tall fescue was not caused by the ingestion of ergot sclerotia due to *Claviceps purpurea* infection of seedheads, despite the similarity of the symptoms. The discovery many years later that ergovaline is present in herbage of tall fescue infected with *N. coenophialum* (Lyons *et al.* 1986; Yates *et al.* 1985) may not be the end of this story.

Recently we have obtained evidence that endophyte-infected adventive tall fescue from Manawatu roadsides contains appreciable levels of dehydroergovaline, in addition to ergovaline, particularly in samples of immature flower heads (Lane *et al.* 1999). In this New Zealand material, dehydroergovaline appears to be present at rather higher concentrations relative to ergovaline than reported by Shelby *et al.* (1997) for US material. Dehydroergovaline is not yet available in pure form for testing. However, as ergopeptides typically show high biological activity (Berde & Schild 1978), this compound may well be a significant contributory factor to the high toxicity of endophyte-infected adventive tall fescue in New Zealand, and to the original discovery of fescue toxicosis by Cunningham (1949). We have yet to identify *aci*-ergovaline (Shelby *et al.* 1997) in the New Zealand material.

Dehydroergovaline does not appear to be present in *N. lolii*-infected perennial ryegrass (Lane *et al.* 1999). We have not detected the compound in seed or other tissues, even when concentrations of ergovaline were very high. Thus the differential in toxicity between endophyte-infected ryegrass and tall fescue (above) may derive at least in part from differences in the chemistry of the associations.

Simple lysergic acid derivatives also contribute to the ergot alkaloid toxicity of some endophyte-infected grasses. Very high concentrations of lysergic acid amides, including ergine, have been found in endophyte-infected sleepygrass (*Stipa robusta*) in the southwestern USA (Petroski *et al.* 1992), and in drunken horse grass (*Achnatherum inebrians*) from China (Miles *et al.* 1996). The most evident biological effect of these compounds is stupor of the grazing animal. Ergine has been reported in *N. coenophialum*-infected tall fescue seed (Shelby *et al.* 1997; TePaske *et al.* 1993), and tentatively identified in *N. lolii*-infected perennial ryegrass seed (TePaske *et al.* 1993), and we have recently confirmed these findings in New Zealand material (Lane *et al.* 1999). It is not clear whether ergine is present in herbage as the HPLC method is confounded by fluorescent interferences in this material.

Porter and co-workers found clavine alkaloids, including chanoclavine-I, in *N. coenophialum* in culture (Porter *et al.* 1981), and they subsequently were also found *in planta* (Lyons *et al.* 1986). Chanoclavine-I has also been reported from endophyte-infected *S. robusta* (Petroski *et al.* 1992). Recently Flieger and co-workers have reported the presence of chanoclavine-I in endophyte-infected perennial ryegrass herbage (Cagas *et al.* in press), and we have recently identified this compound in seed of endophyte-infected tall fescue and perennial ryegrass of New Zealand origin (Lane *et al.* 1999).

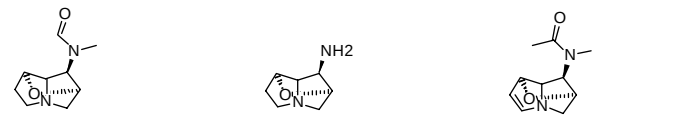
There have been only limited studies of the biological role and impact of the ergot alkaloids of *Neotyphodium spp.* other than ergovaline. Lysergic acid amides appear to be significant in the stupor of animals grazing *Neotyphodium*-infected sleepygrass (*S. robusta*) (Petroski *et al.* 1992) and drunken horse grass (*A. inebrians*) (Miles *et al.* 1996), but their impact in relation to fescue toxicosis is less clear (e.g., Piper *et al.* 1997). Recently, the activity of a range of endophyte metabolites and related compounds as feeding deterrents to black beetle (*Heteronychus arator)* has been investigated (Ball *et al.* 1997). The results suggest ergovaline and other ergopeptides, where present, are likely to be more significant as insect resistance factors than the simpler lysergic acid amides and clavines. However, further investigation of these issues is required.

Other compounds

Pyrrolizidine alkaloids of the loline group were the first class of *Neotyphodium* endophyte metabolites investigated, and are well known as insect toxins from *N. coenophialum*-infected tall fescue and other endophye-infected Pooid grasses (Lane *et al.* In press; Powell & Petroski 1992). As lolines have been recently shown to be produced by *N. uncinatum* in culture under appropriate conditions (C. Schardl, pers. comm.), it is

now clear these are fungal products, although key substrates may be provided by the plant. Typically several variant structures are found together in loline-producing grass–endophyte associations, with or without methylation and acylation of the amino nitrogen (e.g., *N*-formylloline, norloline, Figure 7). A ring-unsaturated variant has recently been reported from endophyte-infected *F. argentina* (Casabuono & Pomolio 1997), tentatively identified as 5,6-dehydro-*N*-acetylloline (Figure 7). Lolines are not usually found in *N. lolii*-infected *L. perenne* (TePaske *et al.* 1993), although they are named for their original discovery in the annual species *Lolium cuneatum* (Yunusov & Akramov 1955). However, a *N. coenophialum* strain produced lolines in perennial ryegrass (Siegel *et al.* 1990), and formation of lolines in endophyte-infected *L. perenne* (presumably with *N. lolii*) under temperature stress has been reported (Huizing *et al.* 1991).

Figure 7 Selected loline alkaloids of *Neotyphodium spp.* fungi.



N-formylloline Norloline 5,6-Dehydro-*N*-acetylloline

In New Zealand, loline alkaloids may not be a general feature of New Zealand ryegrass pastures, but they are present not only in introduced endophyte-infected adventive tall fescue of our road verges and adventive annual *Lolium spp.*, but also in our natural environment. Recently an investigation of samples of the endemic Australasian forest grass, *Echinopogon ovatus* (hedgehog grass) infected with *Neotyphodium spp.* fungi (Miles *et al.* 1998) showed that loline alkaloids were present in some New Zealand accessions. Ergovaline, peramine and lolitrem B were not found in these samples, and their current important role in mediating grass–herbivore interactions in New Zealand may have developed subsequent to the introduction of pastoral agriculture. However, Australian reports early in the century of toxic effects of *Echinopogon spp.* grasses on grazing stock, and recent ELISA evidence for the presence of unknown indole-diterpenes suggests other potent *Neotyphodium* metabolites are part of our natural heritage (Miles *et al.* 1998).

A range of other compounds has been found from grasses infected with *Neotyphodium spp.* fungi or their *Epichloë* relatives, including antifungal sesquiterpenes, and sterols (see Lane *et al.* In press). Recently a flavonoid with mosquito larvicidal activity was isolated from endophyte-infected blue-grass *Poa ampla* (Ju *et al.* 1998). This compound may well be of plant origin. The possible occurrence and significance of these and other *Neotyphodium* metabolites in grasses in New Zealand remains unexplored.

Conclusions

This extended list of metabolites from *N. lolii–L. perenne*, particularly the array of indole-diterpenes, poses a challenge for researchers. Clearly the fungus can synthesise a diverse array of metabolites. How significant are these compounds in the overall biological activity of endophyte-infected perennial ryegrass? Do they account for as yet unexplained or unnoticed effects? Do some of them provide benefits in insect resistance or other beneficial effects of endophyte infection, outweighing any neuro-toxicological hazards?

A primary requirement is a means of measuring these compounds (discovered largely in seed extracts) in herbage samples. To date, the methods we have been using for screening endophyte chemotypes, and analysing lolitrem B, ergovaline, and peramine in pasture samples (Barker *et al.* 1993), have been tightly focused around the physical and spectroscopic properties of the specific molecules of interest. Advances in methodology for identification and measurement of metabolites mean that measurements of a wide range of endophyte metabolites in pasture samples may now be practicable. In particular, recent developments in liquid chromatography–mass spectrometry suggest a more generic approach is now possible. The distinctive molecular masses of endophyte metabolites may provide the selectivity required to measure a range of these compounds simultaneously in a complex plant matrix.

Better understanding of the diversity of endophyte–grass metabolites may open up new prospects for grass–endophyte improvement. Investigations of the biology of endophyte–grass associations continue to reveal interactions where the underlying chemical agent(s) remain to be defined. If Shelby *et al.* (1997) are correct, and some of the metabolic diversification derives from plant modification of endophyte products, then new possibilities and routes for developing valuable associations may be generated.

While nature has been shaping endophyte–grass associations for millennia, deliberate human intervention is still very new. The development of a more complete and complex understanding of the chemical ecology of grass endophytes poses a scientific challenge. It also offers the prospect of new opportunities for endophyte–grass improvement.

ACKNOWLEDGEMENTS

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