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Review

Loline alkaloids: Currencies of mutualism

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Abstract

Several species of *Lolium* and other cool-season grasses (Poaceae subfamily Pooideae) tend to harbor symbiotic, seed-transmitted, fungi that enhance their fitness by various means. These fungal endophytes – species of *Neotyphodium* or *Epichloë* (Clavicipitaceae) – are known for production of antiherbivore metabolites such as the bioprotective loline alkaloids. Lolines are saturated pyrrolizidines with an *exo*-1-amine and an ether bridge between C-2 and C-7. The ether bridge is an unusual feature for a biogenic compound in that it links two bridgehead carbon atoms. Much of the loline-biosynthetic pathway has been elucidated by administering isotopically labeled precursors to fungal cultures and by comparisons of loline biosynthesis genes to known gene families. The first step appears to be an unusual γ-substitution reaction involving an enzyme related to *O*-acetylhomoserine (thiol) lyase, but which uses the secondary amine of L-proline rather than a sulfhydryl group as the nucleophile. The strained ether bridge is added after formation of the pyrrolizidine rings. Lolines with dimethylated or acylated 1-amines have insect antifeedant and insecticidal activities comparable to nicotine, but little or no toxicity to mammals. Considering the surprising abundance of lolines in some grass–endophyte symbiota, possible additional effects on plant stress tolerance and physiology are worth future consideration. In this review, we discuss the history of loline discovery, methods of analysis, biological activities and distribution in nature, as well as progress on the genetics and biochemistry of their biosynthesis, and on the chemical synthesis of these alkaloids.
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Keywords: *Epichloë* species; *Neotyphodium* species; Clavicipitaceae; Epichloe endophytes; *Lolium* species; Poaceae; Ryegrass; Fescue; Fungal metabolism; Symbiosis; Bioprotection; Mutualism; 1-Aminopyrrolizidines; Loline alkaloids; Gene clusters

Contents

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1. History of lolines

Lolium species (family Poaceae) include ryegrasses and broad-leaf fescues from Europe and the Mediterranean that have been established worldwide as extremely popular pasture and forage grasses. However, certain *Lolium* species have been associated with episodes of toxicity to livestock. Occasional references to livestock poisonings by ryegrasses date back to ancient Roman times and sparked 19th-century investigations by chemists searching for the toxic principle and by mycologists who suspected that a parasitic fungus might produce the toxin. By the 1890s, studies of a particularly notorious plant, *Lolium temulentum* L. (=*Lolium cuneatum* Nevski, darnel), yielded the first reports of a novel symbiotic fungus (Guérin, 1898) as well as a novel group of metabolites (Hofmeister, 1892), the lolines. The symbiont, now known as *Neotyphodium occultans* (Moon et al., 2000) (family Clavicipitaceae), is a mutualistic endophyte that transmits exclusively via systemic infections of seeds, such that it is maternally inherited like a plant organelle (Freeman, 1904). Curiously, despite the early discoveries both of this endophyte and of lolines in darnel, more than 70 years passed before clavicipitaceous endophytes and loline alkaloids were explicitly linked in the literature (Bush et al., 1993; Yates et al., 1989). Lolines have since been found in abundance in several pasture and wild grasses, always associated with congeners of *N. occultans* (which are classified as *Epichloë* species if they produce sexual fruiting structures, but otherwise as *Neotyphodium* species) (Craven et al., 2001; Justus et al., 1997; Siegel et al., 1990; TePaske et al., 1993a; Yates et al., 1989). Ultimately, neither the lolines nor *N. occultans* has been tied to livestock toxicosis. Instead, the lolines exhibit strong antiinsect activities (Riedell et al., 1991; Yates et al., 1989), a highly desirable characteristic of grass–endophyte symbiota in pastures.

The earliest report of an alkaloid of the loline class was by Hofmeister (1892), who identified a compound with the elemental formula $C_7H_{12}N_2O$ and named it temuline. Beginning six decades later, similar alkaloids were isolated from other *Lolium* species (Fig. 1), various structures were proposed, and gradually the commonality of their *exo*-1-aminopyrrolizidine-2,7-ether core (**1**) was demonstrated. The term *loline* was first used in 1955 (Yunusov and Akramov, 1955) to describe an alkaloid from darnel seeds (**2**), which was isolated together with a related alkaloid, *N*-acetylloline (**3**). Dannhardt and Steindl (1985) later determined that temuline – now commonly called norloline (**1**) – is related to **2** but lacks the *N*-methyl group.

In a further investigation of the darnel alkaloids, Yunusov and Akramov (1960b) allowed loline to react with HCl and obtained a hydroxylated and chlorinated product (Fig. 2). This result demonstrated that an oxygen atom bridged the two rings of a pyrrolizidine. From this derivative, the chlorine atom and hydroxyl group were selectively removed to yield a mixture of *N*-methylpyrrolizidine and free methylamine and pyrrolizidine. The pyrrolizidine

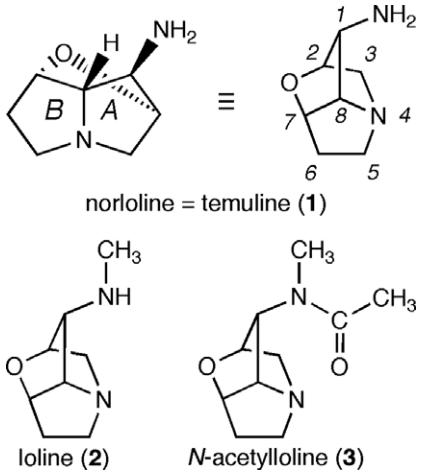

Fig. 1. The first three loline alkaloids discovered from *Lolium temulentum*.

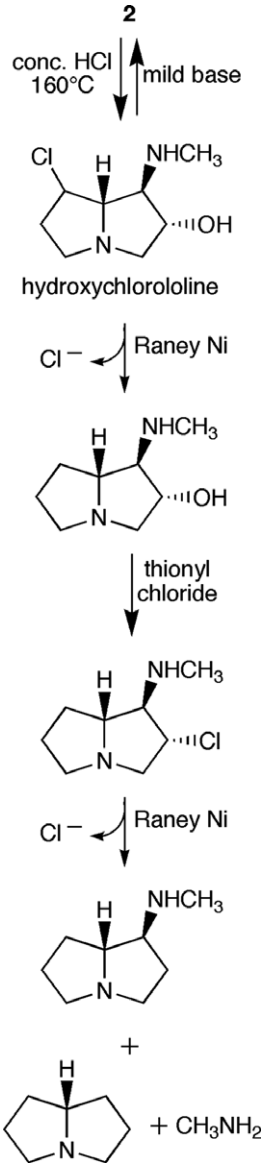

Fig. 2. Scheme showing the chemical treatment by which the ether bridge in lolines was demonstrated.

structure was determined by comparing the properties of their product with the properties of known pyrrolizidines.

Yunusov and Akramov (1960c) first suggested a structure for lolines with the non-ring N and O attached to the same ring C. The same researchers disproved this hypothesis six years later (Akramov and Yunusov, 1966), and instead suggested an *endo*-*N*-methyl-1-aminopyrrolizidine structure with a C-2–O–C-7 bridge. The structure proposed by Yates and Tookey (1965) for festucine from *Lolium arundinaceum* (Schreb.) S.J. Darbyshire (=*Festuca arundinacea* Schreb.; tall fescue) was similar except that the $NHCH_3$ substituent was placed on the *exo* face of the pyrrolizidine. Aasen and Culvenor (1969) confirmed this assignment by IR and proton NMR spectroscopy studies, which, together with melting point and mixed melting point studies, also demonstrated that loline was identical to festucine. The investigators noted that in the NMR spectrum, the H-5 protons were isochronous, which, for pyrrolizidines, was an unusual situation that they attributed to strain in the loline ring system. Without any explicit proposition, Aasen and Culvenor (1969) diagrammed a tricyclic system that was enantiomeric to the currently accepted configuration. The absolute configuration of loline was finally established by anomalous dispersion X-ray diffraction analysis (Bates and Morehead, 1972).

In the few years that followed, several lolines were identified from darnel and tall fescue, with combinations of methyl, formyl and acetyl groups decorating the 1-amine (Fig. 3), compounds **4**–**7** (Batirov et al., 1977a; Robbins et al., 1972). In addition to **7**, several other *N*-acylnorlolines (**8**–**11**) have been identified in *Adenocarpus decorticans* (Fabaceae) (Aasen and Culvenor, 1969; Powell and Petroski, 1992; Veen et al., 1992). Another acylnorloline, *N*-senecionoylloline (**12**) has been reported from horse urine and may be a metabolite of ingested lolines (Takeda et al., 1991). Lolidine (**13**), also reported from darnel, has one of the more unusual proposed structures: a loline alkaloid linked to another saturated pyrrolizidine that, instead of an ether bridge, has a chlorine atom at C-7 and is hydroxylated at C-2 (Batirov et al., 1977b). This pyrrolizidine structure, if confirmed, could suggest a pathway for ether bridge formation, as discussed later.

The biogenic source of lolines in grasses was not established until recently. The fact that cultured *Neotyphodium coenophialum* (Morgan-Jones et W. Gams) A.E. Glenn et al. (an endophyte of tall fescue) produced ergot alkaloids (Bacon, 1988) but not lolines suggested that lolines might be a host product induced by endophyte infection or the result of combined host and endophyte metabolism in the symbiotum (Porter, 1994). Ultimately, *Neotyphodium uncinatum* (W. Gams et al.) A.E. Glenn et al., isolated from *Lolium pratense* (Huds.) S.J. Darbyshire (=*Festuca pratensis* Huds., meadow fescue), was shown to produce lolines in defined-medium fermentation cultures when provided with sugars and either organic or inorganic nitrogen (Blankenship et al., 2001). This finding established that the fungus is fully capable of *de novo* synthesis of these alkaloids, implying that lolines in grasses are products of clavicipitaceous endophytes.

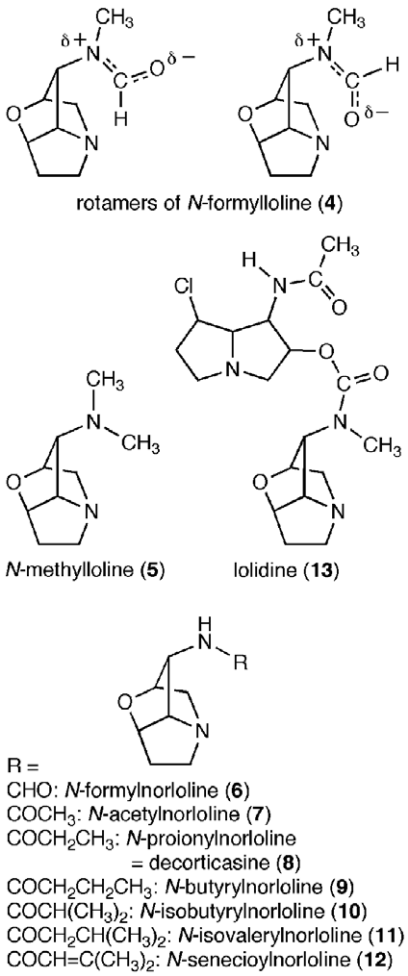

Fig. 3. Additional lolines from grasses (**4**–**7** and **13**) and Adenocarpus species (**7**–**11**), and an apparent metabolite identified in horse urine (**12**).

2. Methods for isolation and analysis of lolines

Extraction and chromatographic methods, as well as NMR and electron ionization (EI) mass spectra (MS), are reviewed by Powell and Petroski (1992). Here we provide highlights and updates of the methods currently in routine use.

Loline alkaloids from endophyte-infected grass tissues are typically extracted by organic solvents under basic conditions. Lolines **2**, **4** and **5** can then be separated on a silica gel column eluted with 5–20% MeOH in $CHCl_3$, and **1** can be purified from **2** on a neutral alumina column eluted with 2% MeOH in $CHCl_3$ (Petroski et al., 1989). Lolines can also be separated by paper chromatography or TLC on silica gel or alumina plates (Blankenship et al., 2001; Robbins et al., 1972; TePaske et al., 1993a,b; Yates, 1963; Yates et al., 1990; Yates and Tookey, 1965). High-speed countercurrent chromatography with a two-phase solvent system

composed of $CHCl_3$–MeOH–H_2O (5:4:3) provides a mild separation condition to minimize decomposition of the lolines (Petroski and Powell, 1991). The lolines are visualized by staining with a chromogenic agent such as potassium iodoplatinate, Dragendorff reagent, or iodine vapor (Casabuono and Pomilio, 1997; Huizing et al., 1991; Petroski et al., 1989; Tofern et al., 1999). Staining with 2-hydroxy-1-naphthaldehyde (Powell and Petroski, 1992) gives different color products with primary and secondary amines, thereby distinguishing **1** (lemon yellow) from **2** (orange), but giving no reaction to the tertiary amines and amides.

For quantitative analyses of lolines from either plant samples or biological fluids, quinoline or phenylmorpholine is added to the extraction solution as an internal standard (Blankenship et al., 2001; Justus et al., 1997; TePaske et al., 1993b; Yates et al., 1990). The samples are analyzed by capillary gas chromatography (GC) with flame-ionization detection or by GC–MS. GC retention times increase with molecular mass for **1**, **2** and **5**, and also for the *N*-acylnorlolines **6** and **7**, and *N*-acyllolines **3** and **4**. The retention times from shortest to longest are in the order **1**, **2**, **5**, **6**, **7**, **4**, **3** (Yates et al., 1990).

Several groups have published characteristic EI-MS data for the lolines (Akramov and Yunusov, 1966; Dannhardt and Steindl, 1985; Petroski et al., 1989; Robbins et al., 1972; Takeda et al., 1991). Takeda et al. (1991) characterized the fragmentation of **2** (*m*/*z* 154). Fig. 4 shows their proposed scheme, with refinements based on MS analysis of **2** from *N. uncinatum* administered ^{13}C- and ^{15}N-labeled precursors (Blankenship, 2004; Blankenship et al., 2005). A base peak at *m*/*z* 82 and peaks at *m*/*z* 95 and *m*/*z* 123 are characteristic of alkaloids in the loline series. The parent ions are *m*/*z* 196 for **3**, *m*/*z* 182 for **4** and **7**, *m*/*z* 168 for **5** and **6**, and *m*/*z* 154 for **2**. Compounds **4** and **7** can be distinguished by an *m*/*z* 154 fragment from **4** and *m*/*z* 139 and 153 fragment ions from **7**. Alkaloids **5** and **6** can be distinguished by the *m*/*z* 140 fragment from **6**.

^1H NMR chemical shifts have been reported for the dihydrochloride salt of **2** (Aasen and Culvenor, 1969), and for **3**, **4**, and **7** (Robbins et al., 1972). Detailed ^1H and ^{13}C NMR of the lolines was reported by Petroski et al. (1989), who listed all ^1H NMR chemical shifts and proton couplings and all ^{13}C NMR chemical shifts. Effects of rotational isomerism of **4** (see Fig. 3) are apparent in the ^1H and ^{13}C NMR spectra (LaPlanche and Rogers, 1963; Petroski et al., 1989; Robbins et al., 1972).

^{15}N NMR chemical shifts for **4** were reported by Blankenship et al. (2005). Each N atom gave two peaks, as expected for the two rotamers. The spectra were also concentration-dependent. As expected, the chemical shift of the amide (formylated) N was far downfield from that of the tertiary N of the pyrrolizidine ring.

3. Biological activities of lolines

In vivo studies employing aphids have consistently supported a role for lolines both in deterrence and insecticidal activity. In a survey of natural and cultivated grass–endophyte symbiota (Siegel et al., 1990), the presence of detectable loline alkaloids correlated with resistance to the bird-cherry oat aphid, *Rhopalosiphum padi* (Homoptera, Aphididae). In this study, all symbiota with lolines also had peramine, a known insect feeding deterrent. Although *R. padi* appeared to be unaffected by peramine, the possibility remained that lolines and peramine might act synergistically. Furthermore, the loline effect on another aphid, *Schizaphis graminum* (greenbug), could not be inferred because peramine effectively deters feeding by the greenbug.

Activities of lolines against insects have been reported consistently. In an early study, large milkweed bugs (*Oncopeltus faciatus*; Hemiptera) (Yates et al., 1989) were fed diets supplemented with alkaloid-containing fractions from *N. coenophialum*-symbiotic tall fescue. Those fractions enriched in lolines (but still containing some ergot alkaloids) were highly toxic to the insect larvae. Lolines **3** and **4** also deterred feeding by larvae (grubs) of Japanese beetle (*Popillia japonica*, Coleoptera) at alkaloid concentrations comparable to those measured in roots of tall fescue – *N. coenophialum* symbiota (Patterson et al., 1991). Such deterrence was not evident in glasshouse and field tests, although some significant negative effects of the endophyte were observed on survival and development of the grubs (Potter et al., 1992).

Riedell et al. (1991) tested **2**, **3**, **4**, and **5** against fall armyworm (*Spodoptera frugiperda*; Lepidoptera) and

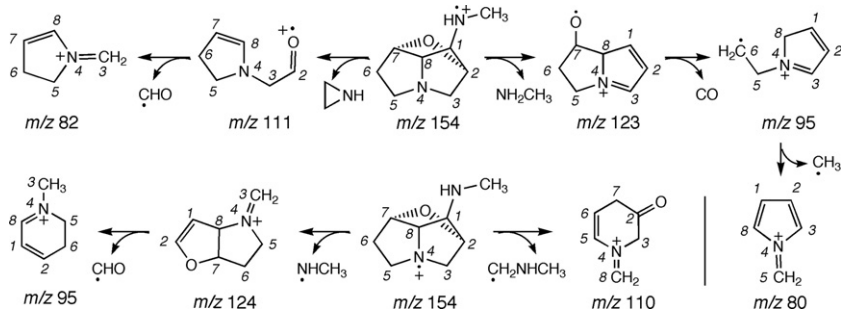

Fig. 4. Major species in electron ionization MS of **2**, and their derivations.

European corn borer (*Ostrinia nubilalis*; Lepidoptera). *N*-Formylloline (**4**) significantly deterred fall armyworm larvae in two-choice tests, and both **3** and **4** reduced larval weight gain in no-choice experiments. *N*-Acetylloline (**3**) also reduced weight gain in European corn borer. These tests did not determine if reduced weight gain resulted from toxicity, antifeedant activity, or both. In the same study, plants infested with the greenbug aphid were sprayed with solutions of lolines or nicotine sulfate, and the LC_{50} values for **3**, **4**, **5**, and the synthetic derivative, *N*-propionylloline, were within 95% confidence levels of the LC_{50} of nicotine. There was no evidence in this test of an antifeedant effect, suggesting that greenbug mortality was due to direct toxicity.

Clearly, the decorations of the 1-amine are important in antiinsect activity. In general, **2** exhibited rather little activity, and **5** was active against aphids but not did not reduce weight gain in *S. frugiperda* or *O. nubilalis* (Riedell et al., 1991). Many of the synthetic derivatives of **2** with larger acyl groups had significant effects on lepidopteran feeding and larval weight. Unfortunately, biological activities of the *N*-acyl derivatives of **1**, such as those reported from *Adenocarpus* species (see Fig. 3), have not been tested.

An interesting multitrophic effect was reported by Bultman et al. (1997), who added lolines to diets of fall armyworms that were parasitized by the wasps, *Euplectrus comstockii* and *Eu. plathypenae* (Hymenoptera) and observed significant negative effects on survival of the parasitoids. Lolines also appear to be involved in a different sort of multitrophic interaction involving the hemiparasitic plant, *Rhinanthus serotinus* (Schönh.) Oborny (Lehtonen et al., 2005). When parasitizing *N. uncinatum*-symbiotic meadow fescue, the hemiparasites exhibited greater resistance to the aphid, *Aulacorthum solani* than when parasitizing aposymbiotic meadow fescue. Lolines produced by *N. uncinatum* in the host are acquired by the hemiparasite, and may be responsible for the enhanced aphid resistance.

Refinement of genetic methods for the *Epichloë* species (Leuchtmann et al., 1994), and intraspecific variation in alkaloid profiles (Schardl, 2001), permitted a Mendelian analysis of the in vivo activity of lolines (Wilkinson et al., 2000). In no-choice tests, meadow fescue symbiota with the loline-producing endophyte, *N. uncinatum*, or with loline-producing isolates of *Epichloë festucae* Leuchtmann et al. (none of which produced the feeding deterrent, peramine) caused a highly significant decline in populations of *R. padi* (Fig. 5). The greenbug aphid appeared to be at least as sensitive to lolines. Two *E. festucae* parents differing in loline alkaloid expression were mated, and progeny were introduced into meadow fescue. These progeny segregated 1:1 for in vivo production of lolines, and only the loline alkaloid producers exhibited activity against the aphids.

Remarkably, wounding of plants induces high levels of lolines (Fig. 6). In this sense, lolines can be likened to typical plant defenses against chewing insects, except that they are fungal rather than plant metabolites. This response has

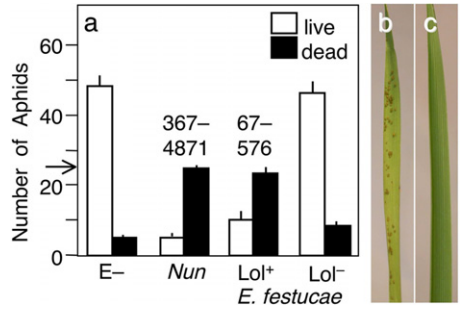

Fig. 5. Anti-aphid activity attributable to loline alkaloids. Panel a: Results of no-choice feeding tests of the aphid *Rhopalosiphum padi* on plants differing in endophyte and loline alkaloid status (from Wilkinson et al., 2000). Meadow fescue 'Predix' plants contained no endophyte (E−), *N. uncinatum* (Nun), or full sibling *E. festucae* progeny that segregated for loline alkaloid production (Lol^+) or nonproduction (Lol^-). The experiment began when 25 aphids (arrow) were placed onto 3 tillers of each plant. Live and dead aphids were counted after 72 h. Error bars = 1 SE. Three tillers of each plant were also assayed for loline alkaloids, and the ranges of alkaloid levels (μg combined **3** and **4** per g dry weight) are indicated above bars. Lolines were undetectable in E− and Lol^- plants. Panels b and c, leaves of tall fescue without endophyte (b) or with the loline-alkaloid-producing endophyte *N. coenophialum* (c) challenged with *R. padi* aphids. Aphids are feeding on the endophyte-free leaf but not on the endophyte-symbiotic leaf. Photographs in panels b and c were provided Dr. Stephen L. Clement.

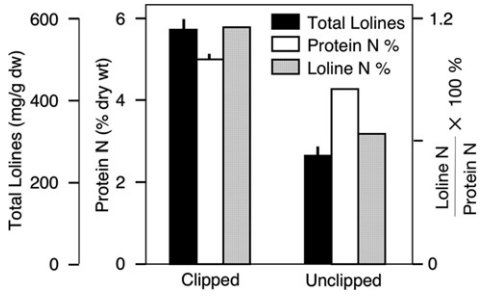

Fig. 6. Loline alkaloid levels from tall fescue-*N. coenophialum* symbiota 14 days following mock herbivory (clipping), and from unclipped control plants. Black bars indicate total **3** and **4**, white bars indicate the protein N as a percent of dry weight, and grey bars indicate loline-alkaloid N as a percentage of protein N. Error bars = 1 SE. Data are from Bultman et al. (2004).

been observed in tall fescue with *N. coenophialum* (Bultman et al., 2004; Kennedy and Bush, 1983) as well as meadow fescue with *N. uncinatum* (Craven et al., 2001). The most dramatic induction was observed in meadow fescue with *Neotyphodium siegelii*, in which total lolines (**3**, **4**, and **7**) increased from 0.1% to 1.9% of plant dry weight from zero to 11 days after clipping (Craven et al., 2001). This induction suggests communication between host and symbiont, so it will be of considerable interest in the future to determine the mechanism of induction. Conceivably, the endophyte might detect a specific signal from the wounded plant, or the alteration of host physiology may affect endophyte metabolism. For example, it is conceivable that precursor amino acids (Blankenship et al., 2005) become more

abundant in the plant apoplast, a possibility that is currently under investigation.

Natural and synthetic *N*-acyllolines have also been investigated for activity against alfalfa and annual ryegrass (Petroski et al., 1990). In tests with natural lolines **3**–**5**, none inhibited seedling growth, none inhibited alfalfa seed germination, and only **4** inhibited germination of annual ryegrass seeds ($ID_{50} = 770$ nmol per seed). Based on data in that study, it can be estimated that annual ryegrass seed mass averaged 1.2 mg, so the ID_{50} of **4** was approximately 640 μmol/g, or 120 mg/g seed mass. This is more than 10-fold higher than total lolines reported in seeds of various annual ryegrass species (TePaske et al., 1993a), so it is very unlikely that lolines significantly affect germination of endophyte-infected ryegrass seeds. Total loline alkaloid levels measured in roots of tall fescue range from 0.01 to 0.70 mg/g dry mass (Burhan, 1984), so concentrations exuded into the rhizosphere should be at least 100-fold lower than the LD_{50} for seed germination. Therefore, there is no evidence in the literature that lolines have ecologically or agronomically relevant activity against plants.

The possibility that lolines might have subtler effects on plant growth, structure and physiology deserves consideration. Growth effects of the tall fescue endophyte, *N. coenophialum*, have been noted consistently (Schardl et al., 2004). This endophyte, which produces abundant lolines, dramatically affects root growth, architecture, and physiology, presumably via growth regulators. Conceivably, lolines could be involved directly if they have growth-regulatory activity, or the loline pathway may alter the balance of other growth regulators such as polyamines (Martin-Tanguy, 2001), with which they share precursor amino acids. This endophyte also enhances plant tolerance of water deficit (Malinowski and Belesky, 2000), and the possible involvement of lolines as compatible osmolites or as scavengers of reactive oxygen species (Schardl et al., 2004) has been discussed. Such hypothetical effects of lolines on plant physiology and morphology remain to be addressed experimentally.

Early studies of loline alkaloid effects on mammalian systems employed crude or partially purified tall fescue extracts that probably had significant amounts of ergot alkaloids (Yates et al., 1989). The ergot alkaloids are extremely potent, and their known toxicities in livestock strongly suggest that they are the primary or sole cause of fescue toxicosis. This syndrome is caused by the tall fescue endophyte, *N. coenophialum* (Bacon et al., 1977; Lyons et al., 1986), which in addition to being one of the best natural sources of lolines, also produces ergot alkaloids at levels well within the known toxic range. Perhaps in part because lolines are so abundant in those symbiota, and in part because they are more easily assayed, loline presence quickly became a proxy for endophyte presence (Jackson et al., 1984). This, and the confoundment of early toxicity and physiological studies by ergot alkaloids in the extracts, has generated an impression even among some researchers that lolines are toxic to livestock. Nevertheless, no such link has been demonstrated, and effects on livestock and mammalian wildlife are likely to be negligible.

Vascular effects of lolines have been reported, but only when applied in vitro at extremely high concentrations (Oliver et al., 1998; Solomons et al., 1989). Lolines also can reduce release of prolactin by rat pituitary cells, but again, only at extreme concentrations (Strickland et al., 1994). In these studies, ergot alkaloids were several orders of magnitude more potent than the lolines. The identification of loline alkaloids in horse and bovine urine indicates that they are absorbed from feed; however, levels in blood plasma have been too low to detect, indicating that lolines are rapidly cleared (Takeda et al., 1991; TePaske et al., 1993b). Interestingly, a 10–1000 pM concentration of **3** exhibited a mitogenic effect on quiescent vascular smooth muscle cells, and also inhibited growing cells at 100–1000 pM (Strickland et al., 1996). These were levels comparable to those of ergot alkaloids with similar effects, and potentially might be physiologically relevant.

Loline alkaloids have been investigated for possible antitumor activities, and semisynthetic derivatives of lolines with acyl chain lengths of 8–12 were cytotoxic to solid tumors and in brine-shrimp assays (Petroski et al., 1994).

4. Loline alkaloid profiles of plants and symbiota

As discussed earlier, grasses with some species of clavicipitaceous endophytes accumulate lolines with simple adducts on the 1-amine: methyl, formyl or acetyl groups. Those isolated from *Adenocarpus* species have longer *N*-acyl groups, but lack *N*-methyl substituents. Lolines **2**, **4**, **5**, and **8** have also been reported from *Argyreia mollis* (Burm. f.) Choisy (Convolvulaceae) (Tofern et al., 1999). An intriguing possibility is that lolines in *Adenocarpus* and *Argyreia* species might be products of as yet undiscovered fungal symbionts. In fact, Clavicipitaceae have recently been discovered on leaf surfaces of *Ipomea asarifolia* and related plants (Convolvulaceae), and have been implicated as the sources of ergot alkaloids in these plants (Steiner et al., 2006).

Surveys have indicated that a minority of the grass species with clavicipitaceous endophytes also possess lolines (Leuchtmann et al., 2000; Siegel et al., 1990). Initially the high specificity of host species–endophyte species compatibility made it difficult to discern whether the presence or absence of lolines was attributable to host or endophyte genotype. Clearly, the presence of the endophyte is required for high loline alkaloid expression (Justus et al., 1997), and occasional claims of trace lolines in endophyte-infected grasses may be attributed to low background levels of endophyte-symbiotic plants in ostensibly aposymbiotic populations (Bush et al., 1993). Until recently, three of the four alkaloid classes associated with symbiotic *Neotyphodium* or *Epichloë* species had been detected in cultures of the isolated fungi, and only the lolines remained undetected in cultures. However, Siegel

Table 1
Endophyte-symbiotic grasses possessing lolines, and the alkaloid profiles of those symbiota

Host species	Host tribe	Endophyte species	Origin	**2**	**3**	**4**	**5**	**7**
Achnatherum robustum (Vasey) Barkworth	Stipeae	*Neotyphodium* sp.	USA	0/2	0/2	1/2	0/2	0/2
Agrostis hiemalis Britton et al.	Aveneae	*Epichloë amarillans* J.F. White	USA	0/3	0/3	0/3	n.t.	3/3
Echinopogon ovatus P. Beauv.	Aveneae	*N. aotearoae* Moon et al.	New Zealand	n.t.	0/1	1/1	n.t.	n.t.
Festuca versuta Beal	Poeae	*Neotyphodium* sp.	Texas	1/1	1/1	1/1	1/1	1/1
Hordeum bogdani Wilensky	Triticeae	*Neotyphodium* sp.	Central Asia	0/3	0/3	1/3	0/3	0/3
H. brevisubulatum (Trin.) Link	Triticeae	*Neotyphodium* sp.	Central Asia	0/2	0/2	1/2	0/2	0/2
Lolium arundinaceum	Poeae	*N. coenophialum*	USA	3/4	4/4	4/4	2/4	2/4
L. arundinaceum	Poeae	*N. coenophialum*	Morocco	5/6	5/6	5/6	n.t.	6/6
L. giganteum (L.) S.J. Darbyshire	Poeae	*E. festucae*	Europe	n.t.	2/2	2/2	n.t.	n.t.
*L. multiflorum*Lam.	Poeae	*N. occultans*	South Africa	0/1	1/1	1/1	0/1	1/1
L. persicum Boiss. et Hohen.	Poeae	*N. occultans*	Iran	0/1	1/1	1/1	0/1	0/1
L. pratense	Poeae	*N. uncinatum*	Europe	n.t.	6/6	6/6	n.t.	1/1
L. pratense	Poeae	*N. siegelii* Craven et al.	Germany	n.t.	1/1	1/1	n.t.	n.t.
Lolium sp.	Poeae	*Neotyphodium* sp.FaTG-3	Tunisia	1/1	1/1	1/1	n.t.	1/1
L. rigidum Gaud.	Poeae	*N. occultans*	Egypt	0/1	1/1	1/1	0/1	0/1
L. temulentum	Poeae	*N. occultans*	Greece	1/1	1/1	1/1	1/1	0/1
Poa alsodes A. Gray	Poeae	*Neotyphodium* sp.	N. Carolina	0/1	1/1	1/1	0/1	0/1
P. autumnalis Muhl.	Poeae	*Neotyphodium* sp.	Texas	n.t.	1/1	1/1	n.t.	n.t.

Shown for each alkaloid is the number of accessions in which the alkaloid was detected over the total number of accessions investigated. Not included are studies in which the alkaloids were not distinguished or insufficient information was provided concerning plant sources. n.t. = not tested.

et al. (1990) provided an important clue that lolines are fungal metabolites. The investigators established symbiota of perennial ryegrass (*Lolium perenne* L.) with *N. coenophialum* and of tall fescue with the perennial ryegrass endophyte, *Neotyphodium lolii*. Plants with *N. coenophialum* had **3** and **4**, regardless of host species, and plants with *N. lolii* always lacked lolines. Thus, the fungal genotype determined the loline-alkaloid production or nonproduction phenotype.

The fungal source of lolines was confirmed when Blankenship et al. (2001) devised culture conditions that induced loline alkaloid production by *N. uncinatum*, an endophyte associated with exceptionally high levels of lolines (up to 20 mg/g plant dry weight) in meadow fescue (Bush et al., 1993; Craven et al., 2001). This finding also enabled precursor-feeding studies (Blankenship et al., 2005; Faulkner et al., 2006) to elucidate the biosynthetic pathway, as described later in this review.

TePaske et al. (1993a) measured levels of alkaloids **2**–**6** in 21 accessions of 11 grass species. Table 1 presents a collation of results from this and other published surveys (Justus et al., 1997; Leuchtmann et al., 2000; Siegel et al., 1990), plus our own unpublished surveys, which demonstrate that lolines are produced in endophyte-symbiotic plants from numerous genera and tribes of the cool-season grass subfamily, Pooideae. Most of the surveyed species and accessions had **4**, which in *N. uncinatum* is produced by dimethylation of the 1-amine, followed by oxidation of one of the methyl groups (Blankenship et al., 2005). Also present in most accessions is the *N*-acetylated, *N*-methylated **3**. Interestingly, endophytes in all three accessions of *Agrostis hiemalis* and one Moroccan accession of *L. arundinaceum* apparently lack the ability to *N*-methylate **1**. These accessions had **7** but none of the other lolines analyzed.

5. Genetics of loline alkaloid biosynthesis

A Mendelian genetic study was initiated in 1994 to investigate the genetic determinants in *E. festucae* governing alkaloid expression (Wilkinson et al., 2000). Typically, *L. giganteum* (giant fescue) is symbiotic with *E. festucae* and possesses lolines (Leuchtmann et al., 2000; Siegel et al., 1990). An isolate from this grass was mated with fruiting structures (stromata) of *E. festucae* on *Festuca rubra*, a symbiotum in which lolines had not been detected (Wilkinson et al., 2000). The progeny were introduced into meadow fescue, and the resulting symbiota were tested for **3** and **4**. In addition, several backcrosses and a sibling cross were performed and analyzed for lolines. The loline-expressing (Lol^+) and nonexpressing (Lol^-) phenotypes consistently segregated in a 1:1 fashion, as would be expected if the determinant is a single locus in this haploid fungus. Parents and progeny were also analyzed by amplified fragment-length polymorphism (AFLP), and a linked molecular marker was identified.

Development of fermentation culture conditions for loline production by *N. uncinatum* (Blankenship et al., 2001) opened the door for a second molecular strategy to identify the loline biosynthesis genes (Spiering et al., 2002). The approach chosen was suppression subtractive hybridization, to selectively amplify by polymerase chain reaction (PCR), then clone, DNA copies (cDNAs) of regulated mRNAs. This method employed test RNA isolated from loline-alkaloid-producing *N. uncinatum* grown in defined minimal medium with 15 mM L-asparagine and 20 mM sucrose, and driver RNA from cultures in a complex medium that suppressed loline alkaloid production (Fig. 7). The basis for induction or suppression remains unknown, and it is to be expected that the different culture conditions would affect expression of genes for many met-

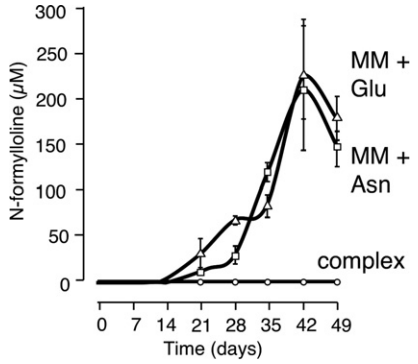

Fig. 7. Kinetics of loline alkaloid expression by *N. uncinatum* grown in three different media, minimal defined medium (MM) with glutamate (Glu) or aspartate (Asp) as sole nitrogen source, or a complex medium (potato dextrose broth). Unpublished data from study by Spiering et al. (2002).

abolic processes. To maximize the chance of finding loline biosynthesis genes, mycelium for test RNA was harvested at a time when the rate of loline alkaloid accumulation was nearly maximal. Two cDNAs identified in this study appeared to be from biosynthesis genes, were found in all loline-alkaloid-producing endophytes, and were absent in nonproducers. The corresponding genes were named *lolA* and *lolC*, in reference to their inferred homologies to aspartate kinase and cystathionine synthase genes, respectively.

The two genetic approaches converged when it was determined that the AFLP marker linked to loline expression in *E. festucae* (Wilkinson et al., 2000) was derived from the *lolC* gene of that endophyte (Spiering et al., 2005). In addition, a PCR test demonstrated that *lolA* and *lolC* were nearby in the *N. uncinatum* genome, and sequencing of the 8-kb PCR product revealed two more genes. Ultimately, a combination of genome-walking techniques revealed two homologous gene clusters (Fig. 8). One cluster (designated *LOL*1) contained nine protein-encoding genes, and close homologues of eight of these were identified in the second cluster (*LOL2*). A homologue of the ninth gene, *lolF* was also identified, but whether it is physically linked to *LOL2* remains to be determined. This second *lolF* gene is linked to another transcribed gene tentatively designated *seqX*, for which the possible association with lolines remains to be determined.

An exhaustive analysis of the mRNA sequences was conducted for each *LOL*-cluster gene in order to infer the polypeptide sequence for each gene product (Spiering et al., 2005). Except for *seqX*, each gene appears to encode a protein within the size range expected from biosynthesis enzymes or regulatory proteins. Furthermore, homology searches have revealed that eight of the nine predicted protein products are related to proteins of known function (Table 2).

The *lolC* gene has been especially intriguing, but also very difficult to study. The gene is related to those encoding γ-class pyridoxal phosphate (PLP) enzymes, of which all well-characterized members are involved in synthesis or interconversion of L-cysteine and L-methionine. Specifically, *lolC* is most closely related to *Aspergillus nidulans cysD* (and homologues in other fungi) encoding *O*-acetylhomoserine (thiol) lyase. However, *lolC* is unlikely to be a functional homologue of *cysD*. When the LolC coding sequence was placed under control of the *A. nidulans alcA* promoter, it failed to complement a *cysD* mutant. In fact, all detectable expression of the *lolC* sequence in *A. nidulans* transformants was lost following one subculture (Spiering et al., 2005). This, and failed attempts to clone full-length *lolC* cDNA in the bacterium, *Escherichia coli*, suggests that LolC or its enzyme product is toxic to cells. The proposed function of LolC is to condense L-proline (Pro) and L-homoserine (Hse), generating a biosynthetic intermediate that is actually toxic to cultured *N. uncinatum* (Faulkner et al., 2006). Presumably, subsequent steps in the pathway minimize accumulation of this toxic intermediate.

An RNAi (RNA interference) experiment provided evidence that LolC is involved in loline biosynthesis (Spiering et al., 2005). A synthetic gene was constructed to generate

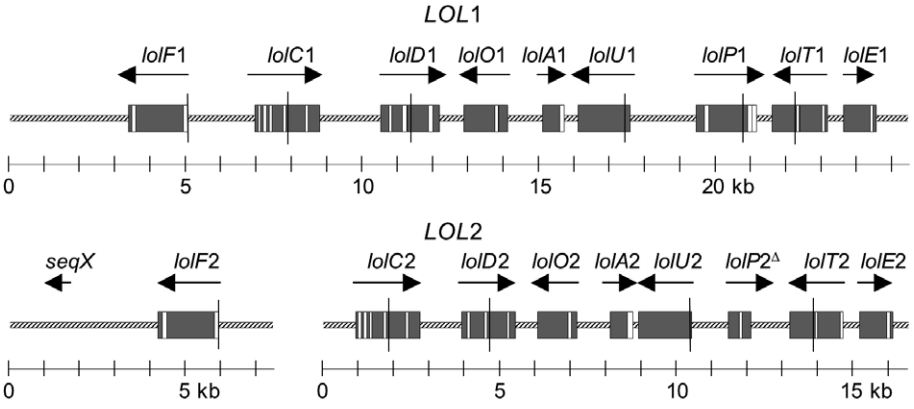

Fig. 8. Diagram of *LOL* clusters in *N. uncinatum*. Shaded rectangles indicate coding sequences, and white rectangles indicate introns. Long vertical lines indicate signature sequences for PLP binding sites in LolC, LolD and LolT, an FAD binding site in LolF, and a Myb DNA binding site in LolU. Arrows indicate directions and minimum lengths of transcripts. No single long open reading frame was predicted from the transcribed *seqX* sequence. The *lolP2*$^{\Delta}$ copy has a deletion that truncates the open reading frame. Data are from Spiering et al. (2005). GenBank Accession Nos. AY723749, AY723750, and AY724686.

Table 2
Characteristics of the predicted products of the *LOL*1 gene cluster of *N. uncinatum*

Gene	Predicted function	Size (amino acids)	Signature in predicted protein[a]	Protein families[b]
lolF	FAD-containing monooxygenase	539	VIVV**GAG**FS**G**ILAV (FAD-binding site)	PF00743.11; COG2072.2; IPR001100
lolC	γ-Type PLP-enzyme	473	DIVVHSAT**K**WIGGHG (PLP-attachment site)	PS00868; PF01053; IPR006235
lolD	α-Type PLP enzyme/group IV decarboxylase	451	FAV**K**SSYDRRLIQTLATCG (family 2 PLP attachment site) ARRVGLNPTVLDI**GGG**YT (family 2 signature 2)	PS00878; PS00879; PF02784; IPR000183
lolO	Oxidoreductase/dioxygenase	362	2-His-1-carboxylate facial triad (**H**222, **D**224, **H**280)	PF03171; IPR005123
lolA	Amino-acid binding	209	nd[c]	COG0527 (partial)
lolU	Possible DNA-binding protein	495	WTRSEDGSL (Myb transcription factor DNA-binding domain repeat signature 1)	PS00037
lolP	Cytochrome P450 monooxygenase	496	FGLGRWQ**C**AG (cysteine heme-iron ligand signature)	PS00086; PF00067; IPR001128
lolT	α-Type PLP enzymes	454	PDFFVSDCH**K**WLFVPRPCAV (class-V PLP-attachment site)	PS00595; PF00266; COG0520; IPR000192
lolE	Epoxidase/hydroxylase	256	2-His-1-carboxylate facial triad (**H**125, **D**127, **H**162)	PF05721; COG5285; IPR008775

[a] Functionally conserved amino acids in the signature patterns are indicated in bold.
[b] PS, prosite; PF, pfam; IPR, interpro; COG, clusters of orthologous groups.
[c] nd, none detected.

an RNA transcript with a region identical to the first exon of *lolC*, followed by an intron, then the reverse-complement of the first exon. This transcript should generate a double-stranded RNA that would trigger degradation of the normal *lolC* mRNA. Indeed, *N. uncinatum* transformants with this gene had only ca. 25% of the *lolC* mRNA, and ca. 50% of the total lolines as *N. uncinatum* transformed with a control plasmid.

Results of another test suggested that LolP catalyzes oxidation of **5** to **4**. The *LOL*2 version of this gene (*lolP*2$^{\Delta}$) has an apparent deletion relative to *lolP*1. This deletion has also shifted the reading frame such that the predicted LolP2 product is much smaller than LolP1, and lacks the key heme-binding site of cytochrome P450 monooxygenases. A marker-exchange mutagenesis removed *lolP*1. Elimination of the only functional *lolP* in *N. uncinatum* altered its loline alkaloid profile. As expected, **4** was absent in the mutant, whereas **5** accumulated in abundance (M.J. Spiering, J.R. Faulkner and C.L. Schardl, unpublished data).

The predicted enzyme activities of *LOL*-cluster gene products fit well with the biosynthetic pathway, as discussed in the next section. Given the available methods for gene disruption and complementation in fungi, including *Neotyphodium* and *Epichloë* species (Tanaka et al., 2005; Wang et al., 2004; Young et al., 2005), combined genetic and biochemical studies should allow a thorough elucidation of the pathway and the mechanisms of some of the more unusual steps such as the condensation of Pro and Hse, and the formation of the ether bridge.

6. Biosynthesis of lolines

The lolines and their biosynthetic pathway have some unique features. For example, an ether bridge that joins two aliphatic bridgehead C atoms is extraordinary for a biological molecule, so the mechanism of its synthesis will be especially interesting to elucidate. Also, the proposed first determinant step in the pathway is a previously unprecedented γ-substitution reaction in which a secondary amine acts as the nucleophile (Blankenship et al., 2005; Faulkner et al., 2006) yielding **14** (Fig. 9). If LolC is responsible for this step, as seems likely, then it will be fascinating to explore its structural relationship with, and evolution from, enzymes of sulfur metabolism.

The proposed second step in the pathway is oxidative decarboxylation of **14** to **15** and a second decarboxylation catalyzed by another PLP-containing enzyme, probably LolD (Faulkner et al., 2006). The product of the second decarboxylation, 1-(3-aminopropyl)pyrrolinium (**16**) lies at the crossroads between the previously proposed pathway from polyamines and the currently favored pathway from Pro and Hse (Bush et al., 1993; Faulkner et al., 2006) (Fig. 9).

Early literature focusing on structural comparisons with plant necines conjectured that lolines might be derived from polyamines (Bush et al., 1993). Whereas homospermidine is the first pathway intermediate to necines (Ober and Hartmann, 1999), the position of the 1-amine in lolines (as opposed to a C at that position in necines) suggested that lolines could be derived from spermine (Spn) or spermidine (Spd) (Bush et al., 1993) (Fig. 9). When it was believed that lolines were plant metabolites, this proposal was particularly attractive because plant polyamine oxidase metabolizes Spn to **16** (Sebela et al., 2001). The polyamine hypothesis was tested and rejected in precursor administration studies that employed loline-producing cultures of *N. uncinatum*. For example, such a pathway was inconsistent with the observation that ^{13}C from C-5 of L-ornithine (Orn) specifically incorporated into C-5 of **4** (Blankenship et al., 2005).

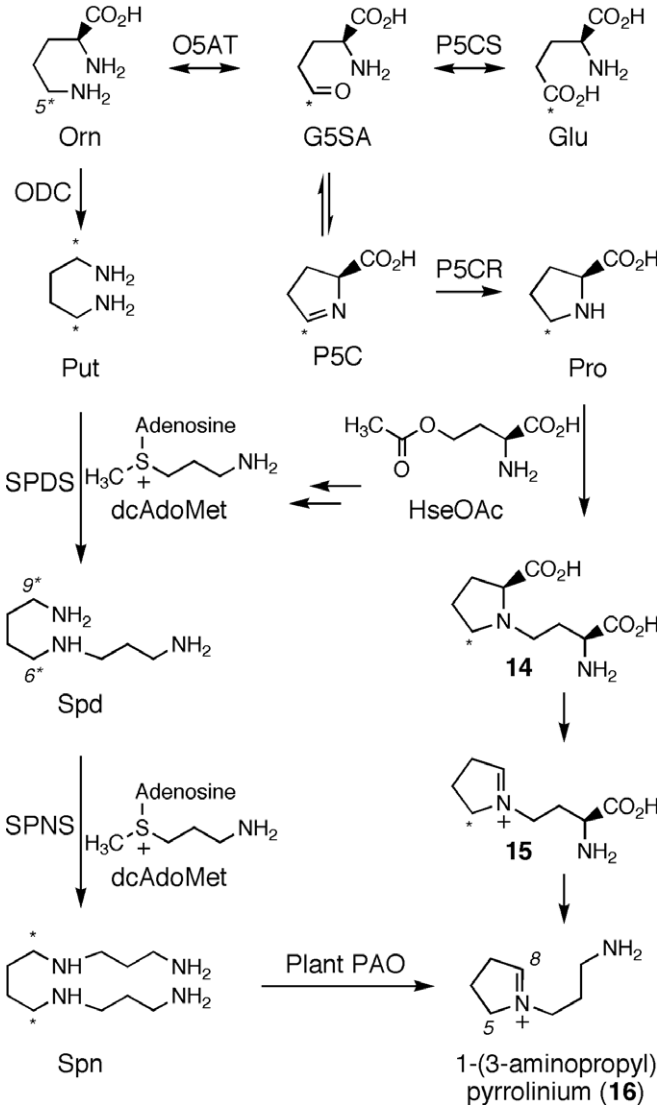

Fig. 9. Polyamine pathway (left) and early steps in the proposed loline pathway (right), showing common precursors and intermediates. Metabolites are: Orn, L-ornithine; Put, putrescine; dcAdoMet, decarboxylated *S*-adenosylmethionine; Spd, spermidine, Spn, spermine; G5SA, L-glutamic acid semialdehyde; Glu, L-glutamic acid; P5C, 1-pyrroline-5-carboxylate; Pro, L-proline; HseOAc, *O*-acetylhomoserine. Enzymes are: O5AT, Orn-5-aminotransferase; ODC, Orn decarboxylase; SPDS, Spd synthase; SPNS, Spn synthase; P5CS, P5C synthetase; P5CR, P5C reductase; PAO, polyamine oxidase. Asterisks (*) indicate the fates of C-5 from Orn.

Orn is decarboxylated to putrescine (Put), a precursor of Spd and Spn (Fig. 9). Because Put is a symmetrical molecule, C-5 of Orn becomes C-6 in half of the Spn molecules, and C-9 in the other half. Therefore, if lolines were derived from Spn, the expectation would be that label from C-5 of Orn would distribute equally to C-5 and C-8 of lolines. The observation that only C-5 was labeled (Blankenship et al., 2005) implied that the positional information from Orn was retained, and that free Put was never an intermediate in the pathway. Furthermore, [^{14}C]Put failed to incorporate into **4**, but incorporated into Spd as expected. In contrast, radiolabeled Orn, Pro, and L-glutamate (Glu) were all incorporated into **4**.

The interesting possibility remains that loline-producing endophytes might incorporate **16** generated from host metabolism of Spn, as well as synthesizing **16** from Pro and HseOAc (Fig. 9).

Orn is one of a group of amino acids that are readily interconverted biologically; it is a precursor and product of L-arginine via the urea cycle, which can also be a precursor of Glu and Pro via L-glutamate-5-semialdehyde (G5SA) (Fig. 9). Transamination of Orn yields G5SA, which is in equilibrium (by loss of H_2O) with L-1-pyrroline-5-carboxylate (P5C). Reduction of the latter is one of the routes to Pro. When loline-producing cultures were fed [^{14}C]Orn, [^{14}C]Glu or [^{14}C]Pro, all universally radiolabeled with ^{14}C, by far the highest specific activity of product was obtained from [^{14}C]Pro (Blankenship et al., 2005). Likewise, when such cultures were administered [^{15}N,U-^{13}C]Pro, label was detected by NMR in the ring N and C-5, C-6, C-7, and C-8 of **4**. GC–MS indicated 51–54% of compound **4** was labeled in cultures fed 5 mM [^{15}N,U-^{13}C]Pro, and 32–33% in cultures fed 5 mM [1,2-^{13}C]Orn. Thus, Pro is the likely donor of the N and four C atoms of the loline alkaloid B-ring.

The sources of the A-ring C atoms and 1-amine were identified in a similar set of experiments (Blankenship et al., 2005) (Fig. 10). Cultures administered ^{15}N-labeled L-aspartate (Asp) or [^{15}N]Hse produced [1-^{15}N]**4** as determined by ^{15}N NMR and GC–MS. Likewise, GC–MS indicated that the isotopic label from [4,4-$^{2}H_2$]Hse was incorporated into **4**, and both NMR and GC–MS demonstrated that label from [4-^{13}C]Asp was incorporated into C-3 of **4**. Such patterns of incorporation would have suggested Spd or Spn as a precursor but for the contraindication from feedings with labeled Orn. Hse is derived from Asp and is a precursor of several amino acids including L-methionine (Met), which in turn donates (via *S*-adenosylmethionine) the aminopropyl groups of Spd and Spn (see Fig. 9). However, there was no detectable incorporation of label from [U-^{13}C]Met in the ring structure of **4**. The possibility that this amino acid failed to enter the cells and contribute to the pool of *S*-adenosylmethionine was deemed unlikely, because the methyl and formyl carbons of **4** were efficiently labeled in this experiment as well as in feedings with [6-^{13}C]Met.

The incorporation of both deuterium atoms from [4,4-$^{2}H_2$]Hse indicates that Pro and Hse were condensed by γ-substitution. Considering that Hse is derived from Asp by two reduction reactions, it was conceivable that Hse is oxidized to L-aspartate-4-semialdehyde or to Asp before introduction into the loline pathway. The former could conjugate with Pro to form an iminium ion (Schiff base), or the latter could form a γ-peptide with Pro. However, retention of the deuterium atoms indicated that C-4 of Hse is never oxidized in the pathway (Blankenship et al., 2005).

Given the evidence for Pro and HseOAc as loline alkaloid precursors, the simplest hypothesis is that a γ-substitution reaction condenses these two molecules to form the

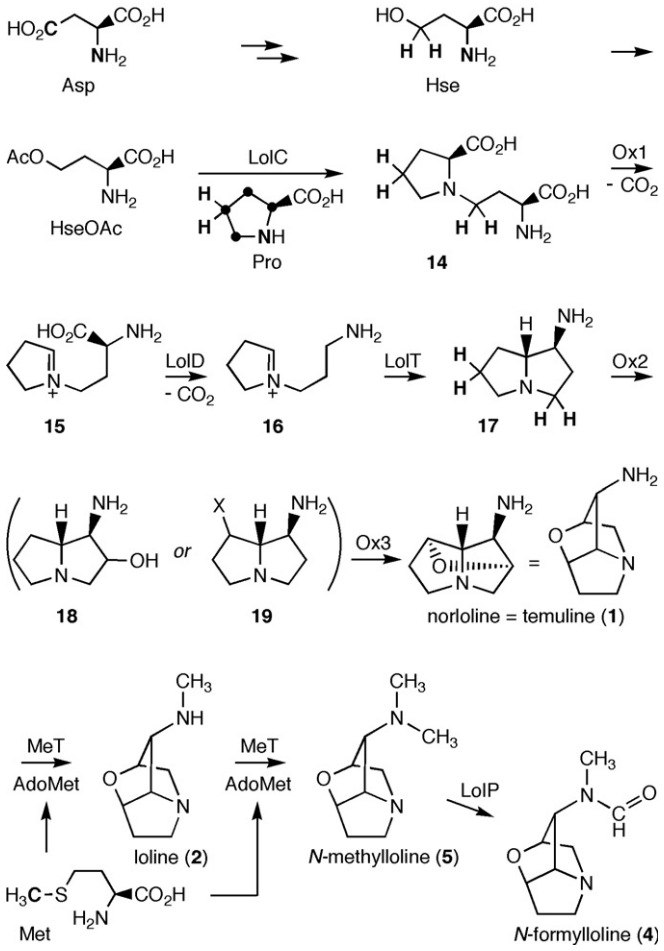

Fig. 10. Proposed loline-alkaloid biosynthetic pathway, and a summary of the results of Blankenship et al. (2005) and Faulkner et al. (2006). Precursors of *N*-formylloline were inferred by feeding studies with labeled L-aspartate (Asp), L-homoserine (Hse), L-proline (Pro), **1**, **4**, or L-methionine (Met). Labeled atoms – indicated by dots or bold letters – were incorporated into lolines as predicted by the pathway shown here. Enzymes likely to catalyze pathway steps are designated above arrows. Genes for LolC, LolD, LolT and LolP are clustered in the *LOL* locus, along with genes for three enzymes (LolE, LolF, and LolO) proposed to carry out the oxidative steps labeled Ox1–Ox3. Functions for LolA, LolU, and LolX – also encoded in *LOL* – are not apparent. No obvious methyltransferase (MeT) gene has been identified in *LOL*. Also unidentified is the gene for an *N*-acetyltransferase likely to catalyze synthesis of *N*-acetylnorloline and *N*-acetylloline (not shown). LolP is also predicted to catalyze formation of *N*-formylnorloline from loline (not shown). The X in compound **6** indicates OH or Cl; AdoMet = *S*-adenosylmethionine.

C-3–N-4 bond. Such a mechanism of C–N bond formation appears to be unprecedented in the biochemical literature. To further test the hypothesis, the proposed condensation product **14** was synthesized in various deuterated forms for labelling experiments (Faulkner et al., 2006). One of these labeled compounds had two deuterium atoms at the equivalent position as the aforementioned [4,4-$^2H_2$]Hse; the other had two deuterium atoms at this position plus two deuterium atoms at the Pro C-4 position. Significant incorporation of label in **4** was detected in both administration experiments, though the levels of incorporation (1.0–2.2%) were far less than in the various amino-acid-administration experiments discussed above. This lower incorporation was probably due to two factors: the necessity to use much less of the labeled **14** (only 1–2 mM), because higher concentrations inhibited fungal growth, and poor uptake of **14** by the fungal cells compared to the uptake of common amino acids. Nevertheless, levels of label incorporated from **14** were significantly higher than (unfed) controls, whereas there was no detectable incorporation from the closely related *N*-(3-aminopropyl)proline or from the 6-hydroxylated form of **14**, indicating that the incorporation of **14** was specific. These results suggested that the pathway-determinant step is a novel γ-substitution reaction (Fig. 10).

Other precursor-administration experiments addressed whether the pyrrolizidine ring system was complete before the ether bridge formed. *exo*-1-Aminopyrrolizidine (**17**) was synthesized with two deuterium atoms either on C-3 or C-6 (loline numbering) and administered to cultures (Faulkner et al., 2006). Incorporation of label from **17** depended on the amount given to cultures, with 1 mM, 2 mM and 4 mM, respectively, giving 2.0%, 3.0% and 5.5% labeled **4**. This result was consistent with the results of administration with L-4-*cis*-hydroxyproline, L-4-*trans*-hydroxyproline, or the correspondingly hydroxylated forms of **14**, none of which gave significant incorporation of label in **4**. Therefore, the loline-alkaloid pathway appears to proceed by formation of the *exo*-1-aminopyrrolizindine, followed by addition of an O atom bridging C-2 and C-7.

The *N*-methyl groups of **2**–**5** derive from the 6-methyl group of Met, as expected if methylation is catalyzed by a typical *N*-methyltransferase. Interestingly, the formyl group of **4** is formed by oxidation of one of these methyl groups (Blankenship et al., 2005). (Compound **6** is much less abundant and has not been investigated, but is probably generated the same way.) Recent evidence discussed in the previous section implicates the cytochrome P450 monooxygenase encoded by *lolP*, which may catalyze two sequential oxygenations (subsequent dehydration would give the formyl group). In this sense, the process is similar to that of plant cytochrome-*a* oxygenase, which, however, has a [2Fe–2S] Rieske center rather than a heme iron (Tanaka et al., 1998).

The loline alkaloid pathway (Fig. 10) has been almost completely elucidated in six years of research, but a few questions remain. For example, mechanisms and timing of decarboxylation reactions remain to be determined. The Pro moiety may be decarboxylated in the first oxidation step, as shown in Figs. 9 and 10, or at the cyclization step forming **17**. Furthermore, decarboxylation at C-1 either precedes or follows A-ring formation. Finally, whereas it is now apparent that the ether bridge is added to the fully formed pyrrolizidine ring system, the enzymes and specific mechanism remain to be determined. The proposed structure of lolidine (**13**) is relevant as evidence that the ether bridge may derive from a C-7-halogenated intermediate. Facile conversion of hydroxychlorololine back to

2 (see Fig. 2) (Yates and Tookey, 1965) lends additional credence to this possibility. This would be an unusual biochemical pathway, but the O-link between bridgehead atoms is, after all, an unusual biological structure.

7. Semisynthesis and total synthesis of lolines

Interconversions between lolines with methyl, formyl or acyl substituents at the 1-amine, followed by chemical and NMR spectroscopic analyses, helped elucidate the natural array of these alkaloids (Petroski et al., 1989; Yunusov and Akramov, 1960a). Loline (**2**) was prepared from mixed lolines (mainly **3** and **4**) by hydrolysis with 1 N HCl at 80 °C for 3 h (Petroski et al., 1989). Norloline (**1**) was prepared by reaction of **2** with $KMnO_4$ in cold 20% H_2SO_4. To prepare **5**, an equimolar mixture of CH_2O and HCO_2H was added to a solution of **1**, and the mixture was allowed to reflux for 4 h. Yunusov and Akramov (1960a) oxidized **5** with aqueous $KMnO_4$ in 20% H_2SO_4 to yield **4**. In contrast, Petroski et al. (1989) formylated **1** and **2** with ethyl formate to yield **6** and **4**, respectively. Reaction of **1** with phenyl acetate (96 h, room temperature) yielded **7**, and reaction of **2** with acetyl chloride (same conditions) yielded **3**. Similar reactions of **2** with the appropriate acyl chlorides yielded a series of synthetic lolines with linear acyl chain lengths of 3, 4, 6, 8, 9, 10, 12, 14, or 16 carbons, and other acyllolines, for toxicity and allelopathy tests (Petroski et al., 1990).

Several attempts to prepare **2** were made before the first successful synthesis was reported in 1986 (Tufariello et al., 1986). Glass et al. (1978) were able to make the loline skeleton **21** via an intramolecular cyclization of amino epoxide **20** (Fig. 11). An independent strategy by Wilson et al. (1981) involved transannular addition of a suitable electrophile to an unsaturated amine **22** to construct **23**. Neither group was able to displace the leaving group at the bridging C atom with a nitrogen nucleophile to obtain a loline derivative.

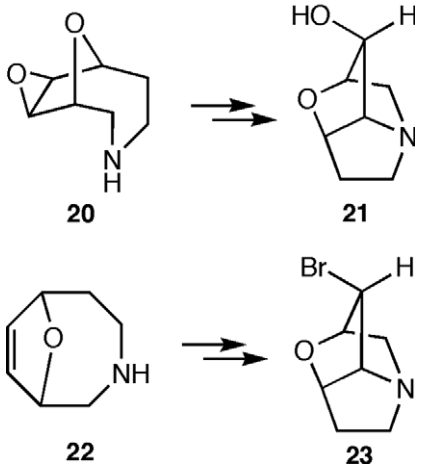

Fig. 11. Early syntheses of the loline ring system.

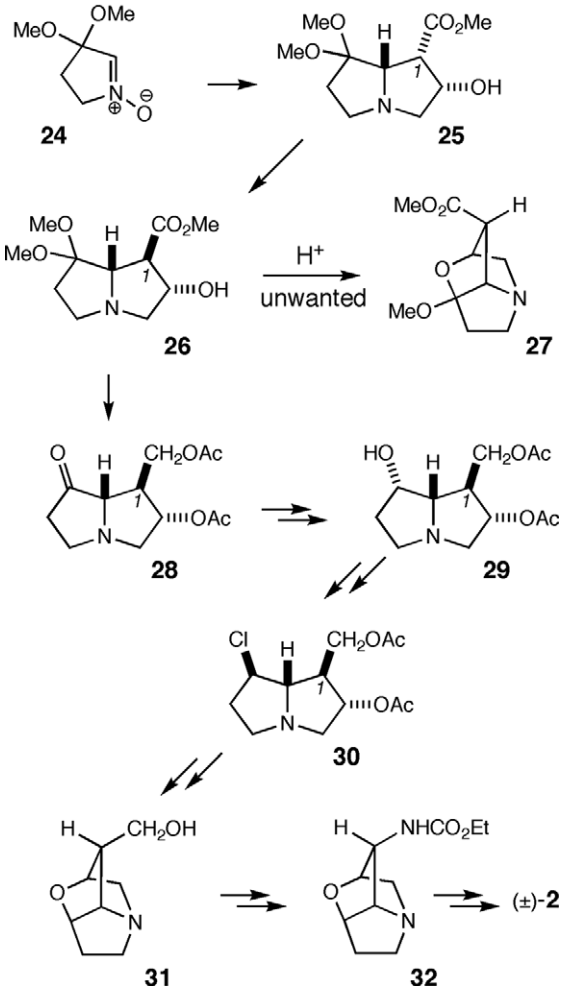

Fig. 12. First total synthesis of (±)-loline (Tufariello et al., 1986).

In the first complete synthesis of **2** (Fig. 12), Tufariello et al. (1986) used nitrone-based methodology, which had been successfully applied to several pyrrolizidine alkaloids (Tufariello and Lee, 1980; Tufariello and Tette, 1971). Dimethoxynitrone **24** was prepared in three steps from *N*-ethyl-3-pyrrolidinone, and methyl 4-hydroxycrotonate was combined with **24** to give **25**. The C-1 stereochemistry in **25** was opposite that required for **2**, but treatment of **25** with NaOMe caused complete epimerization at C-1, providing **26** in excellent yield. The driving force for the epimerization of **25** was removal of a transannular steric compression between the CO_2Me group and one of the MeO groups. Nevertheless, it was somewhat surprising that elimination of the C-2 OH group did not compete with epimerization.

In order to avoid cyclization after hydrolysis of the ketal to form the bridged ketal **27**, hydroxy ester **26** was reduced to a diol and protected as the diacetate before the acetal was hydrolyzed to ketone **28** (Tufariello et al., 1986). Hydrogenation of this ketone by the Adams catalyst in glacial acetic acid gave only alcohol **29**, which was a result of selective hydrogenation from the less hindered, convex, face of the ketone. The alcohol was converted to the

chloride (**30**) with inversion of configuration, and upon deprotection of both OH groups, facile cycloetherification to tricyclic **31** occurred. Finally, a Curtius rearrangement of the carboxylic acid derived from oxidation of **31** gave carbamate **32**, which was reduced with $LiAlH_4$ to complete the synthesis of racemic loline.

An important aspect of this synthesis was the use of a dipolar cycloaddition of appropriately functionalized substrates to produce the pyrrolizidine ring system efficiently. The synthesis also employed an efficient method to reduce ketone **28** to alcohol **29** with very high diastereoselectivity. Furthermore, the synthesis utilized a Curtius degradation to introduce the C-1 amine, avoiding the problems that had plagued previous workers in their attempts to effect nucleophilic substitution at C-1. As a result, the complete total synthesis was accomplished in only 12 steps, which included no poorly selective steps, an impressive accomplishment for this topologically complex and nitrogen-rich small compound.

The first asymmetric synthesis of (+)-loline (Blakemore et al., 2000) was completed in 20 steps from (*S*)-malic acid. The key to the strategy was an intramolecular hetero-Diels–Alder reaction of a reactive acylnitrosodiene to construct the pyrrolizidine framework (Blakemore et al., 2001) (Fig. 13).

The most suitable precursor of **33** was nitrosodiene **34**. Hydroxamic acid **35**, which was prepared from (*S*)-malic acid in 9 steps, was oxidized to **34**, which spontaneously underwent an intramolecular hetero-Diels–Alder reaction (Fig. 14). The most favorable conditions resulted in the formation of the target oxazine **36a** and its diastereomer **36b** in a 57:43 mixture, but **36a** and **36b** were readily separable by silica gel chromatography. Oxazine **36a** was then converted to pyrrolizidine lactam **37** in three steps. The olefinic bond of lactam **37** was then aminohydroxylated under conditions previously described by Li et al. (1996). This oxidation from the *exo* face of **37** provided the desired amino alcohol **38** and its regioisomer in a 3:1 ratio together with a significant amount of diol (Blakemore et al., 2001). Subsequent functional group manipulation yielded hydroxy mesylate **39**, which on direct thermolysis cyclized to *N*-tosylloline **40**. Reductive removal of the tosyl group resulted in (+)-loline (**2**), isolated as its dihydrochloride salt. The ^{1}H and ^{13}C NMR spectra of the synthetic material exactly matched that reported for the natural compound (Petroski et al., 1989).

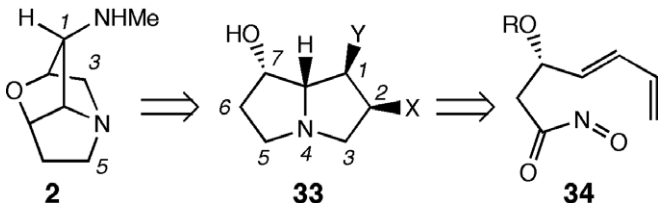

Fig. 13. Retrosynthetic analysis of loline (Blakemore et al., 2001).

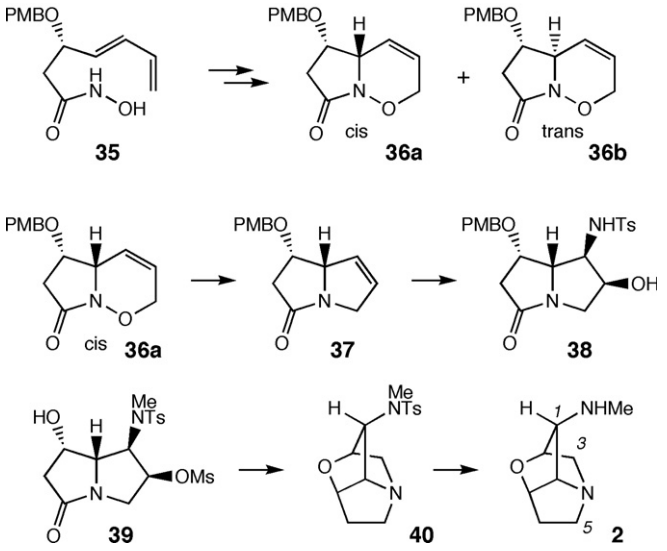

Fig. 14. Total synthesis of (+)-loline (**2**) (Blakemore et al., 2001).

Considering that loline has only a seven-carbon-atom skeleton, a 20-step asymmetric synthesis that includes two key steps with modest diastereo- and regioselectivity is less efficient than one might expect would be possible. Yet, more than 50 years after the loline structure was elucidated, these are the only two complete syntheses in the literature (Blakemore et al., 2001; Tufariello et al., 1986), suggesting that this small natural product is surprisingly difficult to synthesize. Both reported syntheses start with construction of the pyrrolizidine ring system, then construct the ether bridge later in the scheme. Alternatives to this basic strategy will be interesting to explore.

8. Conclusions

Mutualistic symbiosis requires currencies to be contributed by each partner to the benefit of the other partner, such that benefits more than counterbalance costs of the symbiosis. In many grasses, clavicipitaceous symbionts contribute antiherbivore alkaloids that deter or sicken insects, mammals or other animals that may feed on the grasses. Early attention focused on lolines as a possible cause of toxicity to livestock grazing *L. temulentum* or *L. arundinaceum*, but subsequent research has established that these alkaloids are far more active against insects than mammals. Other endophyte alkaloids – namely ergot alkaloids and indolediterpenes – have been implicated in livestock toxicoses. In current efforts to minimize or eliminate such alkaloids in forage cultivars, retention of the lolines is considered desirable for protection from insects (Hunt and Newman, 2005). The facts that lolines are rich in nitrogen and are often produced in abundance even in uncultivated grass–endophyte symbiota (Miles et al., 1998; Siegel et al., 1990; TePaske et al., 1993a) substantiate their roles as currencies of mutualism. Lolines are known to be active against insects in at least four orders

(Patterson et al., 1991; Riedell et al., 1991; Yates et al., 1989), but a comprehensive survey of herbivorous arthropods and assessment of the mode of action remain to be undertaken for these alkaloids.

Although there are several types of pyrrolizidines known in nature (Hartmann, 1999), the lolines are atypical of biogenic molecules in having a strained ether bridge between aliphatic carbon atoms. This feature has sparked interest in their biosynthetic pathway as well as in methods of organic synthesis. Like the synthetic schemes published to date, the fungi apparently add the ether bridge to a completed pyrrolizidine ring system. The specific biosynthetic mechanism is unexplored. A semisynthetic pathway involving a chloride leaving group (Yunusov and Akramov, 1960b), and the reported structure of **13** with chlorine at the equivalent position (Batirov et al., 1977b), suggest intriguingly that halogenation may be involved in the biosynthesis as well.

Early suggestions that the lolines may be polyamine derivatives have been disproved by feeding studies with isotopically labeled precursors (Blankenship et al., 2005; Faulkner et al., 2006). However, loline and polyamine pathways share common precursors. Orn is the immediate precursor of Put and can be converted to the common amino acid and loline precursor, Pro. Hse appears to be a direct precursor of lolines and, via Met and dcAdoMet, contributes the aminopropyl moieties of Spd and Spn. Considering the importance of polyamines in plant development and stress tolerance (Bouchereau et al., 1999; Couee et al., 2004; Walters, 2000) and the high levels of lolines in some symbiota, an interesting question is, does competition for these precursors affect polyamine levels and metabolism? If so, how does this relate to endophyte effects on hosts, such as enhanced drought tolerance and altered root growth (Malinowski and Belesky, 2000; Schardl et al., 2004)?

Another unique aspect of the loline pathway is the γ-substitution by which Pro is condensed with Hse. The predicted protein sequence of the *lolC* gene product suggests that it catalyzes this reaction, because its closest known homologues catalyze γ-substitution reactions of Hse with thiols in the sulfur amino acid biosynthetic pathways (Spiering et al., 2005). As this and other steps are understood in more detail, specific enzymes are definitively associated with specific reactions, and the relationships of their structures and activities are clarified, a greater understanding of the evolution of the biosynthetic pathway and gene cluster will emerge. Also, as more fungal and other genomes are sequenced, the possibility that *lol*-gene homologues are involved in other secondary metabolite pathways can be tested, and metabolomic surveys may uncover related alkaloids in other organisms.

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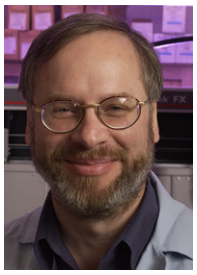

Christopher L. Schardl was born in Oxford, England in 1957, and raised in 13 hamlets, towns and cities in the US and Canada. He has a B.S. in Biochemistry from Cornell University (1978), and a Ph.D. in Biochemistry from the University of California, Davis (1983), where he worked with Clarence I. Kado on nopaline catabolism by *Agrobacterium tumefaciens*. Then, at the Plant Breeding Institute, Cambridge, England, he worked with David M. Lonsdale on the structural dynamics of maize mitochondrial DNA. In 1985, Dr. Schardl joined the University of Kentucky to begin a molecular biology program on plant-symbiotic fungi. He has also been a Visiting Scholar at Massey University, New Zealand (1992–1993), and a Gast-professor at the Swiss Federal Institute of Technology, Zürich (2003). Dr. Schardl's research group and collaborators have described numerous new species of *Epichloë* and *Neotyphodium*, shown that many grass endophytes are interspecific hybrids, cloned and characterized the first ergot-alkaloid biosynthesis gene, identified the loline alkaloid gene clusters, elucidated much of the loline biosynthetic pathway, and sequenced the *E. festucae* genome. Dr. Schardl holds the Harry E. Wheeler Chair in Plant Mycology, directs the University of Kentucky Advanced Genetic Technologies Center, and is a Kentucky Colonel, a Fellow of the American Phytopathological Society, and a Fellow of the Mycological Society of America.

Robert B. Grossman, a native of Long Island, New York, earned his A.B. at Princeton University, where he carried out research under the direction of Robert A. Pascal. After graduating in 1987, he moved to MIT and worked under the direction of Stephen L. Buchwald to develop zirconium- and titanium-mediated and -catalyzed organic synthetic methodology. He earned his Ph.D. in 1992 and moved from Steve's lab in Cambridge, Massachusetts to Steve's lab in Cambridge, England, where he worked in the Ley group on various aspects of the chemistry of azadirachtin. In 1994 he left the UK to join the faculty at UK in Lexington, Kentucky. His research interests are currently focused on synthetic methodology, total synthesis, and biosynthetic pathways. He is the author of *The Art of Writing Reasonable Organic Reaction Mechanisms*, second ed. (Springer, 2002), and one of the cocreators of ACE Organic, a Web-based organic chemistry homework program that provides response-specific feedback to structures drawn by students.

Jerome R. Faulkner was born on July 21, 1978 in Princeton, Kentucky. He obtained a B.S. in Chemistry and a B.S. in Biology in 2001 from Kentucky State University where he was a United States Department of Agriculture 1890 scholar. Upon obtaining his B.S. he worked two years as biologist with the USDA. Natural Resources Conservation Service. Currently he is a Ph.D. student in Plant Pathology at the University of Kentucky, and is investigating the loline alkaloid biosynthetic pathway. He is a father, husband, and proud native Kentuckian.

Padmaja Nagabhyru was born and grew up in Andhra Pradesh, India. She received her B.Sc. degree in Agriculture (1995–1999) from Acharya N.G. Ranga Agricultural University, Bapatla, India. She did her Masters (1999–2001) and Ph.D. (2002–2005) in Plant Pathology from Indian Agricultural Research Institute, New Delhi, India. For her M.Sc. degree she has worked on the biochemical aspects of host pathogen interaction with particular reference to 'Defense enzymes produced in cotton in response to elicitors and *Xanthomonas campestris* pv *malvacearum*', and for her Ph.D. she worked on 'Molecular and pathogenic variability studies in Indian isolates of *Ustilago segetum* fsp *tritici* causing loose smut of wheat'. She joined Dr. Schardl's lab as a Post doctoral scholar in 2006 and is working on 'Tall fescue endophyte effects on environmental stress tolerances, the plant endophyte mutualism and metabolite cross talk.

Uma P. Mallik was born on January 8, 1977, in Bagnan, Howrah, West Bengal, India. He received his B.Sc. with Chemistry Honors, at Bagnan College, Calcutta University in 1999, and his M.Sc. in Organic Chemistry, 2001, from the Indian Institute of Technology, Bombay, where he worked with Prof. K.D. Deodhar. In 2002 he began his Ph.D. studies at the UK Department of Chemistry, working with Prof. R. B. Grossman.