LOLINE ALKALOID BIOSYNTHESIS IN *NEOTYPHODIUM UNCINATUM*, A FUNGAL ENDOPHYTE OF *LOLIUM PRATENSE*

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ABSTRACT OF DISSERTATION

Jimmy Douglas Blankenship

The Graduate School
University of Kentucky
2004
ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

By
Jimmy Douglas Blankenship

Lexington, Kentucky

Director: Dr. Christopher Lewis Schardl, Professor of Plant Pathology

Lexington, Kentucky

2004

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ABSTRACT OF DISSERTATION

LOLINE ALKALOID BIOSYNTHESIS IN NEOTYPHODIUM UNCINATUM, A FUNGAL ENDOPHYTE OF LOLIUM PRATENSE

Some endophytes in mutualistic associations with Festuca, Lolium and other grass species produce insecticidal loline alkaloids (1-aminopyrrolizidines; LA). These loline alkaloids have a saturated pyrrolizidine ring system (two-rings sharing a carbon and nitrogen atom), a 1-amine substituted with methyl, acetyl, or formyl groups, and an oxygen bridge between C-2 and C-7. The development of a reliable system of production of LA in cultures of the Lolium pratense (meadow fescue) endophyte, Neotyphodium uncinatum, facilitated work on the LA biosynthetic pathway. N. uncinatum produced norloline, loline, methyllocline, N-acetylnorloline (NANL), N-formylloline (NFL), and N-acetyllocline as detected in culture filtrates. The total production of the two most abundant alkaloids, NANL and NFL, approached 1000 µg ml⁻¹ of fungal filtrate. ¹H and ¹³C chemical shifts were previously reported for this group of alkaloids. Extraction and synthesis of sufficient quantities of the alkaloids allowed determination of previously unknown ¹⁵N chemical shifts of some LA. Knowledge of ¹³C and ¹⁵N chemical shifts allowed identification of precursors by feeding stable-isotope-labeled compounds. Initially, due to structural similarity to other plant pyrrolizidines, this study examined putrescine and spermidine as possible precursors to LA. Feeding of ¹⁴C putrescine to the fungal cultures failed
to demonstrate any enrichment in the LA, but enriched spermidine. In contrast, cultures fed with positionally labeled $^2$H, $^{13}$C and $^{15}$N amino acids — namely, L-ornithine, L-proline, L-aspartate, L-homoserine, and L-methionine — demonstrated specific isotopic enrichment in NFL.

Determination of the enrichment from the labeled amino acids utilized $^{13}$C and $^{15}$N NMR (nuclear magnetic resonance) and gas chromatography-mass spectroscopy (GC-MS). This study allowed the biosynthetic origins of all carbons and nitrogens of NFL to be determined. NFL incorporated L-proline into the B-ring and L-homoserine into the A-ring and 1-amine. The results strongly indicated that polyamines are not precursors of LA and implicated a novel biochemical pathway for the synthesis of LA.

KEYWORDS: Neotyphodium uncinatum, Fungal Endophyte, Lolium pratense, Loline Alkaloids, $^{13}$C and $^{15}$N NMR
LOLINE ALKALOID BIOSYNTHESIS IN *NEOTYPHODIUM UNCINATUM*, A FUNGAL ENDOPHYTE OF *LOLIUM PRATENSE*

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DISSEPTION

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Chapter 1
Loline alkaloids of fungal endophytes of cool season grasses

Biology of symbiota

Fungal endophytes occur naturally in the many cool season grasses in symbiotic relationships. The two fungal genera, *Epichloë* and *Neotyphodium*, comprise closely related endophytes that live in the apoplasts of their host grasses (Clay and Schardl, 2002; Schardl, 1996; Schardl, 2001; Schardl and Phillips, 1997). These fungi belong to the family Clavicipitaceae, order Hypocreales in the phylum Ascomycota. The *Epichloë* endophytes maintain the capability to reproduce sexually, whereas *Neotyphodium* spp. are strictly asexual. These sexual endophytes, such as *Epichloë typhina*, can in fact be somewhat pathogenic on their host grasses by producing choke disease. Choke disease occurs during the reproduction of the grass when the endophyte shuts down flower maturation. The endophyte produces stromata on the surface of the "choked" tiller where spermatia develop. Symbiotic flies (*Botanophila* spp.) carry fungal spermatia to other stromata for fertilization to occur. These fertilized stromata produce orange perithecia which shoot ascospores that are capable of infecting nearby inflorescences (horizontal transmission). Infected seed develop from these inflorescences and carry the endophyte to the next generation of grass. Some *Epichloë* species exhibit a mixed strategy by choking some tillers while spreading asexually through the seed on other tillers (vertical transmission). The asexual *Neotyphodium* spp. exhibit only vertical transmission. Fungal hyphae grow coincides with tiller elongation and infect seeds for asexual (clonal) propagation of the endophyte.

The fungal endophytes exist solely in the apoplastic region of the plant (Philipson and Christey, 1986). This group of fungi does not penetrate the host cells to produce haustoria, intracellular hyphae, or arbuscules as do mycorrhizal fungi. Since the endophytes live exclusively inside the plant, they must acquire all required nutrients from their host plants. One of the most important of these nutrients is a carbon source, notably sucrose (Lam et al., 1994). Fungal endophytes also obtain nitrogen-containing compounds from their plant host. Mechanisms for delivery to the apoplast of carbon- and nitrogen-containing compounds exist in plants as demonstrated in other symbiotic relationships and some parasitic interactions. Specific mechanisms for export of sugars and amino acids into the apoplast and fungal uptake are
reported (Hall and Williams, 2000; Smith and Smith, 1990). In these symbiotic relationships, the plant also benefits.

Several studies implicate the fungal endophytes in many beneficial effects on the grasses. Endophyte-symbiotic grasses better resist some parasitic fungi and nematodes, and tend to be less frequently infected by certain viruses (probably by deterring insect vectors) than do aposymbiotic (endophyte-minus) plants of the same species (Gwinn and Gavin, 1992; Kimmons et al., 1990; Mahmood et al., 1993). Drought tolerance increases with endophyte infection likely due to osmotic changes, root changes, and more stomatal closure (De Battista et al., 1990; Malinowski et al., 1997a; Malinowski et al., 1997b). Endophyte-infected plants also tend to have greater leaf and root growth than uninfected plants.

Toxins from the fungal endophytes help protect the hosts from both vertebrate and invertebrate herbivores (Bush et al., 1997). Some associations are capable of accumulating ergot alkaloids, lolitrems, peramine, or loline alkaloids (LA) (Figure 1.1). Ergot alkaloids are toxic to both invertebrate and vertebrate herbivores and are most likely the fungal alkaloid responsible for fescue toxicosis (Porter, 1995) suffered by livestock that graze tall fescue (*Lolium arundinaceum = Festuca arundinacea*) with endophyte, *N. coenophialum*. Lolitrems are anti-vertebrate toxins responsible for ryegrass staggers in livestock grazing on perennial ryegrass (*L. perenne*) with the endophyte *N. lolii*. Peramine acts as a feeding deterrent for insect herbivores and is common in many grass-fungal endophyte associations. The LA exhibit insecticidal activity on a wide range of insect herbivores and may be involved in drought tolerance of infected plants (Arechavaleta et al., 1989; Bacon, 1993). The various fungal endophytes produce either one or an array of these alkaloids (Bacon, 1988; Gurney et al., 1994; Porter, 1994; Rowan, 1993).

Culturing these endophytic fungi requires surface sterilization of the grass tissue with alcohol and bleach to remove surface contaminants. Studies on *N. coenophialum*, a common endophyte of tall fescue, indicate optimal growth conditions at ~23°C, pH 6.5, low osmolarity, and aeration (Davis et al., 1986). Studies by Kulkarni et al. (1986) with *N. coenophialum* indicate the requirement of thiamine. These studies also indicate a preference for mannitol, trehalose, sucrose, or fructose as the carbon source. Cultures of *N. coenophialum* grow on medium supplemented with L-proline, L-asparagine, L-glutamine, L-arginine, L-cysteine, ammonium chloride, ammonium nitrate, or more complete but less defined nitrogen sources such
as soytone, yeast extract, peptone, casamino acids, and tryptone. Culture conditions that simulate the apoplastic fluid should promote growth of the endophytes and production of LA since no detectible levels of LA exist under complete medium conditions.

**Loline alkaloids**

The LA are broad spectrum insecticidal metabolites consisting of a saturated pyrrolizidine ring structure with an ether linkage between C-2 and C-7, and a 1-amine. The 1-amine bears various substituents such as formyl, acetyl, and methyl groups as shown in Figure 1.2. Studies show production of the LA in many natural grass-endophyte associations of *Lolium* and *Festuca* spp. grasses. *L. giganteum (=*F. gigantea*) with *E. festucae* (Leuchtmann et al., 2000; Siegel et al., 1990), *L. arundinaceum* with *N. coenophialum* (Leuchtmann et al., 2000; Siegel et al., 1990), *L. pratense (=*F. pratensis*, meadow fescue) with *N. uncinatum* (Leuchtmann et al., 2000; Siegel et al., 1990), *L. pratense* with *N. siegelii* (Craven et al., 2001), *L. multiflorum*, *L. rigidum* and *L. temulentum* with *N. occultans* (TePaske et al., 1993a), *Echinopogon ovatus* with *N. aotearoae* (Moon et al., 2002), endophyte infected *Poa autumnalis* (Siegel et al., 1990), *F. argentina* (Casabuono and Pomilio, 1997), and *Achnatherum robustum* (TePaske et al., 1993a). Recently LA were detected in *Agrostis hyemalis* with *E. amarillans* (unpublished data). LA (Figure 1.2) found in these associations include norloline (NL), loline (L), *N*-formynorloline (NFNL), *N*-acetylnorloline (NANL), *N*-formylloline (NFL), and *N*-acetylloline (NAL). NFL and sometimes NAL tend to accumulate to the highest levels in the associations. Also non-grass plants, *Adenocarpus* spp. (Powell and Petroski, 1992) and *Argyreia mollis* (Tofern et al., 1999), accumulate some LA. The LA possess anti-insect activity as evident in many studies. The alkaloids are toxic to many insects including *Rhopalosipnum padi*—(bird cherry oat aphid) (Siegel, 1990; Wilkinson et al., 2000), *Schizapus graminum*—(greenbug aphid) (Siegel et al., 1990; Wilkinson et al., 2000), and larvae of *Haematobia irritans*—(horn fly) (Dougherty et al., 1998), *Popillia japonica*—(Japanese beetle) (Patterson et al., 1991), *Spodoptera frugiperda*—(fall armyworm) (Riedell et al., 1991), and *Ostrinia nubilalis*—(European corn borer) (Riedell et al., 1991).

Levels of the LA are variable in the grass-endophyte symbiota with levels up to 20 mg g\(^{-1}\) of clipped leaf material (Craven et al., 2001). Leaf clipping greatly stimulated LA production by either *N. uncinatum* or *N. siegelii* in *L. pratense*. Justus et al. (1996) compared LA levels and
growth stages of endophyte (presumably *N. uncinatum*)-symbiotic *L. pratense* plants throughout the growing season. They determined the highest concentration of LA occurred in the developing inflorescences and seeds. Germinating seeds demonstrated high levels of LA and the authors speculated that this could protect the seedling from soil-borne insects.

LA cause lethal neurotoxic effects on a wide range of invertebrate herbivores (Dahlman et al., 1991), although they do not exhibit anti-mammalian activity evident in other types of plant produced pyrrolizidines (PA). These pyrrolizidines consist of five major classes (Hartmann, 1999). Classes I and III include the senecionine and monocrotaline types with $\alpha,\beta$ unsaturated necine bases and a macrocyclic, diester bridge between C-9 and C-7. Class II comprises triangularine type ($\alpha,\beta$-unsaturated necine base with open chained diesters attached to C-9 and C-7). Classes IV and V comprise pyrrolizidines with a single monoester at C-9. These PA, especially the senecionine and monocrotaline types, demonstrate strong DNA binding abilities (Hincks et al., 1991; Kim et al., 1999). PA also act as feeding deterrents for herbivores and produce cytotoxic effects on the liver (Hartmann, 1999). LA contain no $\alpha,\beta$-unsaturation of the necine base or the ester linkages at C-1 or C-7, and lack the cytotoxicity of PA. Intravenous injection of LA induces effects on mammalian herbivores but oral ingestion of LA induces no effect at normal plant levels (Dew et al., 1990; Jackson et al., 1996).

The high levels of LA produced in the endophyte-grass associations, their insecticidal activity, and their lack of anti-mammalian activity make this group of alkaloids very interesting to study. Production of the LA by the fungus in fermentation cultures at levels amenable to feeding experiments facilitates the study of these alkaloids and the work on the biosynthetic pathway.

**Biosynthesis of loline alkaloids**

Although LA produce no detectible anti-mammalian activity in natural circumstances like the PA, they share similarity of the pyrrolizidine ring structure. The structural similarity has led Bush et al. (1993) to propose spermidine as the direct precursor of LA. Homospermidine acts as the precursor to the PA as demonstrated by chemical and genetic work (Boettcher et al., 1993; Hartmann, 1999; Hartmann and Ober, 2000; Ober and Hartmann, 1999). Polyamines, putrescine and spermidine, act as precursors for homospermidine as shown with radioactive and stable isotope labeled compounds (Figure 1.4) (Boettcher et al., 1994; Graser and Hartmann, 1997;
Graser and Hartmann, 2000; Graser et al., 1998; Hartmann et al., 1988). Polyamines are common in fungi (Davis, 1996). The biosynthetic pathway for LA biosynthesis proposed by Bush et al. (1993) involves oxidative deamination at C-1 of spermidine followed by cyclization to form 1-(3-aminopropyl)pyrroline (Figure 1.5). It was proposed that 1-(3-aminopropyl) pyrroline further cyclizes, and that the ether bridge between C-2 and C-7 subsequently forms to yield norloline. The other LA in this proposed pathway originate from the norloline substructure.

Wilkinson et al. (2000) implicate the endophyte is responsible for loline alkaloids. A cross between a LA-non-producing strain of \textit{E. festucae} in \textit{F. rubra} and an LA-producing strain in \textit{L. giganteum} produced a segregation ratio of LA production/nonproduction of LA near 1:1 (after the progeny were introduced into \textit{L. pratense}) (Chung et al., 1997). This result indicates single-locus control of LA production for this haploid organism. A genetic marker identified by amplified fragment length polymorphism (AFLP) in the fungal crosses cosegregated with LA production in the crosses. Also, toxicity to aphids (\textit{R. padi} and \textit{S. graminum}) was only evident in plants with progeny producing LA and containing the AFLP marker. This marker was later shown to be a portion of \textit{lolC}, which shares sequence homology to a homocysteine synthase/O-acetylhomoserinesulfhydrolase gene, a gene involved in methionine biosynthesis in fungi (Spiering et al., 2002).

\textbf{Loline chemistry}

Extraction of LA from endophyte-infected grass tissue for analysis with gas chromatography (GC) is well described and involves organic solvents under basic conditions (Blankenship et al., 2001; TePaske et al., 1993a; TePaske et al., 1993b; Yates et al., 1990). Petroski et al. (1989) extracted the LA from tall fescue seeds and produced the other common LA from extracted loline. Literature references indicate firm establishment of the chemical structures of LA (Aasen and Culvenor, 1969; Akramov and Yunusov, 1965; Akramov and Yunusov, 1968; Bates and Morehead, 1972; Knoch et al., 1993; Petroski et al., 1989; Wilson et al., 1981; Yunusov and Akramov, 1955; Yunusov and Akramov, 1959; Yunusov and Akramov, 1960a; Yunusov and Akramov, 1960b; Yunusov and Akramov, 1960c). First isolation of the LA, temuline (= norloline) from \textit{L. temulentum}, was by Hofmeister in 1898 (Powell and Petroski, 1992). Yunusov and Akramov isolated loline and assigned it an incorrect structure.
(Yunusov and Akramov, 1955). Bates and Morehead assigned the correct structure in 1972. Powell et al. (1989) determined the $^1$H and $^{13}$C NMR chemical shifts and mass spectroscopy profiles of LA extracted from tall fescue. Knowledge of the nitrogen chemical shifts of the LA, presented in Chapter 4 of this dissertation, should facilitate the further study of this group of alkaloids.

Cultures conditions mimicking apoplastic conditions promoted LA production from *N. uncinatum* (Chapter 2). With the development of suitable culture conditions, the feeding of proposed precursors indicates their incorporation into LA (Chapter 3). Determination of isotopic enrichment of LA isolated from cultures fed isotopically labeled precursors utilized techniques such as gas chromatography-mass spectroscopy and nuclear magnetic resonance. Confirmation of the identity of the LA nitrogens utilized $^{15}$N NMR (Chapters 3-4). From this research, a new hypothesis for LA biosynthesis is proposed (Figure 1.6).
ergovaline

peramine

loline alkaloids

where R and R’ = H, CH3, HC=O, or CH3C=O

lolitrem B

Figure 1.1: Examples of alkaloids produced in grass-endophyte associations
Figure 1.2: The common loline alkaloids occurring in tall fescue and meadow fescue symbiotic with *Neotyphodium* species.
Figure 1.3: Examples of plant produced pyrrolizidines (PA).
Figure 1.4: Biosynthesis of plant pyrrolizidines (PA)
Figure 1.5: Previously proposed LA biosynthetic pathway (modified from Bush et al., 1997)
Proposed pathway steps
1. gamma-substitution on L-proline amine
2. oxidative decarboxylation and cyclization
3. oxidations
4. ether bridge formation
5. methylation via SAM
6. methylation via SAM
7. oxidation of methyl group
8. acetylation
9. acetylation

Figure 1.6: Proposed loline alkaloid biosynthesis. Solid arrows = published reactions. Dashed arrows = proposed reactions.
Chapter 2

Production of loline alkaloids by the grass endophyte, *Neotyphodium uncinatum*, in defined media

Introduction

Loline alkaloids (LA, saturated 1-aminopyrrolizidines with an oxygen bridge) (Fig. 1) have been identified in many grass-endophyte associations (Siegel et al., 1990) but are absent in most other organisms. These grasses are naturally infected with symbiotic fungal endophytes, especially *Epichloë* spp. and their asexual derivatives (*Neotyphodium* spp.), which grow in the intercellular spaces (apoplast) of host plants. In these symbioses, an array of other anti-herbivore alkaloids is produced. Three of the alkaloid classes commonly found in endophyte-grass associations — peramine (a pyrrolopyrazine), ergot alkaloids, and lolitrems (indolediterpenes) — are of known fungal origin because these are reported to accumulate in fermentation cultures (Bacon, 1988; Gurney et al., 1994; Porter, 1994; Rowan, 1993). The LA are produced in a number of grass-endophyte symbioses: *Lolium arundinaceum* (=*Festuca arundinacea*) with *N. coenophialum* (Siegel et al., 1990), *L. giganteum* (=*F. gigantea*) with *E. festucae* (Leuchtmann et al., 2000), *L. pratense* (=*F. pratensis*) with *N. uncinatum* (Bush et al., 1993; Justus et al., 1997), *L. pratense* with *N. siegelii* (Craven et al., 2001), and endophyte-infected *F. argentina* (Casabuono and Pomilio, 1997), *L. temulentum* (Dannhardt and Steindl, 1985), *Poa autumnalis* (Siegel et al., 1990), and *Stipa robusta* (TePaske et al., 1993). The plants, *Argyreia mollis* (Convolvulaceae) and *Adenocarpus* spp. (Fabaceae) have also been reported to contain LA, although endophytes have not yet been implicated (Hartmann and Witte, 1995; Tofern et al., 1999).

The LA are secondary metabolites and have an unusual structure consisting of a saturated necine ring with an –NRR' substituent at C-1 and an oxygen bridge between C-2 and C-7 (Powell and Petroski, 1992; Yates et al., 1990). LA thus differ from most plant pyrrolizidines, which are synthesized from homospermidine (Boettcher et al., 1994) and have a –CH₂OR group at C-1. Nothing is known about the biosynthesis of the endophyte-associated LA, but based on this difference, the LA have been suggested to be derived from spermidine or spermine (Bush et al., 1993). The alkaloids are found throughout the plant in these associations and have protective
effects for the plant due to their anti-invertebrate and feeding deterrent activities (Dougherty et al., 1998; Patterson et al., 1991; Wilkinson et al., 2000). LA are broad-spectrum insecticides (Siegel and Bush, 1997); in grasses symbiotic with strains of *E. festucae* segregating for loline LA expression, accumulation of these alkaloids was clearly associated with activity against certain aphid species (Wilkinson et al., 2000). Thus, the LA are of great interest as natural plant protectants. Although studies do indicate immunosuppressive effects in mice (Dew et al., 1990) and depressed feed intake has been observed in rats fed LA (Jackson et al., 1996), it has not been conclusively shown that the LA at *in planta* levels are toxic to mammalian herbivores (Siegel and Bush, 1997). LA do not share the same potent anti-mammalian hepatotoxicity and carcinogenicity as plant pyrrolizidines, which contain C-1,2 unsaturation in the necine ring (Hincks et al., 1991); cytochrome P450 converts these plant pyrrolizidines into more bioactive forms, which to varying degrees are capable of cross-linking of DNA (Kim et al., 1999).

LA expression correlates with fungal genotype, and in some grass-endophyte symbiota levels can exceed 2% plant dry mass (Craven et al., 2001). Until the present study, LA have been the only grass-endophyte associated alkaloid class reported in the literature not observed in fungal cultures (Porter, 1994). Knowledge of the origin of LA would be beneficial for elucidating the biosynthetic pathway and its regulation, identifying biosynthesis genes, and expanding biotechnological possibilities for these alkaloids.

Since LA were only detected in endophyte-infected plants, we hypothesized the endophyte was responsible for their production, and that cultures that adequately mimic the internal environment of the plant may stimulate LA production. In this paper, it is shown that *N. uncinatum* produces LA also found in plant tissue — norloline (NL), loline (L), methylloline (ML), *N*-acetyl-norloline (NANL), both rotamers of *N*-formylloline (NFL), and *N*-acetylloline (NAL) — under certain minimal medium conditions.

**Materials and Methods**

**General experimental procedures:**

For gas chromatography, all samples were run on a Hewlett Packard (Avondale, PA) 5890 Series II Plus GC equipped with a flame ionization detector and a J & W Scientific Inc. (Rancho Cordova, CA) fused silica capillary column (SE 30, 60 m x 0.30-mm inner diameter with 0.25 µm film thickness of dimethylpolysiloxane). Nitrogen was used as the carrier gas at a
flow rate of 20.4 mL min\(^{-1}\). The run parameters were an initial column temperature of 110°C, an injection temperature of 250°C, a detector temperature of 325°C, a 2.2-min purge time, and a 4°C min\(^{-1}\) increase in column temperature to 188°C. Between each sample run, the column was purged at 280°C for 5 min. \(^{13}\)C NMR analysis employed a Varian Gemini 200 equipped with VNMR 6.1B software (Varian Inc., Palo Alto, CA). Samples of purified alkaloids in 750 µl D\(_2\)O (Cambridge Isotopes Laboratories, Inc., Andover, MA) were prepared in 5 mm X 17.8 cm NMR tubes (Wilmad, Beuna, NJ) and 10 µl methanol added as an internal standard. \(^{13}\)C NMR conditions were at 200 mHz, ambient temperature, and proton decoupling. Chemicals for these experiments were obtained from Sigma-Aldrich (Milwaukee, WI).

**Fungal isolate isolation:**

Cultures were obtained by growing *N. uncinatum* (W. Gams Petrini et Schmidt) Glenn Bacon et Hanlin (voucher specimen CBS 102646 at Centraalbureau Voor Schimmelcultures) out of an infected plant (number 167 in our plant collection) of *L. pratense* (Huds.) Darbysh. \([F. pratensis* Huds., realigned with genus *Lolium* by Darbyshire (1993)].* Leaf blades were sterilized in 50-mL Costar tubes with 95% ethanol for 1 min, followed by a rinse of sterile water, then soaked for 2 min in 5.25% sodium hypochlorite. The leaf blades were then rinsed with sterile water, cut into 2-mm sections, and plated on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI) with penicillin (167 units mL\(^{-1}\)) and streptomycin (76.3 units mL\(^{-1}\)). After 14 days, 5 pieces of fungal mycelium grown out (approximately 0.5 cm) of the plant material was removed from the agar and homogenized in 10 mL of the minimal medium in an Omni Homogenizer Model 17105 (Omni International, Waterbury, CT) with six 10-sec bursts. The ground mycelium was drop-inoculated onto PDA and grown for 7 days, then 1.5 to 2 cm plugs were cut out of the agar, homogenized in minimal medium, and 1 mL used to inoculate each 30 mL minimal medium culture for study. Large differences in alkaloid expression between experiments most likely were due to unavoidable differences in inoculum density and physiological condition of the source mycelium, so employing a single source of inoculum for each experiment minimized variation between replicates. Cultures of *E. festucae* Leuchtm. Schardl et Siegel (CBS 102474) from *Lolium giganteum* (L.) Darbysh. (=*F. gigantea* L.), *N. coenophialum* (Morgan-Jones et W. Gams) Glenn Bacon et Hanlin (American Type Culture Collection accession ATCC 90664) from *L. arundinaceum* (Schreb.) Darbysh. (=*F. arundinacea*
Schreb.), and *N. siegellii* K. D. Craven Leuchtm. et Schardl (ATCC 74483) from *L. pratense* were obtained similarly (Craven et al., 2001).

**Culture medium:**

The defined media used in these experiments were adapted from Chung and Schardl (1997) with modifications. The 10X stock solutions of basal salts — 300 mM K$_2$HPO$_4$ and 300 mM KH$_2$PO$_4$ in deionized water— were mixed to the desired pH (4.5 - 8.0 as specified). Then, 2-(N-morpholino)ethanesulfonic acid (MES) was added to the basal salt solution to 300 mM, and pH readjusted with NaOH. MES was used to prevent the drastic pH increase that sometimes occurred in cultures with only phosphate as buffer. Final concentrations in the minimal medium were 30 mM potassium phosphate and 30 mM MES. The carbon sources were added to a conc. of 8.3 mM, and nitrogen sources were added to give 30 mM nitrogen atoms, except where otherwise specified. To the medium were added MgSO$_4$ to 2 mM, thiamine to 0.6 µM, and a solution of trace elements (from 1000 X stock) to give 3.6 µM H$_3$BO$_3$, 1 µM CuSO$_4$, 0.7 µM KI, 0.8 µM FeNa-ethylenediaminetetraacetic acid, 1 µM MnSO$_4$, 0.5 µM NaMoO$_4$, and 0.4 µM ZnSO$_4$ (Kulkarni and Nielsen, 1986). Basal salts and MES in purified water were autoclaved together, and the MgSO$_4$ was autoclaved separately to prevent precipitation of salts. Carbon source, nitrogen source, thiamine, and trace elements were filter-sterilized together then combined with the basal salts and MgSO$_4$ solution. After preliminary trials with cultures in Erlenmeyer flasks proved inconsistent, 100 X 25 mm polystyrene petri plates (Fisherbrand, Fisher Scientific, Pittsburgh, PA) were used for the 30 mL cultures in all experiments reported here. The cultures were incubated at 21°C with 100 rpm rotary shaking.

**Sampling from cultures:**

Aliquots of medium from each culture were transferred into 1.7-mL Eppendorf (Brinkmann Instruments, Inc., Westbury, NY) microcentrifuge tubes that had been pretreated at 100°C for 2 days to remove extractable contaminants that otherwise interfered with the analysis. Prior to day 20 of culture, 1 mL aliquots were sampled; thereafter, aliquots were 0.5 mL. For fungal biomass and LA level determinations, 30-ml cultures were harvested for each time point and filtered through tared Whatmann no. 1 filter paper (Whatmann Limited, England). Then the mycelium on each filter was oven dried (37°C, 2 days) and weighed, and the culture filtrate was frozen and freeze-dried for later analysis of LA.
Alkaloid analysis:

For GC analysis, freeze-dried samples were extracted using a modified procedure from Yates et al. (1990) and Robbins et al. (1972). One-tenth volume of a saturated sodium bicarbonate solution (0.25 g ml⁻¹ in H₂O) was added to each dried filtrate and the tubes were vortexed. Chloroform (1 mL), with 14.2 mg L⁻¹ of quinoline (Rₜ=7.60) as the internal standard was added to each tube. The suspension was vortexed, then shaken 30 min on a platform shaker. The CHCl₃ layer was transferred to a capped glass vial. Levels for LA from GC analysis in these experiments are reported as the total of NANL (Rₜ=16.75) and NFL (Rₜ=17.44), because the levels of L (Rₜ=9.21) were always very low.

For analysis by GC-MS and ¹³C NMR, LA alkaloids were isolated using modifications of the procedure of Petroski et al. (1989). Fungal culture filtrates were freeze dried, then extracted with 1 mL saturated sodium bicarbonate (aq.) and 30 mL CHCl₃. The aqueous layer was discarded and the organic solvent evaporated in a stream of nitrogen gas to yield a brown oil. One mL CHCl₃ was added to the oil and 500 µL of this mixture was spotted onto a K6F Silica gel 60A TLC plate (Whatmann Inc, Clifton, NJ). After drying, the plate was developed in a chromatography chamber with 140 mL of a 50:50:1 (by volume) mixture of CH₃OH, CHCl₃ and saturated NH₃OH. LA were visualized as dark brown spots after exposing the plate to iodine vapor. Rᶠ values for L, NANL, and NFL were determined as 0.26, 0.37, and 0.49, respectively. The visible spots corresponding to the LA on the plate were scraped off individually and alkaloids eluted with purified H₂O, extracted with CHCl₃ and solvent removed by a stream of N₂ gas. These purified samples were then used in GC-MS and ¹³C NMR analysis.

Alkaloid purification and identification:

Loline was isolated by separation of the alkaloids by TLC and extracted from the silica with H₂O, and because of its volatility, conc. HCl was added to yield the salt form, which was recrystallized with EtOH: ¹³C NMR (D₂O, 50 MHz), 29.2 (C6), 34.0 (N-Me), 55.5 (C5), 61.8 (C3), 63.8 (C1), 70.1 (C8), 71.9 (C2), 80.9 (C7); EIMS m/z 154 [M]+ (0), 123(58), 110 (7), 95 (75), 82 (100).

N-Acetylnorloline was purified as a clear oil after being separated from the other alkaloids by TLC, extracted from the plate with H₂O followed by extraction with CHCl₃ which was removed with a stream of nitrogen gas: ¹³C NMR (D₂O, 50MHz), 24.2 (MeC=O), 35.1 (C6),
54.8 (C5), 58.4 (C1), 61.4 (C3), 70.1 (C8), 74.1 (C2), 81.6 (C7), 171.9 (HC=O); EIMS m/z 182 [M]+ (0), 153 [M-29]+ (8), 139 (0), 123 (9), 110 (0), 95 (30), 82 (100), 69 (22).

N-Formylloline (rotamer 1) was purified as a viscous brown oil similar to purification of NaNL: 13C NMR (D2O, 200 MHz), 32.0 (C6), 34.8 (N-Meth), 54.1 (C5), 59.7 (C3), 65.5 (C1), 67.8 (C8), 74.3 (C2), 83.2 (C7), 165.1 (HC=O); Rotamer 2: 30.4 (N-Meth), 31.7 (C6), 54.3 (C5), 60.6 (C3), 63.0 (C1), 68.6 (C8), 73.5 (C2), 81.4 (C7), 167.0 (HC=O); EIMS m/z 182 [M]+ (0), 154 [M-28]+ (25), 123 (13), 110 (13), 95 (28), 82 (100).

Results

This study examined the common endophyte of L. pratense, N. uncinatum, for potential production of LA in culture because grasses symbiotic with this species accumulate LA to high levels. In a minimal medium modified from Chung and Schardl (1997), with mannitol as the carbon source and asparagine as the nitrogen source, N. uncinatum produced NL, L, ML, NANL, NFL, and NAL. Confirmation of the identities of these alkaloids utilized GC-MS and 13C NMR. N. uncinatum in potato dextrose broth (PDB) produced no LA.

The experiments examined various carbon sources typically present in plant apoplasts. Initially, mannitol was chosen because it is a good carbon source for growth of the related endophyte, N. coenophialum (Kulkarni and Nielsen, 1986). Fructose, glucose, and sucrose were then tested as alternative carbon sources because sucrose is a major transport sugar in the apoplast, while glucose and fructose are produced from sucrose by the action of invertase secreted by endophytes (Lam et al., 1994). As shown in Figure 2.2, sucrose was the best of these carbon sources for LA production, giving a range of 500 to 750 µg mL⁻¹ (up to 4 mM) of NANL and NFL, total. This was a 12-fold increase of production over cultures with mannitol. Glucose or fructose also tended to increase production only slightly compared to mannitol cultures, but not to the level obtained using sucrose.

Nitrogen sources tested were asparagine, arginine, ornithine, glutamine, ammonium sulfate, and urea, in cultures at pH 5.5 with sucrose as carbon source. The nitrogen concentration maintained at 30 mM total nitrogen atoms allowed direct comparison between treatments. In this experiment, cultures with urea as the sole nitrogen source yielded the greatest overall level of LA production, ca. 425 µg mL⁻¹ of culture filtrate (Figure 2.3). Ornithine as the sole nitrogen source also gave much greater levels than expressed in cultures with asparagine or arginine. The level
of LA production at day 20 was 5-fold greater when ornithine rather than asparagine was the nitrogen source (Figure 2.3).

Altering concentrations of phosphate between 6.8 and 68 mM, MgSO$_4$ between 0.2 and 4.0 mM, mannitol between 8.3 and 16.7 mM, or asparagine between 15 and 30 mM had only slight effects on LA production levels (data not shown).

To determine how LA production and growth may be related, a time course experiment was conducted with medium containing 16.7 mM sucrose and 30 mM ornithine (Figure 2.4 and 2.5) (these carbon and nitrogen sources were chosen for the time course because they promote LA production and are known substituents of plant apoplasts; Pate 1973). The proportion of NANL to NFL in this experiment (Figure 2.4a and 2.4b) was consistent with levels obtained in similar experiments. (Levels of L were characteristically very low, so they are not included in Figure 2.4). The maximum rate of LA accumulation (Figure 2.4a and 2.4b) consistently occurred after cessation of the fungal growth with onset of stationary phase (Figure 2.5a). A slight increase in pH occurred whether the initial pH was 5.5 or 7.0 (Figure 2.5b), and the change coincided with the onset of LA production (Figure 2.4a and 2.4b) and slowing of fungal growth (Figure 2.5a).

Other grass endophytes known to produce LA in planta — *E. festucae* from *L. giganteum* (Leuchtmann et al., 2000), *N. coenophialum* from *L. arundinaceum* (Siegel, 1990), and *N. siegelii* from *L. pratense* (Craven et al., 2001) — were also tested for production. These fungi were cultured under the preferred conditions established for *N. uncinatum* (including 20 mM sucrose and 30 mM ornithine), but LA were not detected in these cultures.

**Discussion**

Studies implicate the *Epichloë* and *Neotyphodium* species, closely related fungi that live as endophytes of grasses, as the origin of the ergot, lolitrem, and peramine alkaloids (Bacon, 1988; Rowan, 1993, Gurney et al., 1994; Porter, 1994). Previously, production of LA outside the grass-endophyte symbiota did not exist (Schardl and Phillips, 1997). Prior to this study, the origin of the LA (whether the plant or fungus, or the combination of both) was not known. Establishment of culture conditions for production of the LA (specifically NL, L, NL, NANL, NFL, and NAL) by *N. uncinatum* demonstrated that this fungus has the full biosynthetic capacity for many of the LA found in planta. The observed correspondence of maximal LA production
with the stationary phase was in keeping with secondary metabolism in other microorganisms (Campbell, 1984).

Nitrogen sources for these experiments were chosen to test organic and inorganic nitrogen and also based on the known precursors of polyamines and plant pyrrolizidines (Boettcher et al., 1993). Ammonium sulfate as the sole nitrogen source produced levels of production comparable to those with asparagine, glutamine, and arginine. Cultures with urea as nitrogen source yielded the highest levels of LA. Arginine and ornithine are apoplastic substituents in grasses (Pate, 1973), and ornithine is a direct precursor in the polyamine biosynthetic pathway. A hypothesis exists that, based on structures of the LA, that spermidine, spermine, or both could be precursors to the LA (Siegel and Bush, 1997). Arginine and ornithine are closely related, and removal of the urea group from arginine yields ornithine in the urea cycle. A determinant step in polyamine synthesis is decarboxylation of ornithine to form putrescine. Decarboxylated S-adenosylmethionine donates the propylamine group to putrescine to form spermidine. Adding an additional propylamine group from decarboxylated S-adenosylmethionine to spermidine yields spermine. These propylamine groups ultimately derive from aspartate, which biosynthetically arises from asparagine. Preliminary feeding studies with $^{13}$C-labelled ornithine and aspartate confirm that these are likely LA precursors (Chapter 3).

A surprising observation is that no other fungal endophyte that produces the LA in planta produced measurable quantities in culture, even under conditions favorable for production by N. uncinatum. A possibility is that only N. uncinatum has the full competence to produce LA outside its host, whereas in other grass-endophyte symbiota LA production may be accomplished with both symbiotic partners. A more likely possibility, however, is simply that the culture conditions promoting production by N. uncinatum were unsuitable for production by the other endophytes.

Now that production of some common LA exists by cultured N. uncinatum, work is underway on the biosynthetic pathway for LA. The high level of their production in culture will significantly facilitate studies of the pathway and will aid in identifying and characterizing the genes that may be responsible.


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Figure 2.1: Loline alkaloids shown to be produced in fermentation cultures of *Neotyphodium uncinatum*.
Figure 2.2: Effect of carbon source on loline alkaloid production. Carbon sources were ◆16.7 mM mannitol, ▲16.7 mM glucose, ●16.7 mM fructose, or ■16.7 mM sucrose. Asparagine concentration was at 15 mM; pH 5.5. Means (n=5) are indicated with error bars equal to 1 SD. SD smaller than symbol are not shown.
Figure 2.3: Effect of nitrogen source on loline alkaloid production. Nitrogen sources were ▲ 15 mM ornithine, ● 15 mM asparagine, ■ 7.5 mM arginine, or ◆ 15 mM urea. Sucrose concentration was 16.7 mM, and pH was 5.5. Means (n=4, except urea n=3) are indicated with error bars equal to 1 SD.
Figure 2.4: Levels of ▲ N-acetylnorloline and ● N-formylloline in cultures. 
a.) Production at pH 5.5.  b.) Production at pH 7.0. Means (n=4) are indicated with error bars equal to 1 SD.
Figure 2.5: Growth of loline-alkaloid production cultures at pH ◆ 5.5 and ▲ 7.0.
a.) Growth of *N. uncinatum*. b.) Change in pH of culture medium. Carbon and nitrogen sources were 16.7 mM sucrose and 30 mM ornithine, respectively. Means (n=4) are indicated with error bars equal to 1 SD.
Chapter 3

Biosynthetic precursors of loline alkaloids (1-aminopyrrolizidines)

Introduction

Many *Lolium* and *Festuca* species in association with *Epichloë* and *Neotyphodium* species of fungal endophytes produce loline alkaloids (LA) (Leuchtmann et al., 2000; Siegel et al., 1990). The LA accumulate in some *symbiota* to levels approaching 2% plant dry weight (Craven et al., 2001). Although the LA are found primarily in the grass-fungal endophyte associations in the Poaceae (Powell and Petroski, 1992), the alkaloids also occur in non-grass species; namely, some *Adenocarpus* species (Powell and Petroski, 1992) and *Argyreia mollis* (Tofern et al., 1999). The LA group comprises saturated 1-aminopyrrolizidines with various substitutions on the 1-amine (Figure 2.1) and an oxygen bridge between C-2 and C-7. Other plant pyrrolizidines (PA), specifically senecionine and retrorsine, contain α,β-unsaturation and a macrocyclic ring attached between C-1 and C-7 on the pyrrolizidine ring structure, but do not contain the ether bridge between C-2 and C-7 or the 1-amine. While the LA do not exhibit the anti-mammalian activity associated with PA (Hincks et al., 1991; Jones et al., 1983; Kim et al., 1999), LA show insecticidal activity against a wide array of insect herbivores (Dougherty et al., 1998; Riedell et al., 1991; Wilkinson et al., 2000).

The structural similarities of the LA to the necine base of PA implicate polyamines as LA precursors. Khan and Robins demonstrate homospermidine as the precursor of the necine ring structure of the PA (1981a). Studies utilizing 13C and 14C labeled putrescine or spermidine demonstrate both act as precursors to homospermidine in PA biosynthesis (Graser et al., 1998; Khan and Robins, 1981a; Khan and Robins, 1981b; Khan and Robins, 1985a; Khan and Robins, 1985b; Robins and Sweeney, 1981). Based on structural similarity to PA and on the number and arrangement of carbon and nitrogen atoms, Siegel and Bush (1997) have proposed spermidine as the direct precursor to the loline alkaloid ring system.

*Neotyphodium uncinatum*, an endophyte of the grass *Lolium pratense* (= *Festuca pratensis*), produces LA in defined-medium fermentation cultures (Blankenship et al., 2001). The principal LA produced in culture are shown in Figure 2.1. In this study, I feed *N. uncinatum* fermentation cultures with labeled amino acids and amines to demonstrate the origins of all
carbon and nitrogen atoms of N-formyloline, and present evidence that LA biosynthesis involves a unique pathway unrelated to PA pyrrolizidine ring formation.

Materials and Methods

Materials:

L-[U-14C]-Ornithine (>250 µCi mmol-1), [1, 4-14C2]-putrescine (100-120 mCi mmol-1), L-[U-14C]-proline (200-300 mCi mmol-1), and L-[U-14C]-glutamic acid (>200 mCi mmol-1) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). L-[U-14C]-Aspartic acid (130-250 mCi mmol-1) was obtained from Moravek Biochemicals (Brea, CA). L-[5-13C]-Ornithine (99%) and L-[1, 2-13C2]-ornithine (99%) were purchased from Mass Trace (Woburn, MA) and Icon Stable Isotopes (Mt. Marion, NY), respectively. L-[15N]-Aspartic acid (>99%) was obtained from Medical Isotopes Inc. (Pelham, NH). Deuterium oxide (99.9%), L-[15N, U-13C5]-methionine (97-98%), L-[methyl-13C]-methionine (98%), L-[4-13C]-aspartic acid (99%), L-[15N2]-ornithine (98%), and 15NH4NO2 (>98% 15N in NH4+) were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). [1, 2-13C2]-Succinonitrile was obtained commercially from Isotec, Inc. (Miamisburg, OH). DL-α-Difluoromethylornithine (DFMO) was received as a gift from Marion Merrell Dow Laboratories (Kansas City, MO). All other compounds used in the experiments were purchased from Sigma-Aldrich (Milwaukee, WI). LA and polyamines were separated on 20 X 20 cm, 60A K6F Whatman, Inc. silica gel TLC plates (Clifton, NJ) with 100 µm gel thickness.

[1, 4-13C]-Putrescine was synthesized by a modification of the procedure of Samejima et al (1995). K13CN (410 mg) and 1 g of dibromoethane in 10 mL of 75% ethanol was refluxed for 4 hrs. The potassium bromide produced as a byproduct of the reaction was precipitated with acetone. The ethanol was removed by drying to yield 96 mg of 1, 4-13C succinonitrile. Reduction of the [1, 4-13C]-succinonitrile to [1, 4-13C]-putrescine was carried out via modifications of the Khan and Robins procedure (Khan and Robins, 1985a). To the succinonitrile, 10 mL of dry tetrahydrofuran and 10 ml of 1 M borane in tetrahydrofuran was added. This mix was then heated at reflux for 16 hrs and allowed to cool before addition of 20 mL of dry ethanol. After one hr, dry HCl gas was bubbled into the solution to precipitate [1, 4-
$^{13}$C]-putrescine•2 HCl. Yield was 13% (110 mg). Samples were compared to unlabeled putrescine•2 HCl by $^1$H NMR and $^{13}$C NMR.

Other proposed intermediates — namely, L-[4, 4,$^{2}$H$_2$]-homoserine, L-cis-[3-$^{2}$H]-3-hydroxyproline, N-(3-amino-3-carboxy-[1, 1-$^{2}$H$_2$]-propyl)proline (NACPP), and N-(3-amino-[1, 1-$^{2}$H$_2$]-propyl)proline (NAPP) — were synthesized by the Dr. Robert Grossman group in the Department of Chemistry at the University of Kentucky.

Fungal Cultures:

*Neotyphodium uncinatum* (W. Gams, Petrini, Schmidt) Glenn Bacon et Hanlin (CBS 102646 at Centraalbureau Voor Schimmelcultures) was maintained in symbiosis with *Lolium pratense*, from which the fungus was isolated as described in Blankenship et al. (2001). Fungal cultures were maintained on potato dextrose agar (PDA) plates and routinely subcultured by suspending in sterile water and grinding in an Omni Homogenizer Model 17105 (Omni International, Waterbury, CT) followed by drop inoculation of PDA plates. Fermentation cultures of *N. uncinatum* for precursor-feeding were set up using minimal medium (MM) described in Blankenship et al. (2001) with some modifications. The MM consisted of 30 mM phosphate/30 mM 2-(N-morpholino)-ethanesulfonic acid (MES) buffer, pH 5.5, in deionized water with 2 mM MgSO$_4$, 20 mM sucrose, 15 mM urea, 0.6 $\mu$M thiamine, and trace elements (Kulkarni and Nielsen, 1986). All labeled compounds and inhibitors were diluted and filter sterilized prior to addition to MM fungal cultures at day 5 to 7. Fungal tissue was removed from PDA plates and homogenized in MM solution (1 mL MM per plate to inoculate). Cultures were in 100 X 25 mm polystyrene Petri plates (Fisherbrand, Fisher Scientific, Pittsburgh, PA) with 29 mL of MM per plate and 1 ml of ground inoculum, on a rotary shaker at 100 rpm at 21-23°C. Plates were wrapped with Parafilm to prevent desiccation and contamination.

Polyamine synthesis inhibitor studies:

The ornithine decarboxylase inhibitor, DFMO, and the spermidine synthase inhibitor cyclohexylamine (CHA) were added to fermentation culture plates at day 7, after fungal biomass accumulation had ceased. The following treatments in MM were set up to examine the effects on loline production: +/- 5 mM putrescine, +/- 5 mM spermidine, DFMO (1 mM/5 mM with +/- 5 mM putrescine), or CHA (1 mM/2 mM with +/- 5 mM spermidine). Stock concentrations of
polyamines and inhibitors in MM were as follows: putrescine (155 mM), spermidine (155 mM), DFMO (31 mM and 155 mM), and CHA (31 mM and 62 mM). Inhibitors and polyamines were filter sterilized prior to addition. Each treatment consisted of 4 replicates.

**Precursor feeding and analysis:**

Labeled compounds were diluted in MM and filter sterilized, and added to 5-day-old fermentation cultures. \(^{14}\)C-Labeled compounds were added at 8 \(\mu\)Ci per culture plate. Stable-isotope-labeled compounds were added to a concentration of 2-5 mM. Culture filtrates were harvested between day 20 and 25 for TLC, GC-MS, or NMR analysis.

**Gas chromatographic and mass spectroscopy analysis:**

Samples of fermentation cultures (1 mL consisting of medium and mycelium) were harvested from loline producing cultures and placed in 2 mL microcentrifuge tubes (prior to use, tubes were heat-treated at 100°C overnight to remove contaminants). Gas chromatography (GC) was conducted with a Hewlett Packard (Avondale, PA) 5890 Series II Plus GC with a J & W Scientific Inc. (Rancho Cordova, CA) column (SE 30, 60 m X 0.30 mm ID with 0.25 \(\mu\)m thick dimethylpolysiloxane film) and a flame ionization detector. Conditions for GC and extraction of LA were as described in Blankenship et al. (2001). GC-mass spectrometry (GC-MS) employed a Hewlett Packard G1800A GCD equipped with an electron ionization detector, 30 m Restek column (5 Sil MS, 0.25 mm ID, and 0.25 \(\mu\)m film thickness) and data collected via GCD ChemStation (G1074A, Version A.00.00) software. Further analysis of GC-MS data utilized the freeware, WSEARCH32 Version 1.1.2003 (http://minyos.its.rmit.edu.au/~rcmfa/search.htm). Retention times for LA are listed as follows: loline (4.02), methylloline (4.12), N-acetylnorloline (6.19), N-formylloline (6.44), and N-acetylloline (6.55). For GC-MS analysis, 2 ml of culture sample was lyophilized and extracted with 100 \(\mu\)L of 1 M NaOH and 1 mL of chloroform. The aqueous layer was removed, and organic layer concentrated. The residue was brought up to 100 \(\mu\)L with chloroform, placed in glass GC vials, and capped, and 1 \(\mu\)L of each sample was injected onto the GC-MS. Values for enrichment of precursors were calculated from absolute intensity of GC-MS data as:
\[
\left( \frac{[M+n]_{\text{labeled}}}{[M]_{\text{labeled}} + [M+n]_{\text{labeled}}} - \frac{[M+n]_{\text{unlabeled}}}{[M]_{\text{unlabeled}} + [M+n]_{\text{unlabeled}}} \right) \times 100 = \% \text{ enrichment}
\]

Where \( M \) = intensity of a fragment peak, \( M+n \) = the intensity of the peak shifted in amu higher due to isotopic substitution, and labeled and unlabeled represent the spectra of compounds isolated from cultures that were or were not fed labeled precursors.

**TLC separation:**
Fermentation cultures were centrifuged (2000 rpm, 10 min, RT) to remove fungal mycelium, and the culture supernatants were lyophilized. To the residue from 90-120 mL of culture filtrate was added 2 mL of 1 M NaOH and 15 mL chloroform (2X). After vigorous shaking, samples were centrifuged and the chloroform layer removed. The chloroform extract was concentrated to approximately 200 \( \mu \)L and spotted onto a TLC plate. Solvent for development of TLC plates was 49.5% chloroform, 49.5% methanol, and 1% ammonium hydroxide. After exposure to I\(_2\) vapor to stain the alkaloids, the area corresponding to NFL (\( R_f = 0.69 \)) was scraped and placed into a 2 mL microcentrifuge tube. NFL was extracted with 400 \( \mu \)L D\(_2\)O (2X). These extracts were pooled and brought up to 750 \( \mu \)L for NMR analysis.

**NMR analysis:**
NMR analysis was carried out in 5 mm x 17.8 cm Wilmad NMR tubes (Wilmad Glass Co., Inc. Buena, NJ). \(^{13}\)C NMR analysis employed a Varian Gemini 200 with VNMR 6.1B software (Varian Inc., Palo Alto, CA) at ambient temperature and proton decoupling. For \(^{13}\)C NMR analysis, methanol was used as either an internal or external standard with methanol peak set to 49.0 ppm. \(^{15}\)N NMR analysis employed a Varian 400 with VNMR 6.1B software (Varian Inc., Palo Alto, CA) at ambient temperature and proton decoupling. Samples for \(^{15}\)N NMR analysis contained 5 mg of \(^{15}\)NH\(_4\)NO\(_2\) and NH\(_4\) peak set at 20 ppm was used as an internal standard. Sample tubes were washed thoroughly with 95% ethanol (2X) and acetone (2X) and were dried with N\(_2\) gas between sample runs.
Dansylation procedures:

To determine whether $^{14}$C-putrescine entered the fungal cells, fungal mycelium from $^{14}$C-ornithine and $^{14}$C-putrescine fed cultures from above experiment was extracted for polyamines. Using a procedure adapted for tissue or cultured cells for polyamine extraction and detection (Madhubala, 1998), the harvested fungal mycelium was centrifuged to remove remaining supernatant, and washed with deionized water. All supernatants were saved for determination of incorporation of radioactivity into LA (above). Washed fungal mycelium (100 mg) mixed with 1 mL of 2% perchloric acid was ground in a 1.7 mL Eppendorf (Brinkmann Instruments, Inc., Westbury, NY) microcentrifuge tube with a plastic grinder. This mixture was kept at 4°C overnight, after which the homogenate was centrifuged at 13,000 rpm for 10 min. To 200 µL of the supernatant in a 2 mL microcentrifuge tube was added 400 µL of dansyl chloride solution (5 mg ml$^{-1}$ dansyl chloride in acetone). Note: dansyl chloride is light sensitive and the following procedures were carried out with aluminum foil covering. After thorough mixing, 200 µL of saturated sodium carbonate solution was added to the sample before vortexing well and storing at room temperature overnight. L-Proline (15 mg in 100 µL of deionized water) was added and the mixture was vortexed. The proline and excess dansyl chloride were allowed to react at room temperature for 1 hr before extraction with 500 µL toluene. The toluene layer was removed and concentrated to 100 µL, then spotted on a silica gel TLC plate. The plate was developed with 40% cyclohexane:60% ethyl acetate, then air dried in the dark. Plates were exposed to UV light and photographed to determine the positions of dansylated compounds. A Phosphor cassette (35 x 43 cm; Molecular Dynamics, Sunnyvale, CA) was then exposed to the TLC plate to determine the incorporation of the radioactivity with a Molecular Dynamics PhosphoImager 445SI Version 4.0 (Sunnyvale, CA). Spermidine and spermidine spots were examined for radioactive enrichment.

Results

$^{14}$C labeled feedings indicated possible precursors for the LA. As demonstrated in Figure 3.1 (right panel), cultures fed L-$[^{14}\text{C}]$-aspartate, L-$[^{14}\text{C}]$-ornithine, L-$[^{14}\text{C}]$-glutamate, and L-$[^{14}\text{C}]$-proline incorporated label in NFL and NANL. L-$[^{14}\text{C}]$-Proline-feeding produced the strongest enrichment at 5850 cpm mmol$^{-1}$ NFL. Precursors to L-proline, L-$[^{14}\text{C}]$-ornithine and L-$[^{14}\text{C}]$-glutamate, also gave enrichment of 1952 cpm mmol$^{-1}$ and 2771 cpm mmol$^{-1}$, respectively.
Enrichment from L-[14C]-aspartate was 1268 cpm mmol\(^{-1}\). The polyamine, [1,4-14C]-putrescine, gave very low enrichment at 87 cpm mmol\(^{-1}\). L-[U-14C]-ornithine and [U-14C]-putrescine incorporation into spermidine from the fermentation cultures in feeding experiments is shown (Figure 3.2). L-[14C]-Ornithine-feeding resulted in less enrichment in spermidine, but greater incorporation into lolines than putrescine.

Precursors for the A- and B-ring of NFL were determined with the use of stable isotope-enriched amino acids by comparing the natural abundance spectra of NMR (Figure 3.3) and GC-MS spectra (Figure 3.4) of labeled and unlabeled compounds with enrichment (%) listed in Table 3.1. Cultures fed with L-[5-13C]-ornithine gave enrichment in only the C-5 position in the B ring (Figure 3.5). This enrichment was also evident in the GC-MS spectrum (Figure 3.6 and Table 3.1). Cultures fed with L-[1, 2-13C\(_2\)]-ornithine gave NFL specifically enriched in the B-ring position, C-8 (Figure 3.7). One of the nitrogens from L-[15N\(_2\)]-ornithine incorporated into the pyrrolizidine ring system as shown by GC-MS (Figure 3.8 and Table 3.1). Cultures fed with L-[15N, U-13C\(_5\)]-proline gave NFL specifically enriched in the B-ring carbons (C-5, C-6, C-7, and C-8) (Figures 3.9 and Figure 3.10). Enrichment (%) for L-[15N, U-13C\(_5\)]-proline feeding was determined (Table 3.1). Splitting of peaks in the 13C NMR was due to 13C-13C coupling from the labeled L-proline. Loss of the nitrogen of L-[15N, U-13C\(_5\)]-proline and incorporation of the carbon skeleton is indicated with a +4 amu shift (Table 3.1). Also incorporation of only the nitrogen of L-[15N, U-13C\(_5\)]-proline indicated in Table 3.1 is due to transamination reactions. L-[15N, U-13C\(_5\)]-proline demonstrated enrichment of the ring amine nitrogen from L-[15N, U-13C\(_5\)] proline. Enrichment from L-[15N, U-13C\(_5\)]-proline was evident in 82 + 5 peaks and 95 +4 peaks for loline, methylloline, N-acetylnorloline, and N-acetylloleline (Figure 3.11).

Norloline levels in this experiment were too low to determine enrichment. Other unidentified compounds, giving GC-MS peaks at 5.73 min, 5.94 min, 6.27 min, 6.46 min, 7.08 min, 7.23 min, 7.31 min, and 8.41 min (loline eluted at 4.02 min, methylloline at 4.11 min, NANL at 6.12 min, NFL at 6.39, and NAL at 6.53) showed incorporation of L-[15N, U-13C\(_5\)]-proline and contained peaks consistent with LA substructure. Low levels of these compounds made it difficult to determine their identities. The label from L-[4-13C]-aspartic acid was incorporated into the C-3 of the A-ring of NFL exists as demonstrated in (Figure 3.12). Based on comparison with the natural-abundance 15N NMR spectrum (Figure 3.13, top panel), NFL from L-[15N]-aspartate feeding exhibited only 1-amine enrichment (Figure 3.13, bottom panel). NFL exists as a mixture of rotamers in solution.
(Figure 3.14) as can be seen by the presence of two peaks for each carbon and nitrogen in the NMR spectra. This phenomenon has been described for substituted amides (LaPlanche and Rogers, 1963). L-[15N, U-13C5]-methionine demonstrated enrichment in only the N-formyl and N-methyl carbons of NFL, but not in any ring carbons (Figure 3.15). Given the lack of enrichment in ring-carbons — which would have been expected if the propylamine group of methionine was involved via polyamines or otherwise — the fate of the 15N label from L-[15N, U-13C5]-methionine was not investigated. Feeding of L-[methyl-13C]-methionine confirmed that the methyl group of methionine was responsible for the enrichment of the N-formyl and N-methyl carbons (Figure 3.16).

Examination of NFL from the L-[15N]-homoserine-feeding experiment indicated enrichment of the 1-amine (Figure 3.17, Table 3.1). Analysis of NFL from L-[4-2H2]-homoserine fed cultures indicated incorporation of both deuteriums (GC-MS data shown in Figure 3.18 and Table 3.1).

Feeding studies with deuterium-labeled hypothetical intermediates, L-cis-[3-2H]-3-hydroxyproline and NAPP, exhibit no enrichment (Table 3.1), and feeding studies with NACPP exhibits inconsistent results based on the GC-MS data.

To further investigate the possible involvement of polyamines in LA synthesis, I examined effects of the ornithine decarboxylase inhibitor, DFMO, and the spermidine synthase inhibitor, CHA, on LA production by early stationary-phase cultures. At the concentrations tested, neither DFMO nor CHA gave any significant change in loline production (Figure 3.19). Addition of spermidine to all treatments, except 1 mM CHA + 5 mM spermidine, tended to lower LA production, although not significantly. Also, the inhibitors exhibited no significant effects on growth (Figure 3.20).

**Discussion**

This study describes direct precursors and evidence against polyamine involvement in the LA biosynthetic pathway. The use of radioactive and stable-isotope-labeled precursors allows the determination of constituents of the LA pyrrolizidine ring structures, the 1-amine nitrogen and substituents on the 1-amine. Based on incorporation of labels from L-[14C]-proline and L-[15N, U-13C]-proline, I conclude that this amino acid contributes C-5, C-6, C-7, C-8 and the ring N of LA. Precursors of L-proline biosynthesis, L-[14C]-glutamate and L-[14C]-ornithine, also
demonstrated strong enrichment in NFL. These amino acids serve as metabolic precursors of proline, so these results are consistent with the hypothesis that proline is a precursor. With $^{13}$C NMR analysis, positions C-8 and C-5 of NFL showed strong specific enrichment from L-[1, 2-$^{13}$C$_2$]-ornithine and L-[5-$^{13}$C]-ornithine, respectively. These result indicated that the $\alpha$-carbon of proline should correspond to C-8, and the $\delta$-carbon to C-5 of LA. Furthermore, the ornithine-feeding result was inconsistent with a polyamine pathway, because decarboxylation of L-ornithine to putrescine randomizes C-2 and C-5 of L-ornithine into C-1 and C-4 of putrescine, yet the labeled from L-ornithine such demonstrated no randomization in NFL. Thus, I conclude that LA biosynthesis does not involve putrescine.

Results of feeding with labeled L-aspartate and L-homoserine indicated that both L-aspartate and L-homoserine or one of their derivatives are precursors to the A-ring of LA. The number of carbons in the A-ring not contributed by proline and the position of the 1-amine are consistent with the observed incorporation from L-[$^{15}$N] and L-[$^{4-13}$C]-aspartate. Aspartate is the metabolic precursor of homoserine, so the possible involvement of homoserine was also tested. As evident by GC-MS, both deuteriums of L-[4-$^2$H$_2$]-homoserine were incorporated into NFL, and incorporation of L-[$^{15}$N]-homoserine was also indicated. These incorporation patterns would have been consistent with either direct incorporation of homoserine into a LA intermediate or with a polyamine pathway. Homoserine is a precursor of methionine, which via decarboxylated S-adenosylmethionine (dcSAM), donates the propylamine groups of spermidine and spermine. However, label from L-[$^{15}$N, U-$^{13}$C] methionine enriched none of the A-ring carbons, yet the labeled S-methyl groups enriched the N-formyl and N-methyl carbons, demonstrating that sufficient methionine entered the cells. Although it is possible that these results might be explained by compartmentalization of steps in the pathway, it seems unlikely that proline, aspartate, and homoserine would enter the appropriate compartments in abundance but methionine would not. The L-[4-$^2$H$_2$] homoserine feeding experiment indicates that L-homoserine is not oxidized back to the L-aspartylsemialdehyde losing one of its two deuteriums. While the exact precursor that attaches to the L-proline was not determined from this work, it is likely that an activated form of L-homoserine, likely L-$O$-acetylhomoserine, would be involved.

Whether proline or a derivative thereof is a proximate precursor could not be determined. The LA pathway almost certainly involves loss of the $\alpha$-carboxyl group of proline, and this might have occurred before or after condensation to the A-ring precursor. Oxidative
decarboxylation of proline could give Δ4-pyrroline, Δ5-pyrroline, 4-hydroxypyrrolone, or 4-hydroxypyrrolidine, and incorporation of any of these would be consistent with the results of [13C] ornithine and [15N, 13C] proline feeding experiments.

Based on the above considerations, I hypothesize the LA biosynthesis pathway in Figure 3.2.1. This hypothetical pathway involves transfer of activated L-homoserine derivative residue such as L-O-acetylhomoserine which is common in fungi (Marzluf, 1997). This moiety would be directly attached to the α-nitrogen of proline or a proline derivative, via a γ-substitution. The reaction would most likely involve a pyridoxal phosphate (PLP)-containing enzyme. A candidate enzyme is the product of the lolC gene found to be expressed only in LA producing fermentation cultures. This gene shares sequence homology to a O-acetylhomoserinesulfhydrolase gene (Spiering et al., 2002). This gene catalyzes transfer of an aminopropyl group of L-homoserine onto the sulfur moiety of cysteine to form cystathionine. The nitrogen of L-proline could act as the acceptor of the aminopropyl from the activated form of L-homoserine, L-O-acetylhomoserine. The combination of these two amino acid moieties would not proceed via a peptide linkage followed by reduction. A likely mechanism for this reaction would be γ-substitution onto the proline nitrogen.

If proline and activated homoserine are the proximal LA precursors, the first reaction would produce the intermediate, NACPP (Fig. 3.21), which would then be decarboxylated and undergo a series of oxidative cyclizations to form the pyrrolizidine base structure. The lack of enrichment from labeled NACPP argues against this hypothesis, but it might reflect a lack of NACPP uptake into the fungal hyphae or the appropriate cellular compartment.

Once proline and homoserine or their derivatives are linked, I hypothesize a series of reactions by monooxygenases and/or oxidoreductases to generate the basic pyrrolizidine ring structure and the ether bridge between C-7 and C-2, yielding norloline. Methylation of the 1-amine of norloline, by S-adenosylmethionine would give loline. A similar methylation of loline would give methylloline, and oxidation of one of the methylloline 1-amine methyl groups would give NFL. Acetylation of norloline and loline would yield NANL and NAL, respectively.

Studies on the LA biosynthetic pathway such as determining exactly how the other LA are synthesized can be looked at now. Labeled precursors such as norloline-like intermediates without the ether bridge or the basic two ring structure with hydroxyl groups on C-2 and/or C-7 would help to determine the mechanism and timing of ether bridge closure. Further work with
NACPP is also important. This compound may be detectible in fungal mycelium during LA producing conditions. Additional feeding studies with carrier compounds such as DMSO may allow the compound to enter the cell and compartments more readily. Knowledge of the other biosynthetic precursors of LA will facilitate further studies of LA production.
Table 3.1. Isotopic enrichment (%) of NFL due to incorporation of labeled precursors

<table>
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<tr>
<th>labeled precursor</th>
<th>amu shift</th>
<th>82 (%)</th>
<th>amu shift</th>
<th>95 (%)</th>
<th>amu shift</th>
<th>154 (%)</th>
<th>amu shift</th>
<th>182 (%)</th>
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<td>+5</td>
<td>52</td>
<td>+5</td>
<td>55</td>
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<tr>
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<td>a 22</td>
<td>+3</td>
<td>a 19</td>
<td>+4</td>
<td>a 40</td>
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<tr>
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<td>a 18</td>
<td>+3</td>
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<td>a 35</td>
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<tr>
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<td>b 12</td>
<td>+1</td>
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<td>10</td>
<td>+1</td>
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Table 3.1 cont.

\( ^a \) Expected amu shift due to loss of \(^{15}\)N from proline likely due to metabolism and reincorporation (ie transamination).  \( ^b \) Expected amu shift due to transfer of amine from labeled proline and incorporation into another proline.  \( ^c \) Why the % enrichment calculated for this ion is reduced compared to the other ions remains unexplained.  \( ^d \) From the fragmentation that leads to this ion, an ion with this mass is unexpected.  \( ^e \) The loss of one of the hydrogens (deuteriums) at C-3 of NFL should be evident in the 95 (+1) m/z fragment.  \( ^f \) Shift due to one methyl group being incorporated into NFL.  \( ^g \) Shift due to two methyl groups being incorporated into NFL.
Figure 3.1: Precursor feeding for $^{14}$C labelling of NFL and NANL. Panel on left demonstrates location of NFL and NANL by iodine staining of silica gel plate. Panel on right shows radioactive enrichment.
Figure 3.2: Dansylated polyamines (left panel) from radioactive putrescine fed to *N. uncinatum* fermentation cultures. Right panel demonstrates radioactive enrichment in spermidine and spermine.
Figure 3.3: Natural abundance $^{13}$C NMR spectrum of NFL (200 mg in 0.75 ml D2O) with only the major rotamer peaks labeled.
Figure 3.4: Common fragments observed in GC-MS of NFL (modified from Takeda et al., 1991).
Figure 3.5: $^{13}$C enrichment in NFL from L-[5-$^{13}$C] ornithine (5 mM) feeding of fermentation cultures.
Figure 3.6: GC-MS spectrum of NFL (top panel). $^{13}$C enrichment in NFL from L-[5-$^{13}$C] ornithine (4 mM) feeding of fermentation cultures (bottom panel).
Figure 3.7: $^{13}$C enrichment in NFL from L-[1,2 $^{13}$C] ornithine (2 mM) feeding of fermentation cultures.
Figure 3.8: GC-MS spectrum of NFL (top panel). $^{15}$N enrichment in NFL from L-$^{[15]N_2}$ ornithine (5 mM) feeding of fermentation cultures (bottom panel).
Figure 3.9: $^{13}$C enrichment in NFL from L-$[^{15}$N, $^{13}$C] proline (5 mM) feeding of fermentation cultures.
Figure 3.10: GC-MS spectrum of NFL (top panel). $^{15}$N, $^{13}$C$_4$, enrichment in NFL from L-$[U^{13}$C, $^{15}$N] proline (5 mM) feeding of fermentation cultures (bottom panel).
Figure 3.11: GC-MS of other loline alkaloids shown to be produced in fermentation cultures of *Neotyphodium uncinatum* from L-[\(^{15}\text{N}\), U-\(^{13}\text{C}_5\)] proline (5 mM).
Figure 3.12: $^{13}$C enrichment in NFL from L-[4-$^{13}$C] aspartate (4 mM) and L-[5-$^{13}$C] ornithine (4 mM) feeding of fermentation cultures.
Figure 3.13: $^{15}$N NMR of NFL from fermentation cultures without labeled precursors (top panel, 200 mg NFL in 0.75 ml D$_2$O) or with L-$^{15}$N aspartate (5 mM) (bottom panel, ~15 mg NFL in 0.750 ml D$_2$O). Both spectra are referenced to $^{15}$NH$_4$ set at 20 ppm.
Figure 3.14: Rotamer effect of N-formylloline
Figure 3.15: $^{13}$C enrichment in NFL from L-[$^{13}$C, $^{15}$N] methionine (4 mM) feeding of fermentation cultures.
Figure 3.16: $^{13}$C enrichment in NFL from L-[methyl-$^{13}$C]-methionine (4 mM) feeding of fermentation cultures.
Figure 3.17: GC-MS spectrum of NFL (top panel), and $^{15}$N enrichment in NFL from L-$^{[15}$N] homoserine (5 mM) feeding of fermentation cultures (bottom panel).
Figure 3.18: GC-MS spectrum of NFL (top panel), and $^2$H enrichment in NFL from L-[4-$^2$H$_2$] homoserine (5 mM) feeding of fermentation cultures (bottom panel).
Figure 3.19: Effects of polyamine-synthesis inhibitors added at day 7 on loline production of *Neotyphodium uncinatum* in fermentation cultures.
Figure 3.20: Effects of polyamine-synthesis inhibitors added at day 7 on growth of *Neotyphodium uncinatum* in fermentation cultures.
Figure 3.21: Proposed loline alkaloid biosynthesis
Chapter 4

$^{15}$N NMR of loline alkaloids

Introduction

Fungal endophytes of some cool season grasses produce toxic secondary metabolites. The more common toxins include ergot alkaloids, peramine, lolitrem, and loline alkaloids (LA). The LA are an important group of compounds present in some wild and agronomically important Festuca and Lolium species in association with fungal endophytes of the genera Epichloë and Neotyphodium. Whereas most of the research with LA focuses on grasses, LA also occur in non-grass plants, Adenocarpus species (Powell and Petroski, 1992) and Argyreia mollis (Tofern et al., 1999). The LA contain a pyrrolizidine ring structure with a 1-amine (often with methyl and/or formyl or acetyl substituents), plus an ether linkage between C-2 and C-7 (Figure 3.1). LA levels can approach 2% of plant dry weight and accumulation increases with clipping in some endophyte infected grasses (Craven et al., 2001). The insecticidal properties of LA affect a large range of invertebrate but not vertebrate herbivores (Dougherty et al., 1998; Eichenseer et al., 1991; Patterson et al., 1991; Riedell et al., 1991; Wilkinson et al., 2000). Due to the insecticidal properties, knowledge of the LA chemistry would be beneficial. Extraction of LA by any of the published procedures usually involves large quantities of organic solvents, multiple extractions, and time (Petroski et al., 1989; Robbins et al., 1972; Yates et al., 1990). Work by various groups identified characteristic mass spectroscopy data for the LA (Dannhardt and Steindl, 1985; Petroski et al., 1989; Robbins et al., 1972; Takeda et al., 1991). The work of Petroski et al. examines the $^1$H and $^{13}$C NMR spectra of LA and identifies NMR chemical shifts of each hydrogen and carbon of the LA (1989). The present study identifies a facile method for extraction of LA from grass seed, conversion of loline and norloline to the other LA, and the $^{15}$N chemical shifts of the common LA.

Materials and Methods

Chemicals and general techniques:

Chloroform, conc. HCl, NaOH pellets, sodium bicarbonate, silica gel, sodium sulfate, and methanol were purchased from Sigma-Aldrich (Milwaukee, WI). K6F Silica gel 60A TLC plate (Whatmann Glass Co., Inc., Clifton, NJ) were used for small scale separations. Ethanol (95% and
dry) were obtained from AAPER Alcohol and Chemical Co. (Shelbyville, KY). D$_2$O (>99%) and $^{15}$NH$_4$NO$_2$ (>98%) for NMR analysis were obtained from Cambridge Isotopes Laboratories, Inc. (Andover, MA), and 5 mm X 17.8 cm NMR tubes were obtained from Wilmad (Buena, NJ). $^{15}$N NMR was carried out with a Varian 400 with VNMR 6.1B software (Varian Inc., Palo Alto, CA) at ambient temperature and proton decoupling. All loline alkaloid samples were spiked with 5 mg of $^{15}$NH$_4$NO$_2$ for an internal reference. The NH$_4$ peak was set at 20 ppm. Cleaning of sample tubes between samples consisted of thorough washing with 1 mL 95% ethanol (2X), then 1 mL acetone (2X), and drying with N$_2$ gas.

*Extraction of Loline (L):*

For extraction of the loline alkaloids, *Lolium arundinaceum* seed (tall fescue cultivar Kentucky 31, Southern States Cooperative, Inc., Richmond, VA) containing the fungal endophyte, *Neotyphodium coenophialum*, was acquired and extracted with modifications from published papers (Petroski et al., 1989; TePaske et al., 1993; Yates et al., 1990). Seeds were ground with a coffee grinder (Model KSM 2B, Braun Inc., Woburn, MA) to a fine powder (passed through a 2 mm screen). Ground seed (100 g) were then extracted with 1 L of chloroform and 100 mL of 1mM NaOH. The suspension was stirred vigorously for 1 hr and then allowed to stand overnight at room temperature. Seed material was removed by filtering through four layers of cheesecloth in a funnel under vacuum. A sample (1 mL) of this chloroform extract was removed for GC analysis for loline content. LA were efficiently removed from chloroform extract with 100 mL of 1 M HCl (3X) in a separatory funnel. Acid layers were combined and heated at 80°C until concentrated to 50 mL. After cooling, the acid extract was adjusted to pH 7 with sodium bicarbonate, then to pH 10.5 with a saturated NaOH solution. This solution was then extracted with chloroform (5X) at equal volume. Chloroform layers were pooled and the water was removed with dry sodium sulfate. The chloroform extract was subjected to flash chromatography using 20 grams of silica gel (70-270 mesh, 60 A, Sigma-Aldrich, Milwaukee, WI) in a 2 cm wide glass column. The column was washed with 100 mL methanol to elute LA. The eluate was dried with sodium sulfate. To the eluate, 500 µL of conc. HCl and 50 mL of dry ethanol were added. This solution was placed at 4°C overnight, during which loline·2 HCl crystallized as small rods. Yield from 100 g of seed was approximately 165 mg of loline·2 HCl with trace amounts of methylololine·2 HCl. Trace quantities of methylololine were removed by
washing with 95% ethanol. For synthesis of the other LA, loline·2 HCl was converted to free base by dissolving 1 g in 2 mL NaOH and extracting with 20 mL chloroform (10X). After drying down the chloroform extract, approx. 625 mg of loline free base was evident as a clear oil. For loline free base: EIMS $m/z$ 154 [M]$^+$ (4.2), 123 (14.9), 110 (39.0), 95 (32.1), 82 (100). For loline·2 HCl: $^{13}$C NMR (D$_2$O, 200 MHz) 29.2 (C6), 34.0 (N-Me), 55.5 (C5), 61.8 (C3), 63.8 (C1), 70.1 (C8), 71.9 (C2), 80.9 (C7).

Individual LA species were prepared by modifications of the procedures of Petroski et al. (1989) as outlined below.

*Preparation of N-formylloline (NFL):*

Loline (300 mg, free base) in 5 mL of ethyl formate was stirred at room temperature for 4 days. Solvent was removed to leave a brownish oil (275 mg of NFL). EIMS $m/z$ 182 [M]$^+$ (0.6), 154 (19.0), 123 (11.8), 110 (13.6), 95 (29.0), 82 (100.0).

*Preparation of N-acetylloline (NAL):*

Loline (250 mg, free base) was added to 116 µL of acetyl chloride in 2 mL chloroform. This mixture was capped and stirred overnight at room temperature. The mixture was extracted with 1 mL of 0.5 M HCl (2X) and pH of the aqueous acid phase was immediately adjusted to pH 10.5 with saturated NaOH solution. The basic aqueous phase was then extracted with 10 mL chloroform (3X). Upon drying with sodium sulfate and evaporation of the chloroform, 280 mg of NAL was present as oily crystals. EIMS $m/z$ 182 [M]$^+$ (0.3), 167 (4.4), 139 (1.3), 123 (9.7), 110 (2.6), 95 (29.7), 82 (100.0).

*Preparation of methylloiline (ML):*

Loline (300 mg) in 110 µL formic acid and 175 µL formalin was refluxed for 4 h. Then, 1 mL HCl (1 M) was added and solution was extracted with 1 ml diethyl ether (3X). The solution was adjusted to pH 10.5 with saturated NaOH, and extracted with 10 mL chloroform (5X). The chloroform extract was dried with sodium sulfate and subjected to chromatography on a silica gel (10 g) column. ML was eluted from the column with 2% methanol in chloroform. The yield of ML was 225 mg. EIMS $m/z$ 168 [M]$^+$ (2.3), 153 (1.3), 123 (47.1), 111 (8.3), 95 (69.0), 82 (100.0).
Preparation of norline (NL):

Loline·2 HCl (800 mg) was added to 7.2 mL of ice-cooled 20% H2SO4. Potassium permanganate (227 mg) in 7.2 mL H2O was added and the mixture was stirred overnight at room temperature. The mixture was centrifuged to remove any solids and extracted with 10 mL chloroform (2X), then adjusted to pH 10.5 with saturated NaOH. LA were extracted from this solution with 50 mL chloroform (5X). The resulting extract was dried with sodium sulfate and applied to a silica gel column (20 g). L and NL (approximately 50/50 ratio) were separated by washing the column with 2% methanol in chloroform. Fractions of 10 mL each were collected and analyzed for L and NL. Fractions containing NL were combined and dried down to yield 235 mg of NL. This reaction was repeated several times to yield sufficient NL from which to produce other LA. EIMS m/z 140 [M]+ (4.2), 123 (23.8), 111 (15.3), 97 (21.7), 95 (15.1), 82 (100.0).

Preparation of N-acetylnorline (NANL):

To 2.5 mL of phenyl acetate was added 300 mg of NL. The solution was kept at room temperature for 4 days. Solvent was removed with evaporation to yield ~186 mg of NANL. EIMS m/z 182 [M]+ (0.3), 153 (5.0), 139 (1.3), 123 (8.1), 110 (2.8), 95 (26.8) 82 (100.0).

Results

The facile, large scale chloroform-NaOH extraction procedure described in this study yields approximately 1.65 mg loline·2 HCl g⁻¹ ground tall fescue seed. Preparations of NFL, NAL, ML, NL, and NANL generated quantities sufficient for determination of ¹⁵N NMR chemical shifts (Table 4.1). The rotamer effect evident in the ¹H and ¹³C NMR spectra of N-formylloline (LaPlanche and Rogers, 1963; Petroski et al., 1989) was also observed in the ¹⁵N NMR spectrum. Feeding experiments utilizing L-[¹⁵N] aspartate and L-[¹⁵N, U¹³C] proline of LA producing fermentation cultures of Neotyphodium uncinatum provides evidence for the identity of nitrogen chemical shifts of NFL (Chapter 3, this dissertation). Comparison of these chemical shifts to those of other LA helps with the assignment of their nitrogen chemical shifts.
Discussion

LA are abundant in endophyte-infected tall fescue seed when symbiotic with *N. coenophialum*, and the high LA levels in seeds affords facile extraction of these alkaloids. The LA extraction procedure in this study allows the reuse of the organic solvent (chloroform) and reduces steps during the initial LA extraction. The modification of synthetic procedures from Petroski et al. (1989) of the common LA species produces enough of each LA to determine the $^{15}$N NMR shifts reported here. Due to the differences in the chemical environment of the nitrogens of the LA and comparison with published $^{15}$N chemical shifts, assignment of the nitrogens was possible (Martin et al., 1981; Witanowski et al., 1973). Also, feeding experiments from earlier work by this group indicates the additional proof of the chemical shifts of the 1-amine and ring nitrogen of NFL using $^{15}$N labeled precursors. Setting NH$_4^+$ of $^{15}$NH$_4$NO$_2$ at 20 ppm provides a good reference point for the LA. The 1-amine nitrogens of NANL, NFL rotamers, and NAL are in amide linkages to their substituents, and shift far downfield of the NH$_4^+$ internal standard peak. NANL, NFL, and NAL ring nitrogen chemical shifts reside upfield closer to the internal standard. The 1-amine nitrogens in the other tested LA are either primary, secondary, or tertiary amines and should in fact reside further upfield from the *N*-acetyl and *N*-formyl nitrogens and closer to the standard. The 1-amine nitrogens of NL, loline·2 HCl, and ML exhibit chemical shifts closer to the NH$_4^+$ standard. The ring nitrogens of the LA occur in the region occupied by tertiary amines, as is consistent with literature (Martin et al., 1981; Witanowski et al., 1973). With the LA containing two nitrogens, obtaining the $^{15}$N chemical shifts of the known LA species allows further work on the biosynthesis and chemical analysis of this group of compounds.
Table 4.1: $^{15}$N chemical shifts of common loline alkaloids

<table>
<thead>
<tr>
<th></th>
<th>ring N</th>
<th>1-amine</th>
</tr>
</thead>
<tbody>
<tr>
<td>loline·2HCl (2)</td>
<td>70.03</td>
<td>35.02</td>
</tr>
<tr>
<td>N-formylloline (5)</td>
<td>58.89</td>
<td>120.89</td>
</tr>
<tr>
<td>N-formylloline (5')</td>
<td>62.44</td>
<td>117.42</td>
</tr>
<tr>
<td>N-acetylloline (6)</td>
<td>64.73</td>
<td>118.81</td>
</tr>
</tbody>
</table>
Chapter 5
Conclusions

Prior to the studies described in this dissertation, the fungal partners of the *Epichloë/Neotyphodium*-cool season grass interactions were shown to provide three of the known bioprotective alkaloids associated with the symbioses, namely, ergot alkaloids, lolitrems, and peramine. As an essential first step to elucidate the effects and roles of the LA in these symbioses it was important for the origin of the LA — host or symbiont — to be determined. In Chapter 2, I described studies of fermentation cultures with defined culture media, which demonstrated the fungal origin of LA (also see Appendix A). Other feeding experiments were set up with inconclusive or inconsistent results (see Appendix B). The LA species produced by *N. uncinatum* in these fermentation cultures are norloline, loline, methylloline, N-acetylnorloline, N-formylloline, and N-acetylloline (for select GM-MS data, see appendix C), plus very low levels of other compounds of unknown structure but incorporating at least the proline ring structure intact and a 82 or 83 m/z ion (see Appendix D).

The correspondence of onset of LA production with the stationary phase of growth is consistent with production of other secondary metabolites in other fungi (Campbell, 1984). The lag in the accumulation of LA provides ideal timing for precursor feeding experiments because there is likely to be much less movement of the fed compounds into primary metabolism than there would be during the growth phase. Proposed precursors fed at this point would enter a system experiencing a slow-down of primary metabolism and growth, and redirecting much of its resources into secondary metabolism production (ie. LA production).

With the ability to induce production of LA in the *N. uncinatum* cultures, work on the LA biosynthesis was possible, as described in Chapter 3. Results of feeding studies with labeled precursors indicate the involvement of L-proline in the biosynthesis of LA. This involvement of L-proline is intriguing given indications that proline is a component of some ergot alkaloids as well as peramine. Thus, at least three of the four common alkaloid classes in endophyte-grass associations incorporate L-proline into their chemical structures. The role of L-proline in fungi as well as plants is widespread and diverse. Many fungi utilize the amino acid as an osmoregulator as well as storage for excess energy (Davis et al., 2000; Jennings and Burke, 1990). In plants, biotic and abiotic stresses induce L-proline accumulation (Hare and Cress,
1997). Pathogen attack or herbivory stimulate accumulation of L-proline in many plants. Craven et al. (2001) demonstrated that simulated herbivory led to increased LA accumulation. Increased availability of L-proline in host plant tissue due to the feeding stress may explain why LA accumulation increases. High levels of L-proline accumulation also occurs with many abiotic stresses, specifically water deficiency or salt stress as reviewed in Delauney and Verma (1993).

Another possible role of LA in the association is the ability to act as energy storage with the incorporation of L-proline from the plant tissue. Cultures tend to accumulate LA up to day 20-25, and then levels slightly decrease. This is indicative of fungal breakdown of LA or volatilization of the LA. LA are not very volatile at fermentation culture pH of 5.5 to 6.0, and LA levels of culture medium filtered to remove fungal cells do not decrease as quickly. The endophyte is capable of taking up and utilizing L-proline as evident from my feeding experiments (Chapter 3). The endophyte may have developed the LA pathway to allow the fungal cells to store this excess. Comparative studies looking at levels of L-proline and LA co-accumulation in endophyte-free and endophyte-infected grasses due to simulated herbivory would also be interesting.

The other precursor, L-homoserine, incorporated directly into the LA pathway is common in fungi. Most fungi utilize the compound as an intermediate in L-methionine L-threonine, L-serine, and L-isoleucine biosynthesis as follows: L-aspartic acid $\rightarrow$ L-aspartylphosphate $\rightarrow$ L-aspartylesemialdehyde $\rightarrow$ L-homoserine $\rightarrow$ L-O-acetylhomoserine $\rightarrow$ L-cystathionine $\rightarrow$ L-homocysteine $\rightarrow$ L-methionine (Liu et al., 2001). The double deuterium labeling of NFL by L-[4, 4-$^{2}$H$_{2}$]-homoserine (Chapter 3) indicates that the L-homoserine residue is not reoxidized to L-aspartylesemialdehyde before being incorporated into the LA. An activated form of L-homoserine such as L-O-acetylhomoserine or phosphorylated L-homoserine must act as the carbon and nitrogen donor to the A-ring since L-methionine does not. The inconsistent results of some of the proposed intermediates, NACPP and NAPP, may be due to their inability to enter either the fungal cells or cell compartments where LA production occurs. Future work with these compounds could involve the use of enzyme assays with proposed precursors. Detection of these compounds if present in fungal cells should be possible.

Further precursor feeding studies on the LA biosynthetic pathway could involve proposed precursors further down the pathway. Feeding labeled compounds that lack the O-bridge or contain -OH groups at C-2 or C-7 would provide insight into the bridge formation. The
incorporation of the S-methyl group of methionine provides support for the pathway: norloline → loline → methylloline → N-formylloline. It is possible that a methylation of the 1-amine could occur prior to cyclization of the A-ring. Labeled norloline feeding into the other LA would confirm its placement prior to loline in the pathway.

Determination of the $^{15}\text{N}$ chemical shifts allows further study of the other LA. Being able to chemically identify these LA is important, and the determination of $^{15}\text{N}$ chemical shifts gives an additional tool for the determination and study of the LA. Earlier identification of the $^1\text{H}$ and $^{13}\text{C}$ by Petroski et al. and now $^{15}\text{N}$ chemical shifts of some of the LA allows further biosynthetic studies with an array of isotopically labeled compounds.
Appendix A
Minimal medium recipe

The production of medium used for the optimal production of LA consists of the following:

1. Stock solutions of basal salts (mono- and dibasic potassium phosphate) are made individually to 300 mM concentrations in deionized water.

2. The stock solutions of basal salts are mixed together to achieve pH needed and the mixture of this solution (1/10 total volume) is added to deionized water (681 mL H₂O L⁻¹ total volume).

3. MES is added to the basal salts and deionized water solution and pH adjusted again to 5.5. This solution is capped with aluminum foil and autoclaved.

4. In a separate flask, MgSO₄•7H₂O is added to deionized water and autoclaved.

5. Urea, sucrose, thiamine, and trace elements are mixed with the appropriate amount of deionized water and filter sterilized. Trace elements recipe listed in Chapter 2.

6. After autoclaving, basal salts/MES solution, MgSO₄, and solution from step 5 are combined in the sterile transfer hood prior to use.

7. One mL of minimal medium per plate is set aside for grinding of the mycelium for inoculation. Approximately 1 mL of inoculation mix is added to each deep well petri dish containing ~ 29 mL of minimal medium.

8. Plates are wrapped with Parafilm, stacked, and taped together in stacks of 8 plates or less prior to placing on a shaker at 100 rpm for 20-25 days.
Table A.1. Minimal medium recipe for 1000 mL (~33 fermentation plates)

<table>
<thead>
<tr>
<th>Basal salts at pH 5.5</th>
<th>g/ ml</th>
<th>Basal salts at pH 5.5</th>
<th>g/ ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>KPO₄ dibasic</td>
<td>1.369</td>
<td>KPO₄ dibasic</td>
<td>1.369</td>
</tr>
<tr>
<td>KPO₄ monobasic</td>
<td>4.083</td>
<td>KPO₄ monobasic</td>
<td>4.083</td>
</tr>
<tr>
<td>total</td>
<td></td>
<td>total</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

MES 5.856 add to

\[\text{pH basal salts to 5.5 and add MES, pH again to 5.5}\]

<table>
<thead>
<tr>
<th>MgSO₄•7H₂O (2 mM)</th>
<th>0.493 grams in 100 ml deionized H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>urea (15 mM)</td>
<td>0.9 grams in 100</td>
</tr>
<tr>
<td>Sucrose (20 mM)</td>
<td>6.846 grams in 16.7</td>
</tr>
<tr>
<td>Thiamine (0.6 µM)</td>
<td>0.6</td>
</tr>
<tr>
<td>Trace elements</td>
<td>1</td>
</tr>
<tr>
<td>deionized H₂O</td>
<td>681.7</td>
</tr>
<tr>
<td>total volume</td>
<td>1000</td>
</tr>
</tbody>
</table>

For interactive Excel spreadsheet click here.

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Appendix B

Other culture work experiments

To achieve maximal production of LA, I manipulated other components of the minimal medium. Carbon concentration increased LA production in the mannitol fed cultures as shown in Figure A.1, but this was not consistent when utilizing sucrose. Nitrogen concentration (utilizing asparagine as nitrogen source) effects were not consistent (Figure A.2). As seen in Figures A.3 and A.4, magnesium sulfate and thiamine concentrations do not increase production reliably. As can be seen from the figures in Appendix B, conditions that should promote growth also had a trend in increasing LA production although not substantially.
Figure B.1: Carbon concentration effect on LA production.
Figure B.2: Nitrogen concentration effect on LA production.
Figure B.3: Magnesium sulfate concentration effects on LA production
Figure B.4: Thiamine concentration effect on LA production.
LA produced in fermentation cultures of *Neotyphodium uncinatum* can be identified via GC-MS analysis. *N*-Acetylnorloline and *N*-formylloline both have the parent ion of 182 m/z but differ in the presence of a 153 m/z fragment for NANL and a 154 m/z fragment for NFL. Norloline has a parent ion at 140 m/z and loline has the parent ion at 154 m/z. Methylloline fragmentation pattern contains 153 m/z and 168 m/z ions. *N*-Acetylloline contains the 153 m/z 167 m/z ions as well as the parent ion at 196 m/z. All of the LA contain the characteristic 82 m/z ion.
Figure C.1: GC-MS spectrum of norloline.
Figure C.2: GC-MS spectrum of loline.
Figure C.3: GC-MS spectrum of methylloline.
Figure C.4: GC-MS spectrum of N-acetylnorloline.
Figure C.5: GC-MS spectrum of $N$-formylloline.
Figure C.6: GC-MS spectrum of N-acetylloline.
Appendix D

GC-MS of unknown potential loline alkaloids

Compounds shown in Figures D.1 were extracted from L-[U-\(^{13}\)C, \(^{15}\)N]-proline fed Neotyphodium uncinatum fermentation cultures. These compounds do not match any of the common LA but do incorporate the 5-membered ring structure of proline as can be seen by the +5 amu shift as well as the presence of the 82 or 83 m/z ion. These compounds are produced in low concentrations in the cultures but are possible LA with various other substitutions on the \(1\)-amine nitrogen.
Figure D.1: Unknown compounds containing 82 or 83 m/z ion peak and evidence of proline incorporation with +5 amu shift.
References


Vita

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