2011

INTERMEDIATE STEPS OF LOLINE ALKALOID BIOSYNTHESIS

Jerome Ralph Faulkner
University of Kentucky, jfaulkner1@gmail.com

ABSTRACT OF DISSERTATION

JEROME RALPH FAULKNER

THE GRADUATE SCHOOL

UNIVERSITY OF KENTUCKY

2011

INTERMEDIATE STEPS OF LOLINE ALKALOID BIOSYNTHESIS

DISSERTATION

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

By

Jerome Ralph Faulkner

Lexington, Kentucky

Director: Dr. Christopher L. Schardl, Professor Plant Pathology

Lexington, Kentucky

2011

ABSTRACT OF DISSERTATION

INTERMEDIATE STEPS OF LOLINE ALKALOID BIOSYNTHESIS

Epichloë species and their anamorphs, *Neotyphodium* species, are fungal endophytes that inhabit cool-season grasses and often produce bioprotective alkaloids. These alkaloids include lolines, which are insecticidal and insect feeding deterrents. Lolines are *exo*-1-aminopyrrolizidines with an oxygen bridge between carbons 2 and 7, and are usually methylated and formylated or acetylated on the 1-amine. In previously published studies lolines were shown to be derived from the amino acids L-proline and L-homoserine. In addition the gene cluster involved in loline-alkaloid biosynthesis has also been characterized. In this dissertation a survey of plant-endophyte symbioses revealed a phenotype with only *N*-acetylnorloline. This phenotype provided insights into loline alkaloid production. This dissertation focuses on determining the steps to loline biosynthesis after the amino acid precursors. The study involves feeding isotopically labeled potential precursors to loline-alkaloid-producing cultures of *Neotyphodium uncinatum*, as well as RNA interference (RNAi) of *N. uncinatum* genes for steps in the pathway. Synthesized deuterated compounds were fed to loline-alkaloid-producing cultures of *N. uncinatum* to test their possible roles as precursors or intermediates in the loline-alkaloid pathway. *N*-Formylloline was extracted from the cultures and assayed by GCMS for incorporation of the deuterium label. The results indicated that *N*-(3-amino, 3-carboxy)propylproline and *exo*-1-aminopyrrolizidine are intermediates in the loline-alkaloid biosynthetic pathway. Plasmids were also designed for expression of double-stranded RNA homologous to loline-alkaloid biosynthesis genes, and introduced by transformation into *N. uncinatum*. This RNAi strategy resulted in fungal transformants altered in loline-alkaloid profiles. The RNAi results indicated that *N*-acetyl-1-aminopyrrolizidine is the intermediate before oxygen bridge formation. Based on the results of this study and the likely roles of the loline-alkaloid biosynthesis genes inferred from signature sequences of their predicted protein products, I propose a pathway of bond formation steps in loline-alkaloid biosynthesis.

KEYWORDS: Loline, endophyte, Epichloë, biosynthesis, pyrrolizidine

Jerome Ralph Faulkner

Student's Signature

August 16, 2011

Date

INTERMEDIATE STEPS OF LOLINE ALKALOID BIOSYNTHESIS

By

Jerome Ralph Faulkner

Christopher L. Schardl

_____

Director of Dissertation

Lisa Vaillancourt

_____

Director of Graduate Studies

August 16, 2011

_____

Date

RULES FOR THE USE OF DISSERTATIONS

Unpublished dissertations submitted for the Doctor's degree and deposited in the University of Kentucky Library are as a rule open for inspection, but are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but quotations or summaries of parts may be published only with the permission of the author, and with the usual scholarly acknowledgments.

Extensive copying or publication of the dissertation in whole or in part also requires the consent of the Dean of the Graduate School of the University of Kentucky.

A library that borrows this dissertation for use by its patrons is expected to secure the signature of each user.

Name	Date

ABSTRACT OF DISSERTATION

JEROME RALPH FAULKNER

THE GRADUATE SCHOOL

UNIVERSITY OF KENTUCKY

2011

ACKNOWLEDGEMENTS

I would first like to thank my Lord and Savior Jesus Christ because without His grace I would not be the person that was able to do this work. I would like to sincerely thank my major professor and supervisor Christopher Schardl. Thank you for your guidance before, during and after this work. I am positive that your helpful suggestions, assistance and knowledge are the sole reasons that I was able to accomplish this feat. Your kind leadership, encouraging words, and keen research mind has always impressed me and I hope that I take some of those traits along with me in my personal and professional life.

I would like to thank the members of my graduate committee Lisa Vaillancourt, Pradeep Kachroo and Robert Grossman for their assistance in this process and for helping to keep me on the right track to reach this final goal. I would also like to thank George Wagner for being my outside examiner and remaining keeping this commitment.

I would like to express my gratitude and thanks to my family. Thank you Carol, Cameron and Jordan for being there for me and giving me the support I needed. I would like to thank my mom and sister for helping me throughout my college years. I am not good at expressing my feelings but to all of my family thank you for believing and supporting me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FILES

Dissertation_JRF.pdf

CHAPTER 1

Introduction

Endophytes of grasses

Cool-season grasses (subfamily Pooideae) can often be inhabited by symbiotic fungi that grow in the intercellular spaces of their hosts. These fungal endophytes of grasses are from the *Neotyphodium* and *Epichloë* species of Ascomycota. Grass fungal endophytes are provided with nutrients and shelter while providing the grass hosts with numerous fitness benefits. Some of the symbioses can, however, turn antagonistic and result in a disease state on the plant. *Epichloë* species can cause choke disease when fungal stromata overgrow the grass inflorescence, halting seed formation. Ascospores produced in the stromata mediate horizontal (contagious) transmission (Chung et al., 1997). Most of the *Epichloë* species and all of their anamorphic derivatives, the *Neotyphodium* species, are also disseminated along with their hosts via seed (Schardl et al., 2004).

Many associations of the endophytes with grasses constitute a mutualism between the pair in which plant and fungus benefit from the symbiosis. Numerous reports have demonstrated the positive effects of endophyte infection on grasses. Endophyte infection has been shown to increase such factors as drought tolerance, biomass, seed production and root growth (Arachevaleta et al., 1989; Belesky et al., 1989; Hill et al., 1991). The fungi are provided nutrients and shelter while they are responsible for biological protection from herbivores and omnivores (Schardl, 1996; Schardl, 1996). The increased fitness of endophyte-infected plants was one of the main reasons the grasses were cultivated for livestock use. It is still not fully understood how the endophyte confers

some of the benefits to its host or whether the effects are due to a combination of plant and fungal properties.

The *Epichloë* species (sexual stage) use a strategy to disseminate themselves in which they suppress maturation of the developing inflorescence and form a stroma on the affected inflorescence. The stromata are then fertilized by flies of the genus *Botanophila* by the female transferring spermatia between compatible mating types, whereupon perithecia develop, then release ascospores that can infect neighboring flowers and cause new fungal infections (Chung et al., 1997; Brem and Leuchtmann, 1999). The inability of asexual *Neotyphodium* species to produce stromata necessitates that they propagate themselves in association with the plant seeds. The fungi infect the developing ovules of the grass and the hyphae continue to colonize the maturing seed (Schardl, 2001). The seed provides protection for the endophyte and upon germination the *Neotyphodium* species will extend into the region of the shoot apical meristem, and the symbiosis is maintained. Often, this vertical transmission mode is also used by the sexual *Epichloë* species in concurrence with choke disease and horizontal transmission, whereby the different transmission modes are manifested on different tillers.

Alkaloids of grass-endophyte symbiosis

The plant hosts provide the means of transmission for the fungal endophytes and receive fitness benefits from the symbioses. Endophyte-infected plants have long been associated with the alkaloids in chemical classes indolediterpene, ergopeptine, saturated 1-aminopyrrolizidine, and the pyrrolopyrazine peramine (Figure 1.1). These four groups of alkaloids are known to have effects on insects, and the first two also affect mammals.

The highly prenylated indolediterpene forms known as lolitrems are responsible for tremor-inducing ryegrass staggers of sheep. Ergine (lysergic acid amide), ergonovine and the ergopeptines are known as the ergot alkaloids. Ergovaline has been well studied in the symbiosis and is the most frequently encountered ergot alkaloid among the grass-epichloae symbioses (Bush et al., 1997). Ergot alkaloids are the main cause of fescue toxicosis, which results in hyperthermia, vasoconstriction, decreased milk production and low birth weight in cattle grazing on certain endophyte-infected grasses (Thompson and Stuedemann, 1993). Peramine is the only known pyrrolopyrazine to be associated with endophyte infection , and is an insect feeding deterrent (Rowan and Gaynor, 1986; Rowan et al., 1986; Tanaka et al., 2005). The fourth group of alkaloids produced by *Epichloë* and *Neotyphodium* species, 1-aminopyrrolizidines, are also known as the loline alkaloids. They are of great interest because of their insecticidal properties and their unique structures.

Loline-alkaloid history

The loline alkaloids are 1-aminopyrrolizidines with a substituted amino group on carbon 1 and an oxygen bridge between carbons 2 and 7 (Figure 1.2). The first loline alkaloid reported was *exo*-1-aminopyrrolizidine-2,7-ether, isolated by Hofmeister (1892) and given the name temuline. The term loline was first used in 1955 by Russian scientists Yunusov and Akramov (1955) to describe one of three alkaloids that they isolated from seeds of *Lolium temulentum* (reported as *Lolium cuneatum*). In this initial work, loline and *N*-acetylloline (NAL, which they called lolinine) were also isolated. Interestingly, in introducing loline to the world, Yunusov and Akramov noted that

another alkaloid from the same grass species, named temuline, had a related chemical formula. It was later determined that temuline and norloline were the same compound (Dannhardt and Steindl, 1985).

In continuation of their investigation of *L. temulentum* alkaloids, Yunusov and Akramov (Yunusov and Akramov, 1960, 1960) isolated and characterized norloline, determining the pyrrolizidine structure and experimentally demonstrating the presence of an oxygen bridge.

Using the purified norloline, loline, and NAL, Yunusov and Akramov (1960, 1960) erroneous suggested a structure for the lolines with the non-ring nitrogen and oxygen attached to the same carbon; however, they corrected themselves in another paper six years later (Yunusov and Akramov, 1960; Akramov and Yunusov, 1966). Their correction came one year after another alkaloid, termed festucine, was reported from *Lolium arundinaceum* (=*Festuca arundinacea*) (Yates et al., 1990). Yates and Tookey describe the structure of festucine to be a positional isomer of loline, assuming the originally proposed structure of loline (Yates et al., 1990). With the corrected loline structure and the festucine structure published at different times, loline and festucine were determined to be the same alkaloid. However, it took three years for NMR and IR spectra to be published that confirmed that loline and festucine are in fact the same compound (Aasen and Culvenor, 1969).

In addition to festucine and loline, Aasen and Culvenor cite previous work identifying an alkaloid of *Adenocarpus decorticans* termed *N*-methyldepropionyldecorticasine as being festucine (Aasen and Culvenor, 1969). The Aasen and Culvenor (1969) study puts together information showing that lolines are

produced in two different families of plants, the Fabaceae and Poaceae. This laid groundwork for additional studies to determine the prevalence of loline alkaloids in other plant species. However, there are only a few studies that have tried to determine the loline alkaloid profiles of plants containing lolines.

The next analysis of the loline structure in the literature occurred when researchers determined the absolute configuration of the three-ring structure of loline (Bates and Morehead, 1972). The firm establishment of the ring structure allowed the next few years in loline research to be involved in discovering the different forms in seed samples. In *L. arundinaceum,* NAL, *N*-formylloline (NFL), and *N*-acetylnorloline (NANL) were isolated, whereas *N*-methylloline (NML), *N*-formylnorloline, and an *N*-oxide of NAL were described from *L. temulentum* (Robbins et al., 1972; Batirov et al., 1977; Batirov et al., 1977). The latter was compared to a synthesized version but has not appeared in other isolations of loline alkaloids to date. As a result of determining the physical properties of the loline alkaloids, lolines have been discovered in *Lolium* species, *Festuca* species, *Hordeum* species, *Echinopogon ovatus, Poa autumnalis,* and *Achnatherum robustum* (=*Stipa robusta*) (Siegel et al., 1990; TePaske et al., 1993; Leuchtmann et al., 2000). However, it is important to note that most analysis of loline-containing seeds or plant samples looked for the predominant NFL and NAL in associations with the assumption these were the most biologically important versions. It would be beneficial to know more about the complete composition of loline alkaloids in various plant and fungal associations. There has also been a report of loline in association with *Argyreia mollis,* which is a member of the Convolvulaceae family (Tofern et al., 1999). Endophyte infection has yet to be associated with loline production

in the Fabaceae and Convolvulaceae. It has been shown that a clavicipitaceous fungus is associated with Convolvulaceae plants that produce ergot alkaloids (Steiner et al., 2006). However endophyte infection has been linked to loline production in the grasses and the fungal endophyte *Neotyphodium uncinatum* has been shown to produce loline alkaloids in fermentation cultures (Siegel et al., 1990; TePaske et al., 1993; Leuchtmann et al., 2000; Blankenship et al., 2001).

Loline alkaloid effects

The insecticidal effects of loline alkaloids have been demonstrated on many insects. Loline alkaloids have been shown to be feeding deterrents against larvae of large milkweed bug (*Oncopelutus faciatus*), Japanese beetle (*Popillia japonica*) and fall armyworm (*Spodoptera frugiperda*) (Yates et al., 1989; Patterson et al., 1991). The insecticidal effects of lolines have been shown on the bird-cherry oat aphid (*Rhopalosiphum padi*), greenbug aphid (*Schizaphis graminum*), European corn borer (*Ostrinia nubilalis*), and large milkweed bug (Yates et al., 1989; Siegel et al., 1990; Riedell et al., 1991). Also, contact toxicity of loline alkaloids has been reported against the American cockroach (*Periplaneta americana*), cat flea larvae (*Ctenocephalides felis*), and Japanese beetle larvae (Bush et al., 1993). Recently, lolines have been shown to be toxic to the rice leaf bug (*Trigonotylus caelestialium*) in Japan (Shiba and Sugawara, 2008; Shiba and Sugawara, 2009). In the aforementioned instances the lolines used in artificial feedings of the insects were NFL or NAL. Further research should be conducted to determine whether NFL and NAL are the most significant anti-insect loline alkaloids produced in all symbioses. As I will show, NANL is the only detectable loline

alkaloid in several grass-endophyte symbiota, so it would be useful in future to determine the biological activity of NANL compared to NFL and NAL.

The effects of endophyte-produced loline alkaloids are not just directed to pests of grasses. Lolines have been shown to have a multitrophic effects. Loline alkaloids showed a negative effect on parasites of *S. frugiperda* when they were given a diet including lolines (Bultman et al., 1997). A similar, although slightly different effect occurred when a hemiparasitic plant, *Rhinanthus serotinus,* was fed on loline alkaloid-containing plants. The *R. serotinus* plants obtained loline alkaloids from the host plant, enhancing the resistance of the parasitic plants to the aphid *Aulacorthum solani* (Lehtonen et al., 2005). The effects of lolines have also been demonstrated in studies in which loline-alkaloid production was induced by wounding (mock herbivory) or by insect-mediated damage (Bultman et al., 2004; Gonthier et al., 2008). The increase in lolines after damage suggests the endophyte responds to attack of its host plant, increasing the defense and, therefore, fitness of both host and symbiont. However, in meadow fescue (*Lolium pratense*) with *N. uncinatum* or *N. siegelii*, the apparent wounding effect seems to be due to much greater availability of precursor amino acids in younger compared to older plant tissue (Zhang et al., 2009).

Loline production in culture

Loline alkaloids have only recently been definitively shown to be produced by endophytes , once a culture condition that induces loline production by *N. uncinatum* was devised (Blankenship et al., 2001). The culture conditions allowed for molecular genetic analysis to identify gene clusters (*LOL*1 and *LOL*2) associated with loline-alkaloid

production in *N. uncinatum* (Spiering et al., 2002; Spiering et al., 2002; Spiering et al., 2005; Spiering et al., 2005). Homologous *LOL* gene clusters have been characterized in *Neotyphodium* sp PauTG-1, *Neotyphodium coenophialum*, and *Epichloë festucae* (Kutil et al., 2007; Kutil et al., 2007). The *LOL* cluster contains 8-11 genes, depending on the species, with a basic conserved order among the genes in all clusters thus identified, except that *lolF* and *lolC* have not been demonstrated to be linked to some *LOL* clusters. The characterized *LOL*1 cluster of *N. uncinatum* is shown in Figure 1.3. The *LOL* genes are predicted to encode four oxidation enzymes (*lolE, lolF, lolO, lolP*), three pyridoxal-5'-phosphate-dependent enzymes (*lolC, lolD, lolT*), a possible transcription factor (*lolU*), a possible amino acid binding protein (*lolA*) (Spiering et al., 2005), an acetamidase (*lolN*) and an *N*-methyltransferase (*lolM*)(Christopher L. Schardl and Juan Pan, unpubl. data). The predicted functions of the nine predicted enzymes are in line with their prospective roles in hypothesized loline-alkaloid biosynthesis (Faulkner et al., 2006; Zhang et al., 2009). However, the number of oxidation enzymes present in the cluster and the limited possible oxidation steps in loline-alkaloid biosynthesis creates an area for study on the roles of these genes.

The precursors involved in the biosynthesis have been identified by feeding loline-producing *N. uncinatum* cultures with radiolabeled and stable-isotope-labeled amino acids (Blankenship et al., 2005). Results of feedings with radiolabeled L-aspartate (L-Asp), L-ornithine (L-Orn), L-glutamine (L-Glu), L-proline (L-Pro), L-homoserine (L-Hse) and L-methionine (L-Met) provided strong evidence against the earlier hypothesis that loline alkaloids are produced from polyamines (Bush et al., 1993; Bush et al., 1993). Instead the loline-alkaloid carbon and nitrogen backbone was derived from L-Hse and L-

Pro precursors, and the carbons attached to the 1-amine were contributed from the S-methyl carbon of L-Met (Figure 1.4) (Blankenship, 2004). Knowledge of the amino acid precursors establishes a chemical framework to build upon for determining the intermediate compounds that are involved in the loline-alkaloid biosynthesis pathway. The origin of the oxygen and mechanism of cyclization of the third ring in the structure still remains to be elucidated, which is the aim of my dissertation research.

Genetic analysis of *LOL* cluster

Expression of loline alkaloids in culture provides an experimental system for determining the roles of the loline alkaloid biosynthesis (*LOL*) genes. Unfortunately, only *N. uncinatum* is able to produce lolines in culture. The ability to create mutants in *LOL* genes is further hampered by the existence of two *LOL* gene clusters in *N. uncinatum* genome (Spiering et al., 2005; Spiering et al., 2005). As a result, only two studies to date have involved transforming *N. uncinatum* to determine the effects of a particular *LOL* gene. Spiering et al. (2005)(2005) created a RNA interference (RNAi) construct for the *lolC*2 gene of *N. uncinatum* and reduced loline alkaloid levels, as well as *lolC*1 and *lolC*2 mRNA expression levels, thereby correlating expression of the gene with loline-alkaloid production. Spiering et al. (2008) were able to eliminate *lolP*1 from *N. uncinatum* and in that study I confirmed the role of the mutation by complementing it with constitutively-expressed *lolP*1. The results indicated that *lolC* it was necessary for the formation of NFL from NML in culture, and constituted the most direct and conclusive evidence of the role of a *LOL* cluster gene to date. Additional correlative evidence that the other *LOL* genes are involved in the pathway includes the findings that

LOL-gene expression is tightly correlated with alkaloid production during loline expression in stromata, inflorescences and culture (Zhang et al., 2009; Zhang et al., 2010).

This dissertation continues to enhance knowledge of loline-alkaloid production in plants. In Chapter 2, a sampling of plants was analyzed for their loline-alkaloid profiles, and results provided the initial indication for the role of the *lolP* gene, as subsequently determined by Spiering et al.(2008). In Chapter 3, I tested possible steps in the pathway be feeding loline-alkaloid-production cultures with deuterium-labeled compounds predicted to be intermediates according to different hypotheses of the biosynthetic pathway. My molecular genetics experiments, described in Chapter 4, provided additional evidence to further clarify the pathway, and resulted in identification of a previously unsuspected pathway intermediate, *N*-acetyl-*exo*-1-aminopyrrolizidine (NAAP). Finally, I discuss in Chapter 5 how my multidisciplinary studies integrate with each other and with published studies to support a hypothesis for the probable loline-alkaloid production scheme.

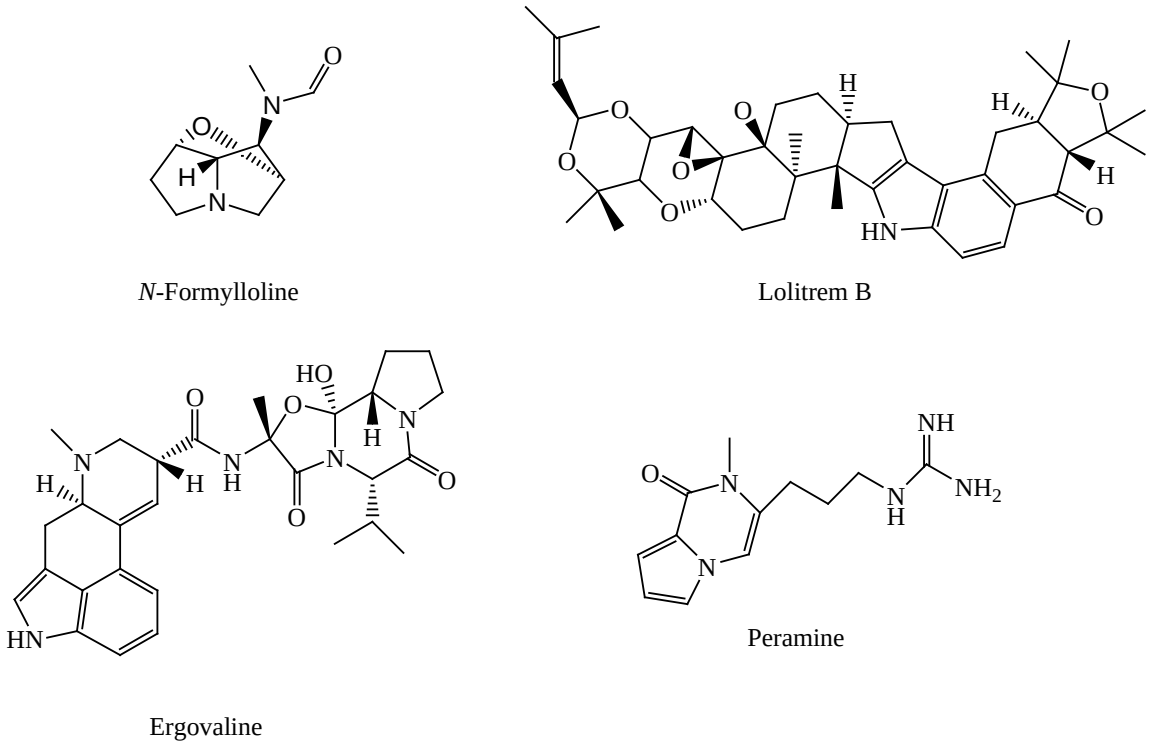
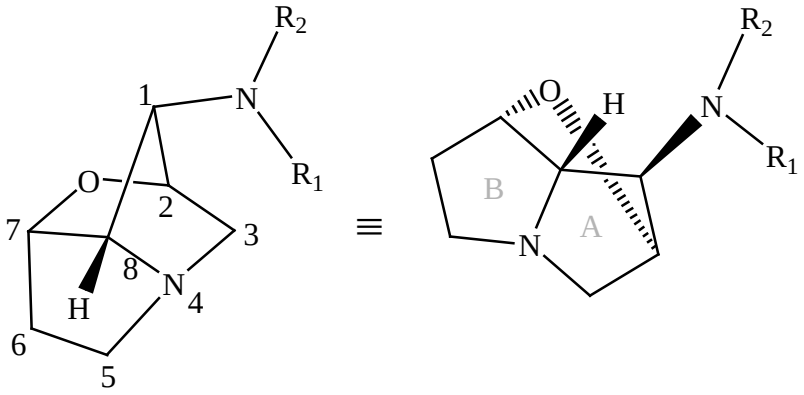


Figure 1.1 Examples of four alkaloid classes found in endophyte grass associations. Shown are *N*-formylloline (a saturated *exo*-1-aminopyrrolizidine), lolitrem B (indolediterpene), ergovaline (ergopeptine), and peramine (pyrrolopyrazine).



Name	R_1	R_2
Norloline	H	H
Loline	CH_3	H
N-methylloline (NML)	CH_3	CH_3
N-formylloline (NFL)	O=CH	CH_3
N-acetylloline (NAL)	O=C—CH_3	CH_3
N-acetylnorloline (NANL)	O=C—CH_3	H
N-formylnorloline (NFNL)	O=CH	H

Figure 1.2 Loline alkaloids found in grass-endophyte associations. Carbons are numbered on the drawing on the left, and A and B rings are labeled on the drawing on the right.

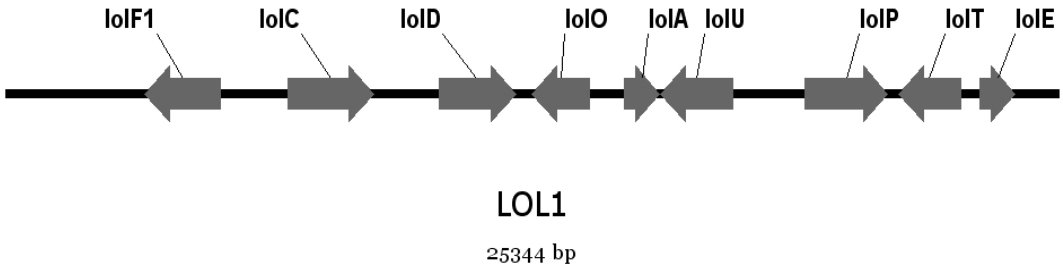


Figure 1.3 *LOL1* gene cluster of *N. uncinatum*. The gene order among *LOL* clusters is conserved although the genes *lolF* and *lolC* are not always demonstrably linked in other endophyte species (Kutil et al., 2007).

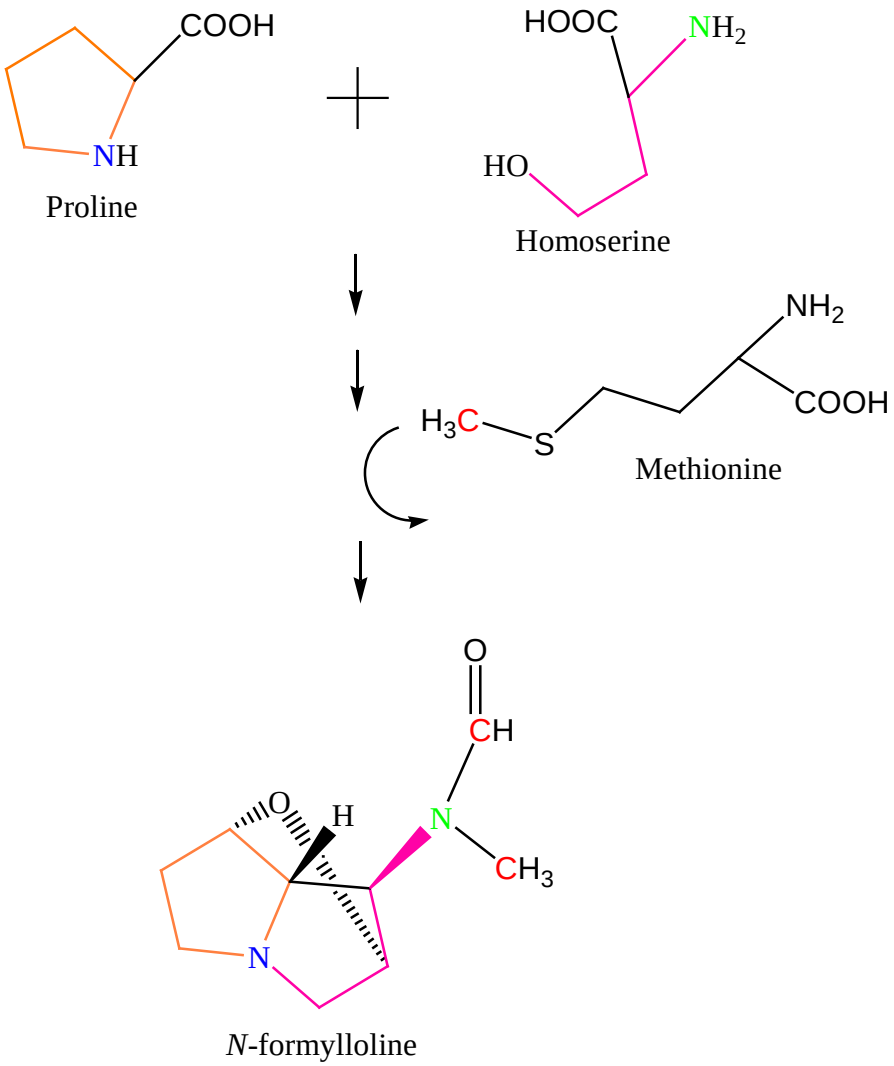


Figure 1.4 Precursors of loline alkaloid production determined by feeding radiolabeled and isotopically labeled amino acid precursors to *N. uncinatum* cultures. Colors indicate carbon and nitrogen contributions of proline, homoserine or methionine to *N*-formylloline.

CHAPTER 2

Loline Composition in Selected Endophytes

INTRODUCTION

Loline alkaloids (1-aminopyrrolizidines) have insecticidal properties and may also increase drought tolerance in plants. Investigations into plant species have associated loline alkaloids with many species of plants in the Poaceae family. In the *Lolium* genus loline alkaloids have been identified in *L. giganteum, L. arundinaceum, L. pratense, L. rigidum, L. multiflorum* and *L. temulentum* (Siegel et al., 1990; TePaske et al., 1993; Leuchtmann et al., 2000). In addition, lolines have also been reported in *Poa autumnalis, Festuca argentina, Festuca versuta, Echinopogan ovatus,* and *Achnatherum robustum* (Siegel et al., 1990; TePaske et al., 1993; Casabuono and Pomilio, 1997; Leuchtmann et al., 2000; Moon et al., 2002). Also, loline alkaloids have been found in association with *Adenocarpus* spp. in the Fabaceae family, and in one member of the Convolvulaceae family, *Argyreia mollis* (Powell and Petroski, 1992; Tofern et al., 1999). Loline alkaloids have been produced in minimal medium cultures of *Neotyphodium uncinatum,* which is an endophyte of *L. pratense* (Blankenship et al., 2001). Endophyte infection has yet to be associated with loline production in the Fabaceae and Convolvulaceae. However, it has been shown that a clavicipitaceous fungus associated with convolvulaceous plants produces ergot alkaloids (Steiner et al., 2006).

Many studies of loline alkaloids in plants consist of determining the presence or absence of *N*-formylloline (NFL) and *N*-acetylloline (NAL), which have been considered the two most predominant loline alkaloids (Bush et al., 1982; Siegel et al., 1990; Leuchtmann et al., 2000; Bultman and Bell, 2003; Koulman et al., 2007). NFL and NAL

are the two most abundant loline alkaloids produced in tall fescue and thus were easy to identify and measure using gas chromatography. In other loline alkaloid studies, the authors have focused on determining the full loline alkaloid profile of a single species of plant (Dannhardt and Steindl, 1985; Yates et al., 1990; Casabuono and Pomilio, 1997; Tofern et al., 1999). In searching through literature related to loline alkaloid production in plant species I only encountered one report that determined the complete loline alkaloid profile associated with more than one plant species: TePaske et al (1993) showed loline alkaloid profiles for *Lolium arundinaceum* (*Festuca arundinacea*), *F versuta, Hordeum bogdanii, L. rigidum, L. temulentum, L. persicum, L. multiflorum, Poa alsodes,* and *Stipa robusta* (Table 2.1), analyzing plants for loline, *N*-methylloline, *N*-acetylnorloline, NFL, and NAL. Since the reports of TePaske et al (1993) it has been shown that loline, *N*-methylloline, *N*-acetylnorloline, NFL, and NAL are produced by fungal endophytes and not are not plant products (Blankenship et al., 2001).

The ability of *N. uncinatum* to produce loline alkaloids has been attributed to two gene clusters designated *LOL*1 and *LOL*2 (Spiering et al., 2002; Spiering et al., 2005). The *LOL* gene clusters contain nine genes encoding enzymes that are indicative of secondary metabolism. The two *LOL* gene clusters contain the same genes in the same conserved order (Spiering et al., 2005; Kutil et al., 2007) except that *lolF*2 has not been mapped relative to *LOL*2. Also, the *lolP*2 gene contains a deletion and resulting frame shift which truncates the reading frame and renders *lolP*2 nonfunctional (Spiering et al., 2008). The *LOL* cluster has been found in other *Neotyphodium* and *Epichloë* endophytes that produce loline alkaloids in symbiosis (Wilkinson et al., 2000; Kutil et al., 2007). The gene order is conserved in the other endophytes; however, *N. uncinatum* is the only

species reported to contain two copies of the cluster. (M.J. Spiering also detected two *lolC* genes in the hybrid endophyte *Neotyphodium chisosum*, apparently donated by two of its three ancestors; unpublished data.)

In the work described in this chapter, I analyzed a worldwide collection of grasses in order to increase the knowledge of loline alkaloid profiles in plant endophyte symbiosis. The discovery of the loline gene cluster would indicate that endophytes with loline genes produce lolines *in planta*. Therefore, I hypothesize that plants without loline production have endophytes without *LOL* genes and the presence of *LO*L genes indicate the ability to produce lolines *in planta*. My goal was to catalog the different loline alkaloid combinations that can be produced *in planta* by endophytes, and I was particularly interested to test if all loline-containing plants contained NFL or NAL, as has generally been assumed (Siegel et al., 1990).

MATERIALS AND METHODS

Plant Material

Plants used in this study were from a wide range of genera and geographical locations. The plants used were *Agrostis hyemalis*, *Elymus* sp., *Agrostis perennans, Cinna arundinacea, L. arundinaceum, L. pratense, Festuca mairei, Sphenopholis obtusata,* and *Sphenopholis* sp. from a variety of geographical areas (Table 2.2). The *L. arundinaceum* plants numbered 4302, 4303, 4304, 4305, 4307, 4308 and 4309 were all from PI598903 collected near Meknes and Boufekrane, Morrocco, plants 4310, 4311 and

4313 were from PI610905 collected near Midelt, Morrocco, and plants 4335, 4335 and 4337 were from PI610904 collected near Zaier, Morrocco.

Tissue print immunoblot (TPIB) assay

Endophyte infection was determined by TPIB,, as previously described (Gwinn et al., 1991) with one modification: after imprinting, the nitrocellulose membrane was air dried at room temperature (not 80 °C) before placing overnight in blocking solution. Antiserum used for TPIB was prepared by W. Hollin (University of Kentucky) as described by An et al. (1993). The antibody used for the TPIB is specific for epichloid endophytes. The immunoblot test indicates the presence of the epichloid endophyte (*Epichloë* or *Neotyphodium* species) by a dark pink color that develops where the tissue had been pressed on the membrane. Plant 18 and plant 19 were used as indicators of non-infected and endophyte-infected material as controls on each nitrocellulose membrane.

Loline alkaloid analysis

Plants were maintained in the greenhouse, and clipped and repotted periodically to control growth. Loline alkaloid analysis was conducted by gas chromatography (GC) using a chloroform extraction of lyophilized plant material, and performed by LaTasha Williams under direct supervision of the author. Loline alkaloids were extracted from 100 mg of lyophilized plant material. Plant material was lyophilized then ground in a Cuisinart Coffee Bar™ coffee grinder Model DCG-20. The ground material was weighed, and to 100 mg was added 100 µL of 1 M NaOH. This basified plant extract was then combined with 1 mL of chloroform. The mixture was then vortexed and

allowed to sit for one to two hours before removing the organic layer for GC analysis as previously described (Blankenship et al., 2001). Loline alkaloids were identified by comparison of retention times to peaks from known loline alkaloid-containing material (tall fescue 'Kentucky 31' seed), analyzed with every batch of samples. Loline extraction for GCMS was performed as follows: 1 g of lyophilized plant material was basified with 1 mL of 1 M NaOH to pH $\geq$ 12 (checked with pH paper). To the basified material, 5 mL of chloroform was added and the solution was vortexed. The mixture was allowed to sit at room temperature for 2 h while vortexing approximately every 15 min. The organic layer was removed. The organic layer was then placed on ice and a stream of air was applied to concentrate the solution to approximately 1 mL, and the sample was then analyzed by GCMS as described below.

Gas Chromatography Mass Spectrometry

Gas Chromatography Mass Spectrometry (GCMS) was performed on samples as needed, using the same extraction procedure as described above. GCMS was conducted using a Hewlett Packard (Avondale, PA) 5890 Series II Plus GC that was equipped with a Restek column (30 m, 5 Sil MS, 0.25 mm i.d., 0.25 m film thickness). Nitrogen at 20.4 mL min^{-1} was used as the carrier gas. The run procedure was an injection temperature of 250°C, detector temperature of 325°C, initial column temperature of 110°C with a 4°C min-1 increase to 188°C. A column purge was conducted of 280°C for 5 min between each sample.

Isolation of endophytes

Cultures of each endophyte analyzed were obtained by growing the fungus out of plant material. Leaves and stems of plants were clipped and placed in a 50 mL Costar tube (Corning, Lowell, MA). The plant material was sterilized by adding 20 mL of 95 % ethanol and gently shaken for 5 min. The ethanol was rinsed away with sterile water, and then a 5.25 % sodium hypochlorite solution was added to the tube and shaken gently for 2 min. The sodium hypochlorite was rinsed away with sterile water, then the leaf and blade material was cut into 2 mm strips. These strips were then placed onto Petri plates containing potato dextrose agar (PDA, Difco Laboratories, Detroit, MI) and incubated until mycelium was observed growing from the plant material (ca. 14 d). The cultures were then transferred onto a new PDA plates and single-spore isolated to obtain a culture for DNA extraction.

Fungal DNA extraction

Two methods were used to obtain fungal DNA for analysis. The first method was a modification of the method described by Spiering et al (2008), whereby 2 µL of 2-mercaptoethanol was added to each 1 mL of lysis solution. The second method of DNA extraction was that of Al-Samarrai et al (2000), and was only used to extract DNA from endophytes that did not yield sufficient high-quality DNA after attempting the first method. In all cases the DNA was quantified and tested by PCR (as described below in PCR primers and conditions) for integrity.

PCR primers and conditions

The primer sequences used in this chapter are given in Table 2.3. PCR was performed (by undergraduate student Erin B. Johnson under my supervision) for *tubB* (β-tubulin), *lolC*, *lolD*, *lolO*, *lolE*, *lolU*, *lolP*, and *lolF*, using primers designed based on sequences of the *N. uncinatum LOL* clusters. PCR was performed with 25 µL reactions containing 0.2 µM of each primer, 2.5 units of AmpliTag Gold, and 200 µM of dNTP's in AmpliTaq Gold PCR buffer (Applied Biosystems, Foster City, CA) with 1.5 mM $MgCl_2$ on a GeneAmp PCR System 2400 thermocycler (Perkin-Elmer, Boston, MA). DNA obtained from endophytes was tested for integrity by PCR amplification of the *tubB* gene using primers 1235 and 11140, with a temperature regime of 95 °C for 5 min, then 35 cycles of 95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 min, with an expected product of about 900 bp. After this verification of the suitability of the DNA preparation for PCR, the *LOL* genes were amplified by PCR using conditions listed in Table 2.4.

Endophytes from grasses that were not analyzed for loline alkaloids but included for phylogenetic analysis were PCR-screened for *lolC* using primers cOAH5'-3'02/22/01 and cOAH3'-5'02/22/01, with PCR conditions of 95 °C for 9 min, then 40 cycles of 95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 2 min, giving an expected product of ca. 1800 bp.

PCR product sequencing

PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) according to manufacturer's protocol. The PCR products were then quantified and used as template for the sequencing reaction. PCR products were

sequenced using the BigDye Version 3 Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) in an 8 µL reaction using 10 to 40 ng of PCR product with the following thermal cycling conditions: 96 °C for 1 min, then 25 cycles of 96 °C for 10 sec, 50 °C for 5 sec, 60 °C for 4 min. The primers used in the sequencing of *lolC* PCR products were lolC5-3seq, lolC3-5seq, cOAH5'-3'02/22/01, and cOAH3'-5'02/22/01. The products were analyzed by capillary electrophoresis on an Applied Biosystems model 3730, in the Advanced Genetic Technology Center (AGTC) at the University of Kentucky.

Sequence Analysis

Sequences were analyzed using Vector NTI 10.3.0 (Invitrogen, Carlsbad, CA). Contigs of individual PCR products were assembled using ContigExpress, and then the sequence was manually checked for any miscalled base pairs, and appropriately annotated. The sequences were then aligned using AlignX to obtain a complete *lolC* sequence. The complete *lolC* sequence was compared with *E. festucae lolC* using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the intron sequences. The intron sequences were taken and combined for each fungus to obtain sequence of only the *lolC* introns. The intron sequences obtained by me were used with *lolC* intron sequences from M. J. Spiering and C.L. Schardl (unpublished). Table 2.5 lists the endophyte species and plant hosts for the endophytes provided by M.J. Spiering and C.L. Schardl. The sequences were aligned with an online version of ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and a phylogenetic tree was

generated from the alignment using the default settings of PhyML ((Guindon and Gascuel, 2003); http://www.atgc-montpellier.fr/phyml/).

RESULTS

Tissue print immunoblot (TPIB) results

TPIB was performed on all plants in Table 2.2 with the exception of plant 4163-20, which was unavailable. The results indicated that plants 19, 57, 167, 273-1, 273-2, 273-3, 4054, 4073, 4118, 4119, 4163-1, 4163-6, 4163-8, 4163-18, 4163-19, 4308, 4310, 4311, 4313, and 4452 contained epichloid endophytes (*Epichloë* or *Neotyphodium* species). The negative TPIB results are contradictory to the previously known endophyte status in most of the grass plants. The plants in Table 2.6 that were previously known to be endophyte negative are plants 18 and plants 909. The remaining plants are known to contain epichloid endophytes (Schardl, unpublished). The TPIB is specific for detection of fungal grass endophytes while in plant material (An et al., 2003).

Loline alkaloids in plant material

Previously, *A. hyemalis* in association with an *Epichloë* sp. was reported to lack NFL and NAL (Siegel et al., 1990). This plant had been given accession number 57 in our greenhouse collection. Anecdotal observations in our greenhouse indicated that *A. hyemalis* plant 57 seemed to be resistant to aphids during outbreaks (Schardl C.L., and Brown J.D. unpublished). This observation led me to investigate whether loline alkaloids may be present in this plant and in other forms or at concentrations too low for detection

in the previous survey. Out of 72 individual plant samples, 25 showed positive GC peaks for one or more loline alkaloids (Table 2.6). Of the positive plants, nine contained only GC trace peaks corresponding to NANL, of which five were *Agrostis hyemalis* numbers 57, 273-1,273-2, 273-3, and four were *L. arundinaceum* plants numbers 4054, 4118, 4307, 4309, and 4452. The five *A. hyemalis* plants were all collected from North America in the states of Texas, Alabama and South Carolina (Table 2.2). Three of the *L. arundinaceum* plants originated from Morocco, whereas the fourth was from the commercial cultivar Georgia Jessup.

In order to confirm the identity of the lone GC peak from plant 57 (Figure 2.1), GCMS was conducted on extract from 1 g of leaf material from plant 57. GCMS identified the peak that *E. amarillans* produced was NANL in symbiosis with *A. hyemalis* (Figure 2.2). GCMS results for the loline peak in plant 57 corresponded to the mass spectra previously published for NANL (Petroski et al., 1989). Using a standard curve based on the NFL standard, I calculated that this symbiotum produces 48 μg *N*-acetylnorloline /g dry weight. The retention time obtained from the NANL peak of plant 57 was used to determine that NANL was the only loline present in plants 273-1,273-2, 273-3, 4054, 4118, 4307, 4309, and 4452.

LOL cluster genes PCR results

PCR was performed on endophytes isolated from plants 19, 57, 167, 273-1, 273-2, 273-3, 4054, 4142, 4163-19, 4303, 4304, 4305, 4309, 4427, 4429, 4441, 4451,and 4452 (see Tables 2.2 and Figure 2.3). The primers were based on loline gene cluster coding sequences obtained from published *LOL* sequence from *N. uncinatum*. Some of

these plants were positive and others were negative for loline alkaloid production. I was unable to obtain fungal mycelium for DNA analysis from the other plants analyzed in Table 2.2 due to bacterial contamination or lack of endophyte growth from the plant material. Figure 2.3 lists the PCR results for the *LOL* genes analyzed. Isolates from plants 4303, 4304, 4305, and 4427 were negative by PCR for the all of the *LOL* genes analyzed. Endophyte from plant 167 tested PCR positive for each of the *LOL* genes analyzed, as expected for *N. uncinatum*. The endophytes from plants 144, 273-1, 273-3, 4429 and 4451 each showed PCR positive for only one of the *LOL* genes. The endophyte of plant 4451 only showed PCR positive using the primers for *lolP1*. The endophyte from 4429 was PCR positive for *lolD*2. The remaining endophytes analyzed by PCR were positive for more than one *LOL* gene.

Phylogenetic analysis of *lolC*

The *lolC* intron sequences of endophytes from plants 57, 273-2, 19, 4054, 4074, 4163-19, 4309, 4142 and 167 were obtained by sequencing the *lolC* PCR products obtained. The sequences were aligned with the sequences provided from Table 2.5 and a phylogenetic tree was generated from the alignment. The phylogenetic tree (Figure 2.4) showed that the *lolC* genes of endophytes from plants only producing NANL did not group together in a distinct clade.

DISCUSSION

The analysis of several plants revealed that loline alkaloid production in plants occurs in a wide distribution of plants from a range of locations. Lolines were found in plants from Europe, Africa, and North America. Endophyte-plant associations contained a wide range of lolines including loline, NML, NANL, NFL, and NAL. However, the presence of *LOL* genes by PCR of endophyte DNA did not always indicate the ability of the endophyte to produce loline alkaloids in planta. Analysis of the plant material provided a more accurate determination of loline presence or absence compared to PCR. A novel loline-alkaloid phenotype was reported; namely, associations possessing NANL but lacking loline, NML, NFL and NAL. Phylogenetic analysis of the endophyte *lolC* genes from NANL-only plants indicated that the phenotype does not indicate a relationship between the endophytes. Also, the PCR results for *LOL* genes in NANL-only endophytes suggested a lack of *lolU* and *lolP* in the endophytes. The use of a wide range of endophyte and plant associations led to determining new loline alkaloid phenotypes that provide for more clues to roles of specific genes in production of different forms of loline alkaloids.

The presence or absence of NAL or NFL has previously been used to determine the capability of plants to contain loline alkaloids (Bush et al., 1997), whereas presence of the other known loline alkaloids in cool-season grasses (specifically NANL, loline, and NML) have not been investigated in the same study with many plant species. The overall assumption is that the lesser lolines are not involved in plant protection as much as NFL and NAL, which are the two most abundant lolines in some important forage

plants. The assumptions are supported by studies demonstrating the effects of NAL and NFL on insects (Yates et al., 1989; Patterson et al., 1991; Riedell et al., 1991; Shiba and Sugawara, 2008). Nevertheless, I wanted to determine if all loline containing plants contained NFL or NAL, and further, how the sets of *LOL* genes present in the endophyte related to alkaloid profiles of the symbiota.

Two different plant species contained the phenotype of only producing NANL. It is interesting to note that previously *A. hyemalis* was reported as not containing loline alkaloids (Siegel et al., 1990). It is likely that previous loline analysis of *A. hyemalis* did not look for NANL as it has been the general method among determining loline alkaloid content to look for NFL or NAL. Previous loline analyses used CH_2Cl_2, MeOH or a mixture of the two solvents for loline alkaloid extraction from plant material (Bush et al., 1982; TePaske et al., 1993; Leuchtmann et al., 2000). The use of $CHCl_3$ in this work could have provided a better extraction to determine a wider range of loline alkaloids from the plants.

In all *A. hyemalis* plants analyzed in this work, NANL was the only loline alkaloid detected. Casual observation of the *A. hyemalis* plants in the greenhouse indicates that these plants do not contain aphid populations during periodic infestations (Schardl unpublished). The *A. hyemalis* analyzed plants were obtained from three separate states in the United States. I speculate that the *A. hyemalis* plants originating in North America could all have an endophyte with the ability to produce only NANL. Three of the *L. arundinaceum* plants originating from Morocco contained NANL as the only loline alkaloid, demonstrating that the ability to make only NANL is not a specific trait to the endophytes of *A. hyemalis*. This was evident by the phylogenetic analysis

grouping the Moroccan endophyte in a separate clade than the *Agrostis* endophytes. The differences in geographic origins of the plants and gene phylogeny of the endophytes would suggest that the plants containing only NANL have some added benefit to the plants that is not specific to just one region of the world.

The nine plants identified as producing only NANL had identified endophytes in association with the plants. The *A. hyemalis* plants were infected with *E. amarillans* while the L. arundinaceum plants were infected with *N. coenophialum*. This shows that the sexual and asexual phases were both able to produce the NANL only phenotype in their hosts. The two different species show that the NANL only phenotype is more than just an isolated endophyte that has developed the inability to make the other loline alkaloids. The two endophytes species have independently developed this phenotype which alludes to the possibility of NANL as being a loline active in plant defense.

The sample plants analyzed contained a large geographic range from Africa and Europe to North America. The presence of loline alkaloids was identified in plants originating from Morocco, France, Sardinia, Switzerland, and the United States of America. The diversity of the plants analyzed illustrates that loline alkaloids are produced in plants native to many different regions of the world. This is a notable occurrence because the endophyte produces lolines yet it relies on the plant for its protections. The protection provided by the plant causes the endophytes to become specific to their particular host. The far spread dispersal of loline production and types of lolines highlights to necessity for the alkaloids in the symbiosis. The *lolC* phylogenetic tree generated from a selected subset of the loline producing endophytes also indicated that the loline genes are dispersed among a wide geographic area of endophytes. The

loline alkaloid phenotype has been shown to be beneficial to the plant host and this work provides additional information that lolines are a valuable component of the endophyte symbiosis (Riedell et al., 1991).

TPIB can be used as a means to determine endophyte infection. The TPIB results for this chapter were not consistent with some of the endophyte statuses for the plants analyzed. As an example, the TPIB results for the plants producing only NANL show endophyte infection for all plants except 4307. I am uncertain why the immunoblot assay for this particular plant did not indicate endophyte infection because I did see fungi growing from the plant material of plant 4307. The culturing out of the endophyte from 4307 was not successful because of bacteria that also grew from leaf and stem samples of the plant together with the fungi, so DNA analysis was not conducted. It is also possible that the endophyte in plant 4307 is not present throughout the plant material as has been documented in some artificial endophyte infections (Christensen, 1995). Therefore, the tiller used in the TPIB may have not contained the endophyte. This reason would also explain why known endophyte infections were TPIB negative. The inconsistent results illustrate the need to use many methods such as loline analysis, culture techniques, and PCR.

PCR analysis of the endophytes confirmed that the *LOL* genes and loline alkaloid production coincided. The plants 273-1, 273-2, and 273-3 all produced only NANL and the plants were isolated from a single location. However, the PCR results showed a difference in the *LOL* genes contained in the endophytes. The difference in PCR results could be due to a difference in the genotypes of the endophytes infecting these three plants. This indicates the plants may be a collection of diverse individuals and therefore

have a diverse group of endophytes, which would yield different PCR results. The PCR results could be a result of variations among the individuals at the primer binding site causing the primers not to bind at the annealing temperatures used. However, the possibility that such variable results were due to variation in quality of the DNA preparations cannot be ruled out.

The PCR results of all of the NANL-producing endophytes showed positives for all *LOL* genes except *lolU* and *lolP* genes. These results led to the hypothesis that the role of the *lolP* product — a predicted cytochrome P450 — is involved in methyl to formyl conversion in making NFL. This prediction was subsequently confirmed (Spiering et al., 2008). The role of *lolU* is unknown and the predicted protein has sequence similarity to a transcription factor (Spiering et al., 2005). A possibility is that the transcription factor may be involved in regulation of other genes, yet to be identified, leading to production of the methylated loline alkaloids. A more in depth understanding of the roles of the loline genes could help to determine the role of *lolU*.

The endophytes from *Cinna arundinacea* (plant 4451), *Lolium arundinaceum* (plant 4429) and *Elymus* sp. (plant 4441) were PCR positive for *LOL* genes and negative for loline alkaloids *in planta*. The loline analysis did not detect lolines in the plants. The PCR analysis did indicate that the endophytes from those plants contained *LOL* genes. It is likely that the endophytes do not possess all of the necessary genes to make lolines. The endophytes were all three negative by PCR for *lolC* which encodes the proposed first committed step in loline biosynthesis (Spiering et al., 2005). Without a *lolC* gene the endophytes may not be able to synthesize the needed precursors to loline alkaloids. These results indicate that PCR alone is not an adequate determination of the endophytes

ability to produce lolines. It also shows that having positive PCR for a *LOL* gene does not indicate that the endophyte will produce lolines in the plant. Ultimately to do chemical analysis of a plant for loline alkaloids is required for chemotyping its endophyte.

In summary, my analysis of multiple plants from several plant species for loline alkaloids indicated that some associations can produce a single version of loline alkaloids. It was determined that some endophyte and grass associations can produce only NANL. The PCR analysis of selected endophytes in these associations were PCR positive for all known *LOL* genes except *lolP* and *lolU*. The plant species and locations of the plants indicated a wide taxonomic and geographical distribution of loline alkaloid-producing endophytes. The survey of loline alkaloid production and *LOL* genes in the endophytes provides a framework to determine the roles of *LOL* genes in the loline alkaloid production.

Table 2.1 Loline alkaloid profile of plants analyzed by TePaske et al 1993. + indicates presence of the alkaloid by GC analysis; – indicates absence; NANL = *N*-acetylnorloline; NFL = *N*-formylloline; NAL = *N*-acetylloline; NML = *N*-methylloline.

Plant	**NANL**	**NFL**	**Loline**	**NAL**	**NML**
Festuca arundinacea	-	+	-	+	-
Festuca arundinacea	-	+	+	+	-
Festuca arundinacea	+	+	+	+	+
Festuca arundinacea	+	+	-	+	+
Festuca versuta	+	+	+	+	+
Hordeum bogdanii	-	+	-	-	-
Hordeum brevisubulatum	-	+	-	-	-
Lolium rigidum	-	+	-	+	-
Lolium temulentum	-	+	+	+	+
Lolium persicum	-	+	-	+	-
Lolium multiflorum	+	+	-	+	-
Poa alsodes	-	+	-	+	-
Stipa robusta	-	+	-	-	-

Table 2.2 Plant species, identification numbers and origins of plant materials used in this study. Ploidy of *Lolium arundinaceum* indicated in parenthesis under plant species column. *indicates plants artificially inoculated with endophyte.

Plant Species	Number	Origin	Endophyte Status
Agrostis hyemalis	57	Texas, USA	Epichloë amarillans
	273-1	Alabama, USA	Epichloë amarillans
	273-2	Alabama, USA	Epichloë amarillans
	273-3	Alabama, USA	Epichloë amarillans
	4452	South Carolina, USA	Epichloë amarillans
Agrostis perennans	906	South or North America	Epichloë amarillans
Agrostis sp.	4429	Kentucky, USA	Epichloë amarillans
Cinna arundinacea	4451	North Carolina, USA	Neotyphodium sp.
Elymus sp.	4441	Kentucky, USA	Epichloë elymi
Lolium arundinaceum (6x)	18	KY-31 cultivar	E-
	19	KY-31 cultivar	Neotyphodium coenophialum
	4054	Morrocco	Neotyphodium coenophialum
	4118	Georgia Jessup cv	Neotyphodium coenophialum
	4119	Georgia Jessup cv	Neotyphodium coenophialum
	4121-4	*	Epichloë festucae
(4x)	4163-1	France	Neotyphodium coenophialum
(4x)	4163-2	France	Neotyphodium coenophialum
(4x)	4163-3	France	Neotyphodium coenophialum
(4x)	4163-6	France	Neotyphodium coenophialum
(4x)	4163-7	France	Neotyphodium coenophialum
(4x)	4163-8	France	Neotyphodium coenophialum
(4x)	4163-18	France	Neotyphodium coenophialum
(4x)	4163-19	France	Neotyphodium coenophialum
(4x)	4163-20	France	Neotyphodium coenophialum
	4179-2	*	Neotyphodium coenophialum
	4307	Morrocco[a]	Neotyphodium coenophialum
	4308	Morrocco[a]	Neotyphodium coenophialum
	4309	Morrocco[a]	Neotyphodium coenophialum
	4310	Morrocco[b]	Neotyphodium coenophialum
	4311	Morrocco[b]	Neotyphodium coenophialum
	4313	Morrocco[b]	Neotyphodium coenophialum
	4326	Sardinia	Neotyphodium coenophialum
	4327	Sardinia	Neotyphodium coenophialum
	4335	Morrocco[c]	Neotyphodium coenophialum
	4336	Morrocco[c]	Neotyphodium coenophialum

[a] Plants from PI598863 [b] Plants from PI610905 [c] Plants from PI610904.

Table 2.2 (continued)

Plant Species	Number	Origin	Endophyte Status
Lolium arundinaceum (6x)	4337	Morrocco[c]	Neotyphodium coenophialum
	4327	Sardinia	Neotyphodium coenophialum
	4335	Morrocco[c]	Neotyphodium coenophialum
	4336	Morrocco[c]	Neotyphodium coenophialum
	4337	Morrocco[c]	Neotyphodium coenophialum
Lolium sp.	4288	Tunisia	FaTG-2
	4289	Tunisia	FaTG-2
	4290	Tunisia	FaTG-2
	4296	Tunisia	FaTG-2
	4297	Tunisia	FaTG-2
	4301	Tunisia	FaTG-2
	4302	Morrocco[a]	FaTG-3
	4303	Morrocco[a]	FaTG-4
	4304	Morrocco[a]	FaTG-5
	4305	Morrocco[a]	FaTG-6
	4314	Sardinia	FaTG-2
	4315	Sardinia	FaTG-3
	4316	Sardinia	FaTG-4
	4318	Sardinia	FaTG-5
	4319	Sardinia	FaTG-6
	4320	Sardinia	FaTG-7
	4321	Sardinia	FaTG-8
Agrostis sp.	4429	Kentucky, USA	Epichloë amarillans
Lolium arundinaceum	4140	Morrocco	Neotyphodium coenophialum
susbp. Atlantigena (8x)	4142	Morrocco	Neotyphodium coenophialum
	4148	Morrocco	Neotyphodium coenophialum
Lolium pratense	167	Switzerland	Neotyphodium uncinatum
Lolium mairei	909	Morrocco	E-
Sphenopholis obtusata	721	Georgia, USA	Epichloë amarillans
	722	Georgia, USA	Epichloë amarillans
	862	Georgia, USA	Epichloë amarillans
	4448	Unknown	Epichloë amarillans
	4478	North Carolina, USA	Epichloë amarillans
Sphenopholis sp.	4427	Kentucky, USA	Epichloë amarillans
	4431	Kentucky, USA	Epichloë amarillans

[a] Plants from PI598863 [b] Plants from PI610905 [c] Plants from PI610904.

Table 2.3 PCR primers used in this study.

Name	Sequence
1235	GTTTCGTCCGAGTTCTCGAC
11140	GAGAAAATGCGTGAGATTGT
cOAH3'-5'02/22/01	GGTCCAAATAAGTCTTCATTGATC
cOAH5'-3'02/22/01	ATGACAGTAGATACGATTACTTCG
lolC13'-5'	CCATGCCACTTCTGGTAGAACAT
lolC15'-3'	CTTGTTTCAATATTGTCGTCGAGC
lolC2cDNA3-5	GTGAATTACGGGTCTATTAGGAAAAC
lolC2cDNA5-3	CTTGTTTCAATATTGTCGTCGAGA
lolD1cDNA3-5	CAGGGACCCAAAAACGAAAAGG
lolD1cDNA5-3	CCTGTCAGCCGAGTGAATTGC
lolE1fwdcDNA	TCATGACCGCTGCTTCTTCC
lolE1revcDNA	TCAAGTCTGCGCTTCCACTG
lolE2cDNA3-5	GTTCTTGTCAAGTCTGCGCTC
lolE2cDNA5-3	GGTGAAGCCCTCTGTTTCCTG
lolF1fwdcDNA	TATCGACTCGATATACGACAGAAAGTG
lolF1revcDNA	TTCTGCATATGATTATTTAATTCTTCTTC
lolF23-5amp/seq	TCCCAATCTTGGAGAAGCTCT
lolF25-3amp/seq	GACATTTCTGATACAACAAAATTTGG
lolO1fwdcDNA	TGACCGACTGGCATAAGTGC
lolO1revcDNA	ATATCACGATTCCTCACTCTTTCC
lolP1cDNA3-5	ACCCCTGCCATGTGTATCCCT
lolP1cDNA5-3	TTGCCGCCGACGGTTCATACAC
lolP2cDNA3-5	ACCACTGCCATGTGTATTCCC
lolP2cDNA5-3	TTGCCACTGACGGCTCATACAG
lolU3-5seq	GAAGTGGACGCAGGTTC
lolU5-3seq	GTTTCCACGTAAACCAAGG
lolU23-5 amp/seq	CATTGTAGCTACAAGCAGCTTTATC
lolU25-3 amp/seq	AGGTGGCTTGCCTATGGTGCTA

Table 2.4 PCR primer pairs, PCR conditions and expected product sizes for *LOL* genes used in this chapter.

Gene	Product Size (bp)	Primers	Initial Time (95°C)	Cycles	Separation (°C)	Anneal (°C)	Extension Time (Minutes)
lolC	1800	lolC13'-5' lolC15'-3'	9	35	95	60	2
lolC	1800	lolC2cDNA5-3 lolC2cDNA3-5	9	35	95	60	2
lolD	1500	lolD1cDNA5-3 lolD1cDNA3-5	9	40	95	58	2
lolD	1500	lolD2cDNA5-3 lolD2cDNA3-5	9	40	95	58	2
lolE	800	lolE1fwdcDNA lolE1revcDNA	5	35	95	55	1
lolE	800	lolE2cDNA5-3 lolE2cDNA3-5	5	35	95	58	1
lolO	1200	lolO1fwdcDNA lolO1revcDNA	5	35	95	55	1.5
lolU	400	lolU3-5seq lolU5-3seq	5	35	95	52	1
lolU	1700	lolU25-3amp/seq lolU23-5amp/seq	5	40	95	64	2.5
lolF	1800	lolF1fwdcDNA lolF1revcDNA	5	40	95	55	2.5
lolF	350	lolF25-3amp/seq lolF23-5amp/seq	5	35	95	56	1
lolP	1700	lolP1cDNA5-3 lolP1cDNA3-5	5	35	95	64	2
lolP	1700	lolP2cDNA5-3 lolP2cDNA3-5	5	35	95	59	2

Table 2.5 Endophyte number, genus, species and plant hosts for *lolC* intron sequences used in phylogenetic analysis. * indicates provided by M. J. Spiering and C. L. Schardl

Number	Endophyte	Host
19	*Neotyphodium coenophialum*	*Lolium arundinaceum*
55*	*Neotyphodium sp.*	*Poa autumnalis*
57	*Epichloë amarillans*	*Agrostis hyemalis*
167	*Neotyphodium uncinatum*	*Lolium pratense*
277*	*Epichloë glyceriae*	*Glyceria striata*
507*	*Neotyphodium sp.*	*Festuca altissima*
899*	*Neotyphodium aotearoae*	*Echinopogan ovatus*
900*	*Neotyphodium aotearoae*	*Echinopogan ovatus*
901*	*Neotyphodium aotearoae*	*Echinopogan ovatus*
915	*Neotyphodium siegelii*	*Lolium pratense*
999*	*Neotyphodium occultans*	*Lolium multiflorum*
1031*	*Epichloë baconii*	*Calamagrostis villosa*
1040*	*Epichloë typhina*	*Dactylis glomerata*
1125*	*Epichloë brachyelytri*	*Brachyelytrum erectum*
2368	*Epichloë festucae*	*Lolium pratense*
3609*	*Neotyphodium chisosum*	*Unknown*
4054	*Neotyphodium coenophialum*	*Lolium arundinaceum*
4074	*FaTG-3*	*Lolium arundinaceum*
4142	*Neotyphodium coenophialum*	*Lolium arundinaceum*
4309	*Neotyphodium coenophialum*	*Lolium arundinaceum*
4452	*Epichloë amarillans*	*Agrostis hyemalis*
273-2	*Epichloë amarillans*	*Agrostis hyemalis*
4163-19	*Neotyphodium coenophialum*	*Lolium arundinaceum*

Table 2.6 Loline alkaloid profile of selected grass plants. *N*-acetylnorloline (NANL), *N*-formylloline (NFL), *N*-acetylloline (NAL), *N*-methylloline (NML). + indicates presence of the compound by GC analysis; – indicates absence.

Plant	NANL	NFL	Loline	NAL	NML	Plant	NANL	NFL	Loline	NAL	NML
18	-	-	-	-	-	4179-2	-	-	-	-	-
19	+	+	+	+	+	4288	-	-	-	-	-
57	+	-	-	-	-	4289	-	-	-	-	-
167	+	+	+	+	+	4290	-	-	-	-	-
273-1	+	-	-	-	-	4296	-	-	-	-	-
273-2	+	-	-	-	-	4297	-	-	-	-	-
273-3	+	-	-	-	-	4301	-	-	-	-	-
721	-	-	-	-	-	4302	-	-	-	-	-
722	-	-	-	-	-	4303	-	-	-	-	-
862	-	-	-	-	-	4304	-	-	-	-	-
906	-	-	-	-	-	4305	-	-	-	-	-
909	-	-	-	-	-	4307	+	-	-	-	-
4054	+	-	-	-	-	4308	+	+	+	+	+
4070	-	-	-	-	-	4309	+	-	-	-	-
4074	+	+	+	+	+	4310	+	+	+	+	+
4078	-	-	-	-	-	4311	+	+	+	+	+
4082	-	-	-	-	-	4313	+	+	+	+	+
4083	-	-	-	-	-	4314	-	-	-	-	-
4084	-	-	-	-	-	4315	-	-	-	-	-
4089	-	-	-	-	-	4316	-	-	-	-	-
4090	-	-	-	-	-	4318	-	-	-	-	-
4118	+	-	-	-	-	4319	-	-	-	-	-
4119	+	+	-	+	+	4320	-	-	-	-	-
4121-4	-	-	-	-	-	4321	-	-	-	-	-
4140	-	-	-	-	-	4326	+	+	+	+	-
4142	+	+	-	+	+	4327	+	+	+	+	-
4148	-	-	-	-	-	4335	+	+	+	+	-
4163-1	+	+	+	+	+	4336	+	+	+	+	-
4163-2	-	-	-	-	-	4337	+	+	+	+	-
4163-3	-	-	-	-	-	4427	-	-	-	-	-
4163-6	+	+	+	+	+	4429	-	-	-	-	-
4163-7	-	-	-	-	-	4431	-	-	-	-	-
4163-8	+	+	+	+	+	4441	-	-	-	-	-
4163-18	+	+	+	+	+	4448	-	-	-	-	-
4163-19	+	+	+	+	+	4451	-	-	-	-	-
4163-20	-	-	-	-	-	4452	+	-	-	-	-
						4478	-	-	-	-	-

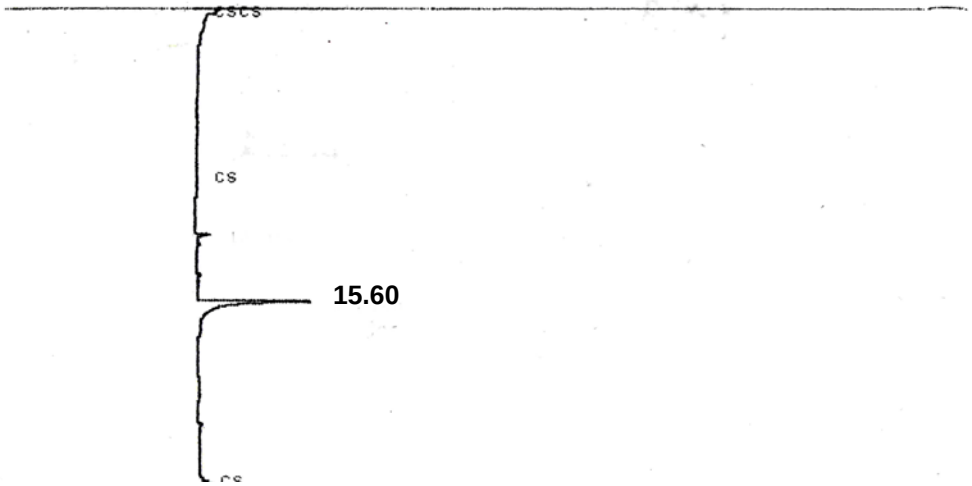


Figure 2.1 GC analysis of *E. amarillans*-infected *A. hyemalis* dried plant material extracted with chloroform, revealed a peak at 15.60 min, a similar retention time to *N*-acetylnorloline (NANL).

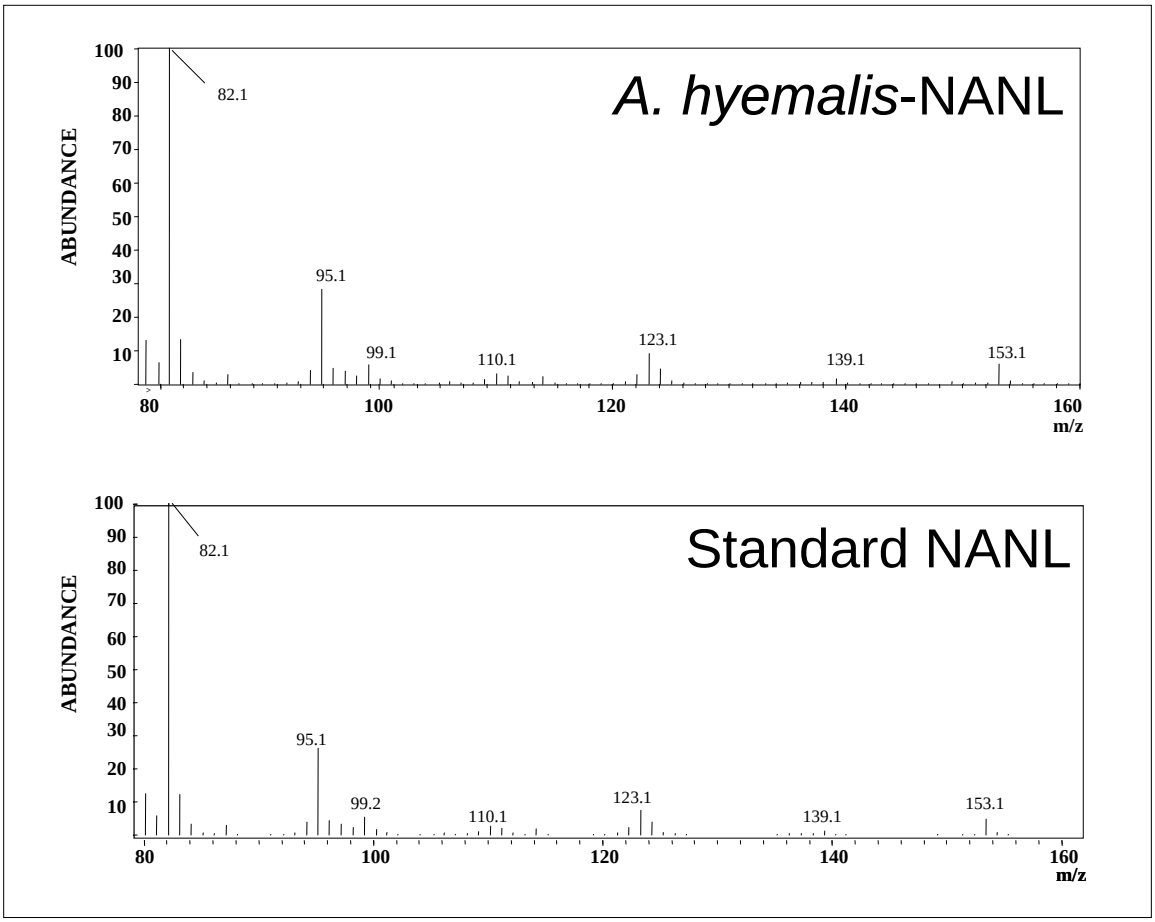


Figure 2.2 GCMS analysis of peak corresponding to the 15.60 peak on the GC. The peak is compared to a standard *N*-acetylnorloline (NANL) extracted from a 'Kentucky-31' tall fescue seed (Standard NANL). The 82, 95, and 110 m/z peaks are indicative of fragments of the loline alkaloid. The standard (tall fescue seed) NANL and the unknown from *A. hyemalis-E. amarillans* contain the same diagnostic mass spectrum peaks, which identifies the compound as NANL.

Plant	lolC1	lolC2	lolD1	lolD2	lolO1	lolU	lolU2	lolP1	lolP2	lolE1	lolE2	lolF1	lolF2
19	O	X	O	X	O	X	O	O	X	O	X	X	X
57	X	O	O	O	X	X	O	O	O	O	O	O	X
144	O	O	O	O	O	O	O	X	O	O	O	O	O
167	X	X	X	X	X	X	X	X	X	X	X	X	X
273-1	O	O	O	O	O	O	O	O	O	O	O	O	X
273-2	X	O	O	O	X	X	O	O	O	X	O	O	X
273-3	O	O	O	O	O	O	O	O	O	O	O	O	X
4054	O	X	O	X	O	O	O	O	X	X	X	X	X
4142	O	X	O	X	O	X	O	O	X	O	X	X	X
4163-19	O	X	O	X	O	X	X	O	X	O	X	O	X
4303	O	O	O	O	O	O	O	O	O	O	O	O	O
4304	O	O	O	O	O	O	O	O	O	O	O	O	O
4305	O	O	O	O	O	O	O	O	O	O	O	O	O
4309	O	X	O	X	O	X	X	O	X	O	X	X	X
4427	O	O	O	O	O	O	O	O	O	O	O	O	O
4429	O	O	O	X	O	O	O	O	O	O	O	O	O
4441	O	O	O	O	O	X	O	X	O	X	O	O	O
4451	O	O	O	O	O	O	O	X	O	O	O	O	O
4452	X	O	X	X	X	X	O	O	O	X	O	X	X

Figure 2.3 Loline gene PCR results from endophytes isolated from indicated plants. X and green color indicates PCR positive, O and red color indicates no PCR product.

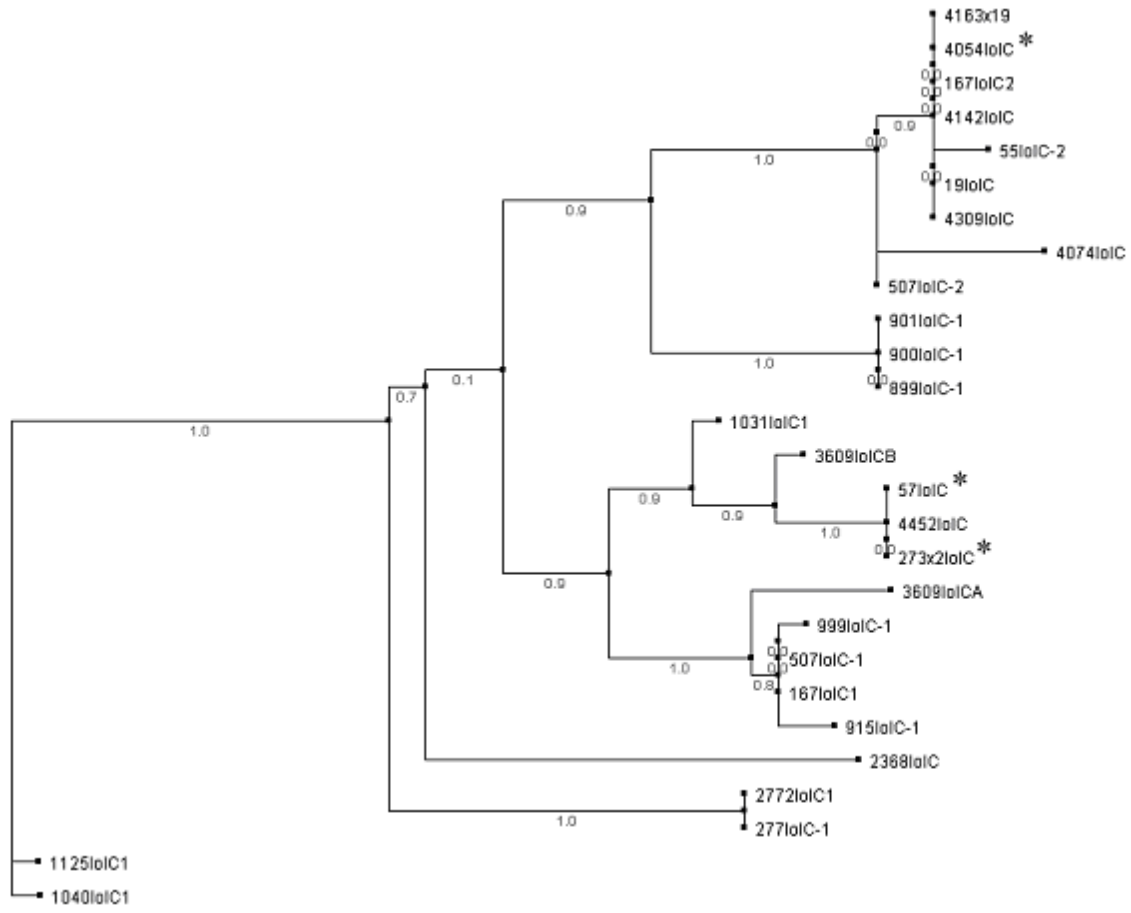


Figure 2.4 Phylogenetic tree generated from intron sequences of *lolC* obtained from PCR for indicated endophytes. Labels for endophytes are numbers described in Table 2.2 and Table 2.6. * indicates NANL only detected from endophyte infected plant.

CHAPTER 3

Incorporation of Potential Biosynthetic Intermediates in Loline Alkaloid-Producing Cultures

INTRODUCTION

Some *Neotyphodium* (asexual) and *Epichloë* (sexual) species are capable of producing loline alkaloids in symbiosis with grass plants. The loline alkaloids are saturated 1-aminopyrrolizidines with an oxygen bridge between C(2) and C(7) of the pyrrolizidine rings. Lolines are differentiated by the groups attached to the non-ring nitrogen. These alkaloids have insecticidal properties and act as antifeeding deterrents (Bush et al., 1993; Schardl et al., 2007). The similarity between lolines and plant pyrrolizidines, such as the necines (Figure 3.1), led others to propose that lolines and necines have a similar route of biosynthesis from polyamines (Bush et al., 1993). The ability to induce *Neotyphodium uncinatum* to produce lolines in culture allowed that hypothesis to be tested and rejected, and determination of the actual amino acid precursors for loline alkaloid production (Blankenship et al., 2001).

In a published study, loline alkaloid-producing cultures were given potential precursors labeled with stable isotopes or radiolabeled to identify which are the biosynthetic precursors of lolines (Blankenship et al., 2005). The incorporation of the isotope or label into *N*-formylloline (NFL; **1**) was assayed by mass spectrometry. The results indicated that homoserine and proline were precursor amino acids. This was in contrast to necine biosynthesis, for which putrescine and spermidine are precursors (Boettcher et al., 1993), indicating that loline alkaloid biosynthesis is via a novel pathway.

The loline alkaloid-production culture conditions also allowed for genetic analysis of *N. uncinatum* loline production (Spiering et al., 2002). A nine gene cluster associated with loline alkaloid production encoded predicted proteins that included a FAD containing monooxygenase (LolF), a cytochrome P450 monooxygenase (LolP), an amino acid binding domain protein (LolA), a putative transcription factor (LolU), three pyridoxal-5´-phosphate (PLP) containing enzymes (LolC, LolD, and LolT), and nonheme-Fe oxidoreductases (LolE and LolO) (Spiering et al., 2005). The predicted functions of the gene products along with identification of the loline alkaloid precursors provide a basis for hypotheses for loline alkaloid biosynthesis, which I test in this chapter.

The pyrrolizidine ring structure of lolines is formed from the carbons and nitrogen of L-homoserine (Hse; probably via *O*-acetylhomoserine, OAH) and L-proline (Pro) (Figure 3.2; (Blankenship et al., 2005)). Therefore, I wanted to determine the first committed step in loline alkaloid biosynthesis, i.e., the first after synthesis of amino acid precursors OAH and Pro. The enzyme likely to catalyze the condensation of Pro with the (1-amino, 3-carboxy) propyl moiety of homoserine is encoded by *lolC*. The LolC protein sequence has the signatures of a gamma-type pyridoxal-phosphate- (PLP) containing enzyme that is similar in amino acid sequence to *O*-acetylhomoserine(thiol)-lyase encoded by the *cysD* gene of *Aspergillus nidulans* (Spiering et al., 2005). *O*-Acetylhomoserine(thiol)-lyase catalyzes a gamma-substitution in methionine biosynthesis that utilizes *O*-acetylhomoserine and H_2S as the substrates (Figure 3.3; (Sienko et al., 1998)). I hypothesize that LolC performs a similar reaction using the secondary amine of Pro to combine with the gamma-carbon of Hse (or likely *O*-acetylhomoserine) (Figure

3.4) to form *N*-aminocarboxypropylproline (**2**). This is one of the hypotheses to be tested in this study.

The oxygen bridge of lolines is biologically unusual because the oxygen atom links two bridgehead carbons (Schardl et al., 2007), so the manner in which this ether bridge is formed is also of interest. The geometric configuration of loline alkaloids shows the ether bridge to be in a highly strained position in the loline molecule. The carbons in the bridgehead positions are unable to form carbocations therefore the oxygen bridge formation step in loline biosynthesis is interesting to determine. The evidence that proline is contributes one of the rings in loline biosynthesis shows that the oxygen bridge cannot be formed through and epoxide intermediate. I hypothesize that hydroxylation generates an intermediate, but there are several possible points in the biosynthesis pathway where hydroxylation might occur. For example, there are two separate possible reactions that could result in a hydroxylated **2** (Figure 3.4). One possibility is that Pro is hydroxylated prior to being used in the pathway. The hydroxyl group from hydroxylated proline would then be carried through loline alkaloid biosynthesis and eventually involved in formation of the oxygen bridge (Figure 3.4). The *cis* and *trans* versions of 3-hydroxyproline (**3**) are components of telomycin, a cyclopeptide antibiotic (Mauger, 1996), indicating that 3-hydroxyprolines can be utilized in secondary metabolite biosynthesis. Alternatively, the hydroxylated **2** could be formed after proline and homoserine condensation. The oxygen from the hydroxylated **2** would later become the oxygen bridge. It is also possible that the oxygen atom is not introduced until farther along in the building of the loline structure. The pyrrolizidine ring could be formed prior to introduction of the oxygen atom (Figure 3.5). Therefore, I test the hypothesis that the

homoserine and proline carbons and nitrogen combine to form the aminopyrrolizidine ring before the ether bridge occurs.

Previous studies by Blankenship et al (2005) demonstrated the proline-derived carbons and nitrogen constitute the B-ring of lolines. Assuming that the proline ring remains intact in the final product, the ring of proline only undergoes three new bond formations. The proline ring nitrogen forms a bond to C(4) from homoserine, the proline C(2) forms a bond with C(3) from homoserine, and the proline C(3) bonds with oxygen in the ether bridge. The postulated first step, condensation of proline with homoserine, would not remove the hydrogen attached to any ring carbon of proline (Figure 3.6). The A-ring closure could proceed with or without exchange of a hydrogen atom at C(7) (the alpha position of proline) depending on whether it occurs before or after decarboxylation at that position. Oxygen bridge formation could entail exchange of a hydrogen atom at C(7), C(6), C(2), or more than one of these positions. Because exchange at C(2) potentially occurs at more than one pathway step (the initial condensation, A-ring closure, or O-bridge formation), that possibility is not addressed here. But the possibility of exchange at C(7) or C(6) is addressed in my study.

In this chapter I describe studies in which I applied to *N. uncinatum* cultures putative loline-biosynthesis intermediates that were positionally labeled with deuterium (D), in order to test various hypotheses for the biosynthesis of the heterocyclic ring structure that forms the core of loline alkaloids. The putative intermediates predicted from the various hypotheses outlined above were synthesized in deuterated forms by researchers in the laboratory of R. B. Grossman, and applied to the cultures at the onset of loline alkaloid production. The alkaloids were then analyzed by GC-MS to identify

instances of significant enrichment of the products with deuterium. These tests, as well as application of [U-D] proline and GC-MS analysis of the loline alkaloid product, helped identify the order, and eliminate some possible mechanisms, for bond formation in the pathway.

MATERIALS AND METHODS

Deuterated compounds

The following deuterated compounds (Figure 3.7) were synthesized by R. B. Grossman and coworkers (University of Kentucky), and provided for this study: N-(3-amino-3-carboxy-1,1-dideuteropropyl)proline (**2a**), N-(3-amino-3-carboxy-1,1-dideuteropropyl)-4,4-dideuteroproline (**2b**), cis-3-deutero-3-hydroxyproline (**cis-3a**), trans-3-deutero-3-hydroxyproline (**trans-3a**), N-(3-amino-3-carboxy-1,1-dideuteropropyl)-3-hydroxyproline (**4a**), exo-3,3-dideutero-1-aminopyrrolizidine (**5a**), exo-6,6-dideutero-1-aminopyrrolizidine (**5b**) (Faulkner et al., 2006). In addition 2,3,3,4,4,5,5-deuteroproline (**6a**) was purchased from Cambridge Isotope Laboratories (Andover, MA) and D_6[DMSO] was purchased from Sigma-Aldrich Corp. (St. Louis, MO).

Fungal culture conditions and application of labeled compounds

Neotyphodium uncinatum CBS 102646 was isolated from plant 167 from our greenhouse using the method described in Chapter 2. The fungus was maintained on potato dextrose agar (PDA) plates at 21 °C and subcultured as necessary. Minimal

medium (MM) for loline alkaloid production (Blankenship et al., 2001) contained urea (15 mM) and sucrose (20 mM) as the carbon and nitrogen sources, respectively. A fungal colony of *N. uncinatum* was scraped from PDA plates using a sterile blade and homogenized with an Omni homogenizer model 17105 (Omni International Marietta, GA, USA) in minimal medium. The homogenate was used to inoculate 28 mL of MM with 1 mL of fungal suspension, in 100 x 25 mm polystyrene Petri plates. The cultures were shaken at 21 - 22 °C and shaking at 100 rpm on a rotary shaker. Sample of culture filtrate were then monitored for loline alkaloid accumulation, and were given the labeled compounds 6 to 8 days after inoculation, when loline production was first detected by GCMS. The stock solutions of deuterated compounds were diluted with water to form a 1-mL feeding solution that was added to the cultures, to give a final volume of 30 mL the desired concentration. Experiments included replicates with each deuterated compound, and three replicates of cultures without deuterated compounds.

Loline alkaloid extraction

After 20 days of growth in minimal medium the cultures were extracted for lolines as follows. The cultures were lyophilized and the dry residue was basified by adding 1 M sodium hydroxide to pH 12. Chloroform (5 mL) was mixed with basified solution and allowed to sit for 30 min. The chloroform was removed from the aqueous phase and the aqueous phase was extracted again with 5 mL of chloroform for 30 min. The chloroform extracts were then combined and concentrated by putting them on ice and blowing with a stream of air, or were stored at -20 °C for later GCMS analysis.

GCMS analysis of lolines

GCMS analysis employed a Hewlett-Packard (Avondale, PA) G1800 A GCD or a Varian Inc. (Palo Alto, CA) CP-3800 gas chromatogram interfaced to a Varian Saturn 2200 mass spectrometer. The Hewlett-Packard GCMS was equipped with a 30 m Restek (Bellefonte, PA) column with 5 Sil MS, 0.25 mm i.d., 0.25 µm thickness and a quadrupole mass analyzer with data collected using GCD ChemStation G1074A Version A.00.00. The Varian GCMS was equipped with a 30 m DB-5MS column (J & W Scientific, Rancho Cordova, CA) with 0.25 mm i.d., 0.25 µm film thickness and an ion trap mass analyzer with data collected using Varian SaturnView Version 5.52. The Hewlett-Packard GCMS was run with the conditions described by Blankenship (2001). The Varian GCMS contained helium as the carrier gas at a flow rate of 1 mL/min. The Varian GCMS used a splitless 1 µL injection with run parameters as follows: injection temperature of 250 °C, initial column temperature of 75 °C ramping to 225 °C at 8 °C/min, burn parameters of 300 °C for 2 min between samples. Results were analyzed for the enrichment of the NFL ions of 82 and 154 m/z. The enrichment of NFL was calculated using the percent enrichment formula as previously described (Blankenship et al., 2005). Statistical analysis was conducted on the percent enrichment used Statistical Program for Social Sciences (SPSS Inc., Chicago, IL) and Statistical Analysis System (SAS Institute Inc., Cary, NC) software. Means were compared using ANOVA with Tukey's post-hoc test for pairwise comparisons.

In this chapter the GCMS analysis of NFL was performed on a Hewlett-Packard (HP) and a Varian GCMS. The two instruments utilized different types of mass analyzers. The HP contained a quadrupole mass analyzer whereas the Varian contained

an ion-trap mass analyzer. The two machines both detected the same loline alkaloids from cultures (NML, NANL, NFL, and NAL). The differences in the machines caused slight differences in the spectrum obtained for NFL (Figure 3.9). In the Varian GCMS the peak for 153 m/z was greater than the 154 m/z peak, whereas the HP GCMS 154 m/z peak was higher than the 153 m/z peak. Therefore, the 153 m/z peak was used to determine enrichment in the Varian GCMS samples, whereas the 154 m/z peak was used to determine enrichment in the HP GCMS samples. Another difference between results from the GCMS instruments occurred at the molecular ion of 183 m/z peak. The HP always indicated a 183 m/z peak having 1 % or less of the total abundance of ions, whereas the 183 m/z peak from the Varian was 10 % of the total abundance of ions or higher depending on the amount of NFL in a sample. Figure 3.10 shows a typical GCMS trace of NFL from the HP GCMS and the Varian GCMS for comparison.

RESULTS

Feeding Results

Application of three of the deuterated compounds to *N. uncinatum* resulted enrichment of the isotopic label in NFL. NFL from cultures fed deuterated compounds was compared to controls without any compounds given to cultures and controls with [D_6]DMSO supplemented to cultures. The NFL extracted from cultures fed with compounds **2a**, **2b**, **5a**, **5b**, and **6a** all showed significant enrichment, compared to controls, of the peaks expected from incorporation of the deuterium atoms (Table 3.1). The enrichment showed that each of these compounds contributed to deuterated NFL.

The feedings indicate that two compounds (**2** and **5**) are likely intermediates in the loline alkaloid biosynthesis pathway.

To test the possibility that condensation of proline and homoserine could form **2** as a step in the pathway, a dideuterated and tetradeuterated form of **2** were synthesized and fed to cultures of *N. uncinatum* (Figure 3.4). The cultures fed **2** had decreased growth when compared to unfed controls, suggesting **2** may be toxic. Cultures fed 4 mM **2** had little new growth and contained no detectable lolines. Cultures fed 1 mM and 2 mM **2a** had a 2.05 % and 1.87 % enrichment, respectively, of the 154 + 2 amu peak, which was significantly different compared to controls ($p < 0.05$). Feeding 2 mM of **2b** to cultures also resulted in a significantly different 1.25 % enrichment in the 154 + 2 amu peak of NFL (Table 3.1). Cultures fed 1 mM of **2b** showed 1.06 % enrichment in the 154 + 2 amu peak of NFL, which was not significantly different from controls. Applications of 1 mM and 2 mM **2b** resulted in 0.95 % (1mM) and 2.72 % (2mM) enrichment in the 154 + 4 amu peak of NFL, which was significantly different from the controls because no 154 + 4 amu peak was detected from the controls. The results of applying dideuterated and tetradeuterated **2** to *N. uncinatum* would suggest that **2** is the first pathway-specific intermediate of loline alkaloid biosynthesis.

The ether bridge of lolines is unusual for a biological molecule. Experiments were performed to determine the when the oxygen atom for the ether bridge is introduced into the pathway. In order to test the possibility that hydroxylated proline could introduce oxygen into the loline alkaloid biosynthetic pathway, **cis-3a** and **trans-3a** were fed to *N. uncinatum* cultures. The results of applying 2 mM and 4 mM **cis-3a** and **trans-3a** were not significantly different from nonfed controls when comparing the 154 + 1

amu peak of NFL. The results indicate that a hydroxylated proline is not involved in loline alkaloid biosynthesis, suggesting that oxygen is not introduced into the pathway until after production of **2**.

It is possible that the hydroxylation of **2** after formation to yield **4** could incorporate an oxygen atom that would. To test this possibility, **4a** was synthesized and fed to *N. uncinatum*. Cultures fed **4a** had no significant enrichment of the 154 + 2 amu peak of NFL. This argues against the hydroxylation of **2** as an intermediate step in loline alkaloid biosynthesis.

The pyrrolizidine, *exo*-1-aminopyrrolizidine (**5**), was synthesized to provide a test for the formation of the pyrrolizidine ring prior to oxygen atom incorporation. The results of the **2a**, **2b**, and **4a** feedings suggest that the pyrrolizidine ring structure may be formed prior to ether bridge formation. This hypothesis was tested by feeding a deuterated *exo*-1-aminopyrrolizidine (**5a** and **5b**) to cultures. Three feedings of **5a** or **5b** were conducted and in all three experiments, results were significant ($p < 0.05$) when compared to unfed *N. uncinatum* and *N. uncinatum* fed [D_6]DMSO as controls. Application of 4 mM **5a** resulted in a 4.48 % enrichment of the 154 + 2 amu peak of NFL, whereas application of 2 mM **5a** resulted in 1.76 % enrichment of the 154 + 2 amu peak of NFL. The enrichment of NFL suggested that the pyrrolizidine **5** is an intermediate, and that the oxygen bridge is formed following pyrrolizidine ring formation.

The proline ring contributes the carbons and nitrogen for the B ring (Blankenship et al., 2005). In order to determine if there is any exchange of hydrogen on proline ring carbons during loline alkaloid biosynthesis, **6a** was fed to *N. uncinatum* cultures, and

NFL was extracted and analyzed by GCMS. In the GC chromatogram a shoulder on the peak corresponding to NFL was determined by MS to be deuterated NFL. The mass spectrum showed that the + 6 amu NFL was the main component of this shoulder (Figure 3.9). The + 6 amu enriched NFL exhibited a faster retention time and was largely separated from the non-enriched NFL on the GC column. This occurs because the deuterium to carbon bond is shorter than the hydrogen to carbon bond, which creates a smaller molecule that moves through the column faster. The feeding of 4 mM **6a** resulted in a 5.82 % enrichment of the 153 + 6 amu peak of NFL and a 4.94 % enrichment of the 153 + 5 amu peak of NFL. The 153 m/z peak was used to calculate enrichment because the Varian GCMS was used to analyze the NFL mass spectrum. The results indicate that six deuterium atoms from the heptadeuterated proline were retained in the labeled NFL. The loss of one deuterium atom was expected in the formation of the ether bridge.

DISCUSSION

Previous work has indicated that the carbons and nitrogen atoms of loline alkaloids are derived from homoserine and proline (Figure 3.2; (Blankenship et al., 2005)). Based on the known amino acid precursors as well as *LOL* gene homologies, possible intermediates in the loline alkaloid biosynthesis pathway were postulated. Several of these were synthesized by R.B. Grossman and coworkers (University of Kentucky), or purchased, in deuterated forms and applied to *N. uncinatum* cultures to test for incorporation of the deuterium atoms into NFL. The results indicate that **2** and **5** are likely intermediates of loline alkaloid biosynthesis. The oxygen bridge formation was

also investigated and the data suggests that the pyrrolizidine ring system is completed before ether bridge formation. Based on these findings I have refined the proposed biosynthetic pathway for loline alkaloids (Figure 3.10).

The *LOL* gene cluster contains a gene, *lolC*, whose predicted product has sequence similarity to *O*-acetylhomoserine (thiol) lyase and cystathionine-γ-synthase (Spiering et al., 2005). These enzymes are involved in γ-substitution reactions. It has been previously shown that γ,γ-deuterated homoserine retained both deuteriums in NFL when fed to *N. uncinatum* cultures (Blankenship et al., 2005). LolC and CysD are both gamma type PLP enzymes and may be involved in similar reactions (Figure 3.3). I observed that all deuterium atoms from **2a** and **2b** are incorporated into NFL, indicating that **2** is involved in loline alkaloid biosynthesis. I speculate that LolC catalyzes the formation of **2** via condensation of proline with a form of homoserine (likely *O*-acetylhomoserine) linking the γ-carbon of homoserine with the secondary amine of proline. This reaction would be the first committed step in the biosynthesis of loline alkaloids.

The second step in loline alkaloid biosynthesis is likely to be the decarboxylation of the proline-derived ring of **2** to form the imminium ion **8**. It is unlikely that **2** is hydroxylated to form **4** because feeding results indicated that **4** did not incorporate into NFL and the incorporation of **5** corroborates the results of feeding **4**. Alternatively the next step might be removal of the carboxyl group originating from homoserine to form **7,** but this is also unlikely because application of deuterated **7** did not result in enrichment of deuterated loline alkaloid (Blankenship, 2004; Faulkner et al., 2006). However, it is possible that **4** and **7** did not incorporate into NFL because the compounds could not

enter fungal cells. Additionally, the similarities in structure between **2**, **4**, and **7**, and the fact that **2a** and **2b** were taken up and incorporated into NFL, suggest that **4**, and **7** would also be taken up by fungal cells.

After formation of imminium ion **8**, there are two alternative scenarios that appear reasonable. The A ring could be formed to generate **9**, or decarboxylation could generate **10**. There are currently no data that can support one of these alternatives over the other. However, additional evidence for the decarboxylation to form **9** comes from sequence similarity of LolD. LolD contains a pyridoxal phosphate (PLP) site and is similar to the PLP-containing enzyme ornithine decarboxylase (Spiering et al., 2005). The structural similarity between ornithine and **2** suggests that LolD could act on **2** in a manner similarly to ornithine decarboxylase (Figure 3.11). Therefore the sequence would suggest LolD could be involved in decarboxylation of imminium ion **8** which would then lead to the imminium ion **10**. These alternatives were not tested in this study.

The possibility was considered that proline is hydroxylated prior to being used in loline alkaloid biosynthesis. The hydroxyl group from hydroxylated proline would then be carried through loline alkaloid biosynthesis and eventually become incorporated in the oxygen bridge or act as a leaving group during oxygen bridge formation (Figure 3.4). The *cis* and *trans* isomers of 3-hydroxyproline (**3**) are components of telomycin, a cyclopeptide antibiotic (Mauger, 1996), lending credence to this possibility. However, the results of feeding *cis* and *trans* **3** in my study suggest that a hydroxylated proline is not involved in loline alkaloid biosynthesis. Furthermore, my finding that *exo*-1-aminopyrrolizidine is an intermediate strongly argues against involvement of hydroxyproline.

Following the formation of **10**, the pyrrolizidine ring is hypothesized to be formed prior to introduction of the oxygen bridge (Figure3.5). The pyrrolizidine *exo*-1-aminopyrrolizidine (**5**) was synthesized to provide a test for this hypothesis. The evidence of incorporation of **5** into NFL supported this hypothesis. This along with the apparent inability of **3a** to incorporate would indicate that the ether bridge is formed after the pyrrolizidine ring. . It is possible that the B ring closure to form **5** is catalyzed by LolT, which is predicted to be a PLP enzyme (Spiering et al., 2005).

The results in this chapter indicate that *exo*-1-aminopyrrolizidine **5** is an intermediate in the pathway. The application of deuterated compounds **5a** and **5b** significantly enriched deuterated NFL. This indicates that the pyrrolizidine ring is formed before the oxygen atom is introduced. This corroborates results of application of hydroxylated compounds **cis-3a**, **trans-3a** and **4a**, which failed to give significant enrichment of deuterated NFL. In addition the results from applying **6a** to cultures indicate that the hydrogen atoms in the proline ring are not exchanged, since feeding [D_7]proline resulted in enrichment of the M + 6 peaks detected by MS. This was in keeping with the expected substitution of a single D atom with the O-link to C(7), without any other loses of D atoms by exchange. The aforementioned results provide evidence which taken together substantiates the claim that **5** is an intermediate of the loline alkaloid pathway.

Following formation of **5** the next steps in loline alkaloid biosynthesis are hypothesized to be addition of the oxygen atom to form the ether bridge. The mechanism of oxygen bridge formation has not been addressed by my experiments. In Chapter 4, I will present evidence that *N*-acetyl-1-aminopyrrolizidine (NAAP) is acted on by LolO

and LolE to form *N*-acetylnorloline (NANL). It is possible that NAAP could be hydroxylated at C(2) and C(7) and then cyclized with one of the hydroxyls serving as the leaving group, to give NANL. Alternatively, NAAP could be converted directly to NANL by a single enzymatic process perhaps with a LolO-LolE complex. The steps following **5** at this point cannot be determined based on the sequence similarity of the remaining *LOL* cluster genes. After accounting for the PLP-catalyzed steps involving LolC, LolD and LolT, the remaining enzymes encoded by the *LOL* cluster genes include four oxidation or oxygenation enzymes. Of these, LolP is a cytochrome P450 monooxygenase that has been shown to be involved in methyl to formyl conversion to make NFL, and is unnecessary for ether bridge formation (Spiering et al., 2008). Of the remaining oxidation and oxygenation enzymes encoded by *LOL* genes, one of these, LolF, is likely involved in oxidative decarboxylation of **2**, and the other two, LolE and LolO, are likely to be involved in formation of the oxygen bridge. The use of molecular techniques could provide some useful insights into the function of the *lolE, lolO* and *lolF*, as described in Chapter 4.

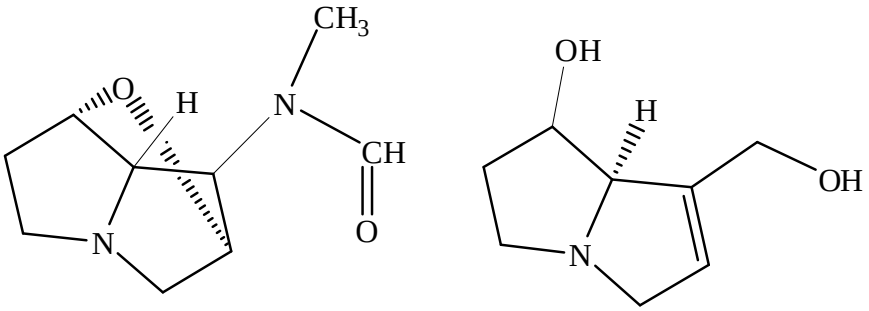
The oxygen bridge in loline alkaloid is in a unique position for a biological molecule because it is attached to unactivated two bridgehead carbons. The mechanism for formation of the ether bridge would provide insights in how such a bond could be formed in nature. It is likely that lolE or lolO are involved in forming the oxygen bridge. Based on sequence signatures (Spiering et al., 2005), these two enzymes are non-heme iron oxygenases and as such, are prime candidates for this process. LolE has sequence similarity to *Penicillium decumbens* epoxidase which is a member of the FeII/2-oxoglutarate-dependent hydroxylases (Watanabe et al., 1999). LolO has sequence

similarity to *Emericella nidulans* isopenicillin N synthetase which is also a member of the FeII/2-oxoglutarate-dependent superfamily of proteins (Spiering et al., 2005). The FeII/2-oxoglutarate-dependent enzymes can catalyze multiple steps in pathways and are involved in reactions such as ring closures, desaturation, and ring expansion (Hausinger, 2004). The gene similarities between enzymes related to LolE and LolO would indicate they could act individually or together in the oxygen bridge. Further study should be conducted to determine if 1-aminopyrrolizidine **5** is a substrate for LolE or LolO.

In summary, this chapter provides evidence that supports the proposed hypothesis for loline alkaloid biosynthesis outlined in Figure 3.10. Applying to cultures deuterated compounds representing possible intermediates of the loline alkaloid biosynthesis pathway has provided evidence that the first committed step is the condensation of proline and homoserine or (more likely) *O*-acetylhomoserine in a γ–substitution reaction to produce **2**. Further steps supported by results of these experiments involve decarboxylation of **2** and cycliziation to form 1-aminopyrrolizidine **5**. The incorporation of **5** into NFL indicates that the oxygen bridge is formed after the pyrrolizidine structure of loline alkaloid. Then, **5** is further acted on by oxidation or oxygenation enzymes via one or more reactions to form loline alkaloids.

Table 3.1 Incorporation of deuterium atoms into N-formylloline produced by *N. uncinatum* fed labeled compounds, as measured by GCMS. Shown are means of three replicates of experiments in which the deuterated compounds indicated were applied to the cultures. The enrichment was calculated using the MS scan from the top of the NFL chromatogram and the fourth MS scan from the beginning of NFL chromatogram for comparison. The fourth MS scan column shows increased enrichment because deuterated NFL elutes before non-deuterated NFL. * indicates significantly different from controls [a] indicates enrichment measured using 153 m/z peak

			Unlabeled NFL ions			
			Top of chromatogram		Fourth MS scan	
			82 m/z	154 m/z	82 m/z	154 m/z
Compound	Conc (mM)	amu shift	% increase	% increase	% increase	% increase
2a	1	2	1.63	2.05*	1.91	2.06*
2a	2	2	1.18	1.87*	1.73	1.92*
2b	1	4	0.04	0.04*	1.05	0.95*
2b	2	4	0.02	0.04*	2.17	2.72*
2b	1	2	0.73	1.06	1.05	0.95
2b	2	2	0.58	1.25*	2.17	2.72*
cis-3a	2	1	0.08	0.41	0.20	0.61
cis-3a	4	1	0.04	0.07	0.22	0.45
trans-3a	2	1	0.01	0.24	0.31	0.27
trans-3a	4	1	0.05	0.44	0.74	0.25
4a	1	2	0.19	0.12	0.29	0.00
4a	2	2	0.15	0.26	0.98	0.00
5a	2	2	0.93	1.76*	9.32	4.86*
5a	4	2	3.41	4.48*	10.55	8.28*
5b	1	2	1.31	1.69*	1.47	3.29*
6a[a]	4	6	27.25	28.73*	n/a	n/a
6a[a]	4	5	11.78	15.22*	n/a	n/a
D_6[DMSO]	2	1	0.08	0.08	0.07	0.00
D_6[DMSO]	2	2	0.06	0.11	0.19	0.00
D_6[DMSO]	2	4	0.03	0.05	0.10	0.00



N-formylloline (**1**) Retronecine

Figure 3.1 Comparison of the loline alkaloid and necine alkaloid structures. Shown are the loline alkaloid *N*-formylloline (NFL) and the senecio alkaloid retronecine.

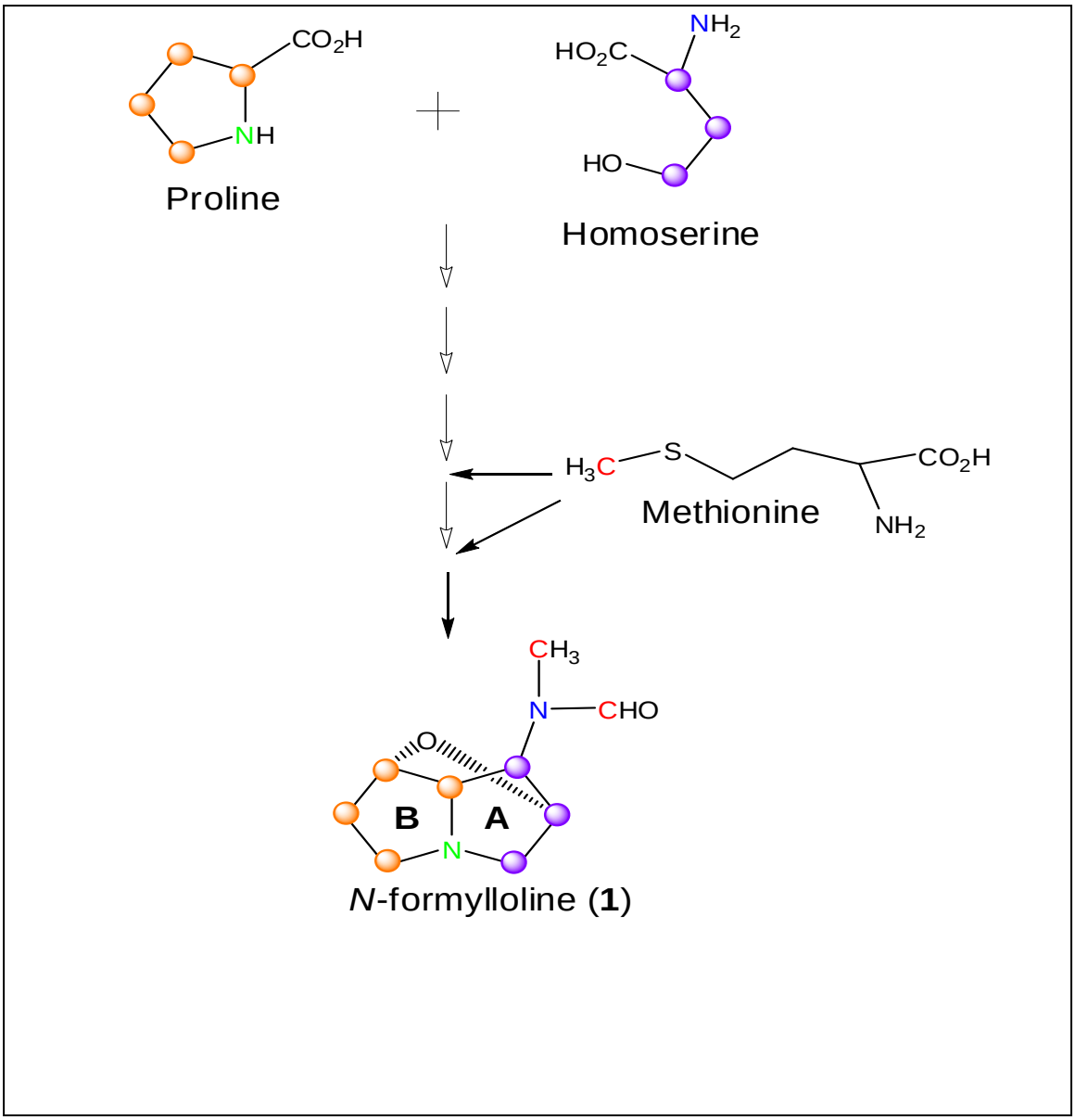


Figure 3.2 Amino acid precursors of loline alkaloids. Colored balls indicate atoms derived from proline and homoserine, colored atoms indicate carbons and nitrogen derived from the amino acid precursors. The A and B ring of loline alkaloids are labeled in N-formylloline.

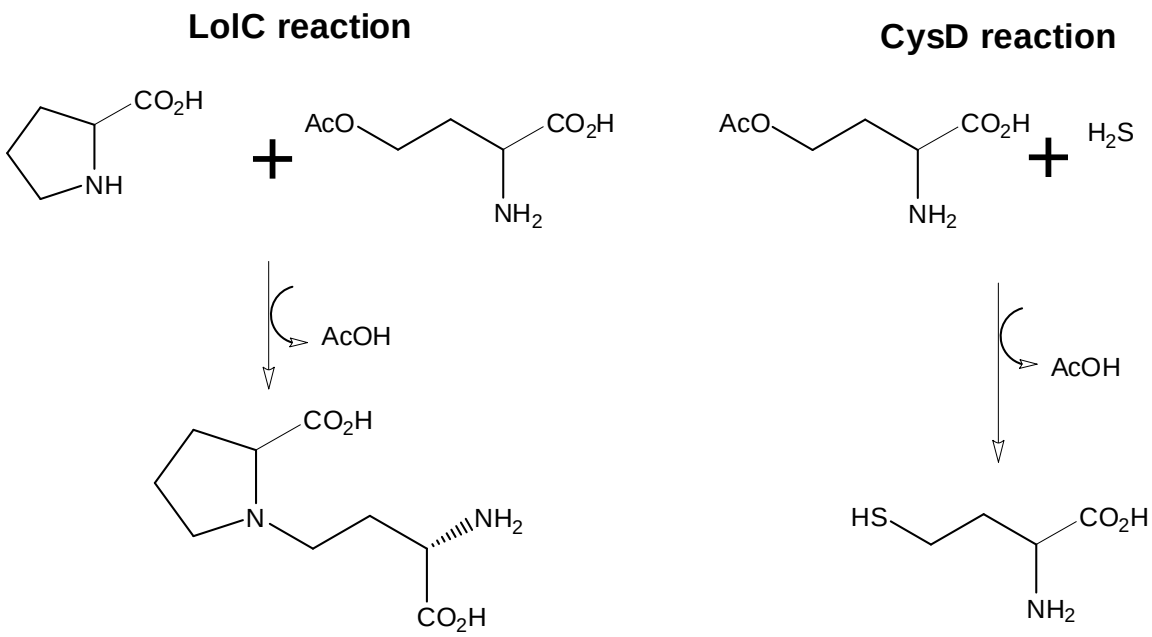


Figure 3.3 Proposed function of LolC and function of the *Emericella* (*Aspergillus*) *nidulans cysD* gene product. *cysD* encodes *O*-acetylhomoserine (thiol)-lyase which is a gamma-type pyridoxal phosphate (PLP) enzyme. LolC has sequence similarity to CysD and is a predicted gamma-type PLP enzyme

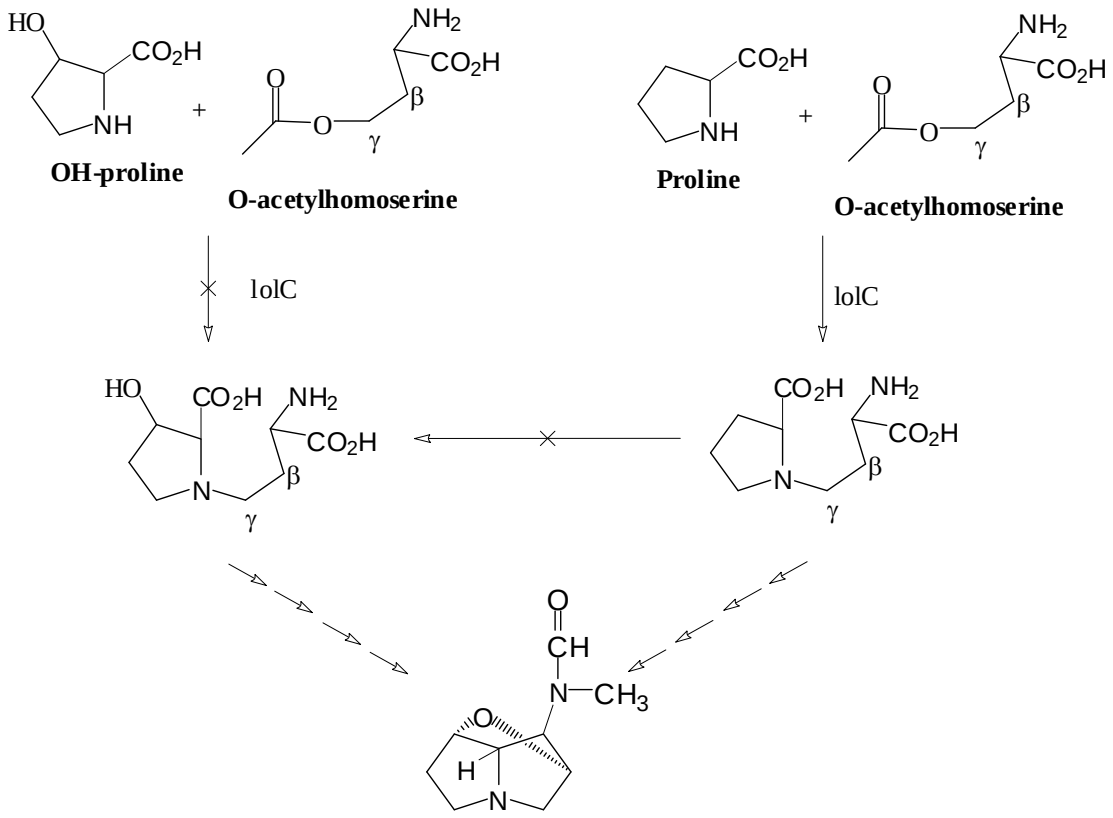


Figure 3.4 Proposed initial steps in loline biosynthesis catalyzed by LolC. LolC likely catalyzes a reaction involving *O*-acetylhomoserine. Possibilities that were examined include a hydroxyproline precursor acted on by LolC (left), or hydroxylation of the LolC product (arrow left to right), or later addition of the bridge O atom. The possibilities not supported are crossed out.

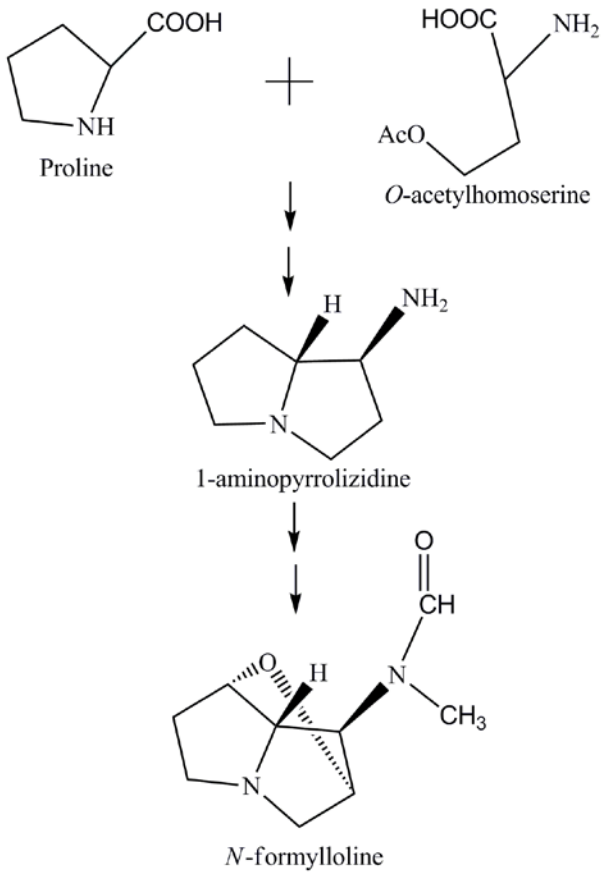


Figure 3.5 Proposed involvement of *exo*-1-aminopyrrolizidine in loline alkaloid biosynthesis. The carbon and nitrogen of proline and O-acetylhomoserine would be converted in several steps to *exo*-1-aminopyrrolizidine. The oxygen bridge would then be formed on the pyrrolizidine structure to give lolines.

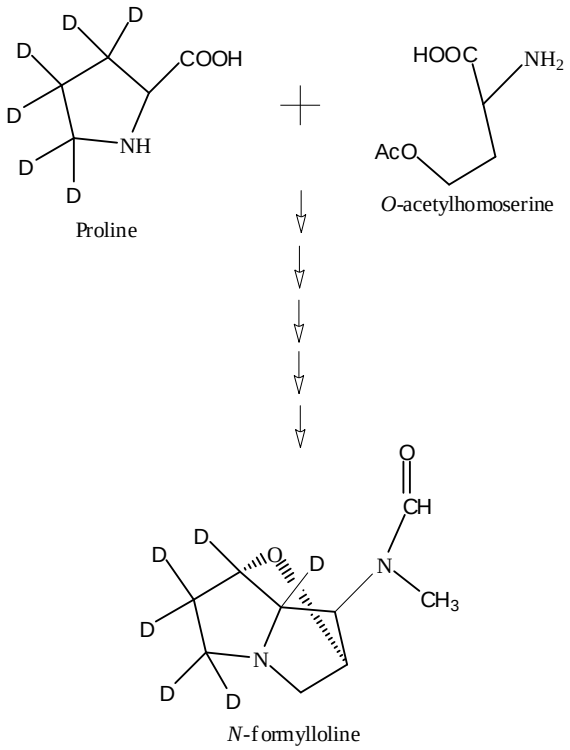


Figure 3.6 Proposed product of feeding heptadeuterated proline to *Neotyphodium uncinatum* loline producing cultures. Results indicated that the proline ring only loses a single C(7) deuterium atom as expected due to the oxygen bridge formation.

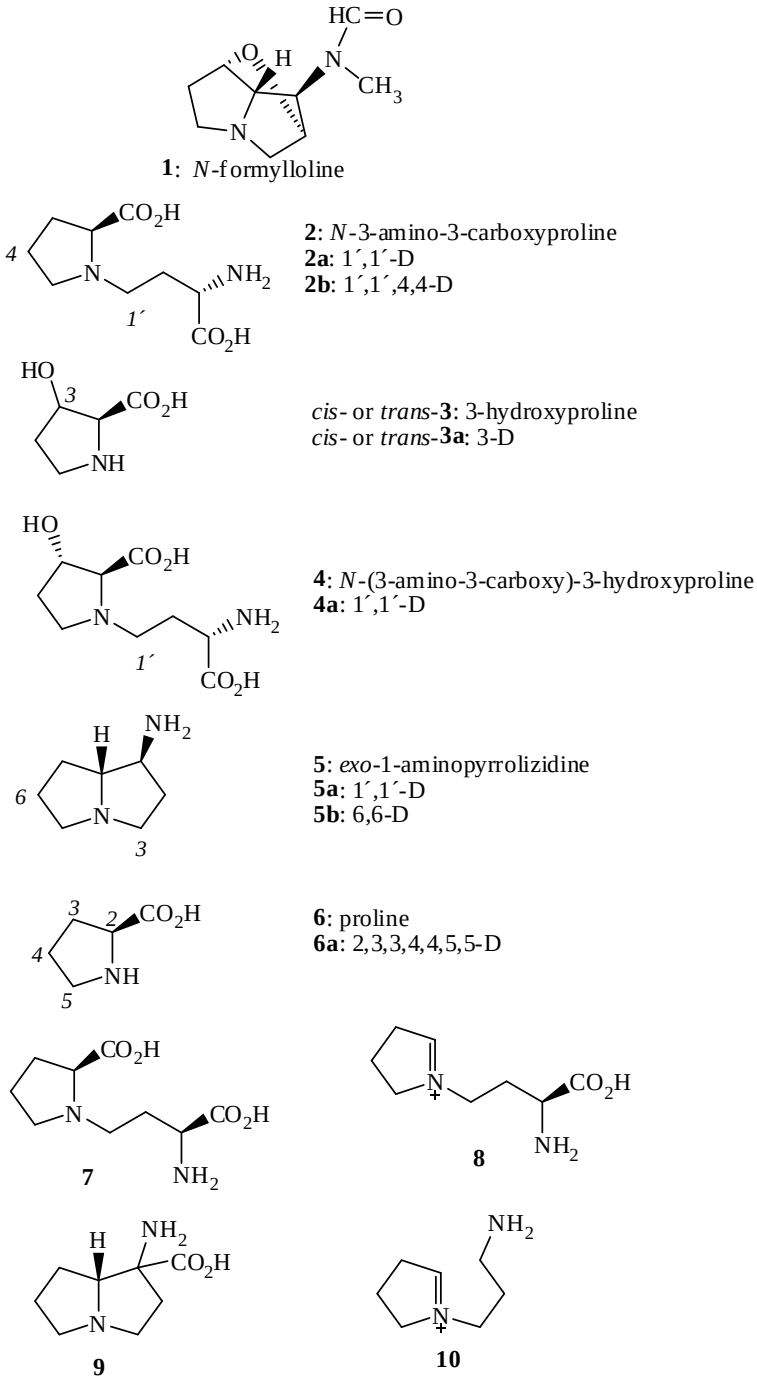


Figure 3.7 Assigned compound numbers for this chapter. Compounds 2 thru 6 were synthesized and fed to *N. uncinatum* loline producing cultures. Deuterium atoms are indicated with D. Compounds 7 thru 10 are intermediates steps in proposed biosynthesis pathways

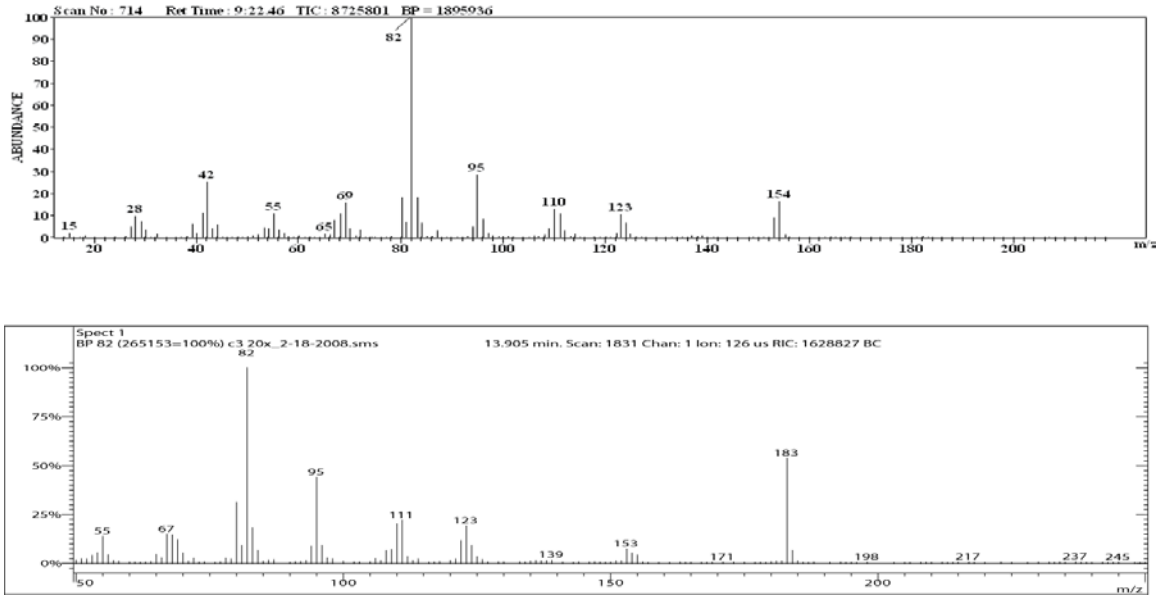


Figure 3.8 GCMS of NFL obtained using different GCMS machines. The top panel is a spectrum obtained from the Hewlett-Packard GCMS equipped with a quadrupole mass analyzer, whereas the bottom panel is the output from a Varian GCMS with an ion-trap mass analyzer.

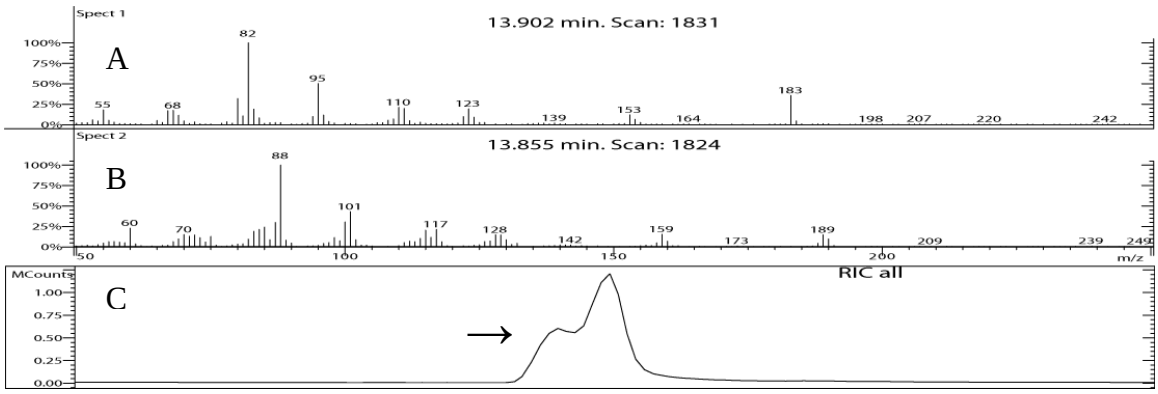


Figure 3.9 Mass spectrum and chromatogram of NFL extracted from heptadeuterated proline application to *N. uncinatum* culture. The enrichment in deuterated NFL is observed in the mass spectrum and the chromatogram by the separation of the deuterated NFL (+6 amu) from the non-deuterated NFL. A. Mass spectrum of NFL B. Mass spectrum of NFL enriched with deuterium from heptadeuterated proline and showing +6 amu shift in spectrum C. Chromatogram of NFL extracted from heptadeuterated proline with arrow showing shoulder in peak from separation of deuterated (+6 amu shift) and non-deuterated NFL.

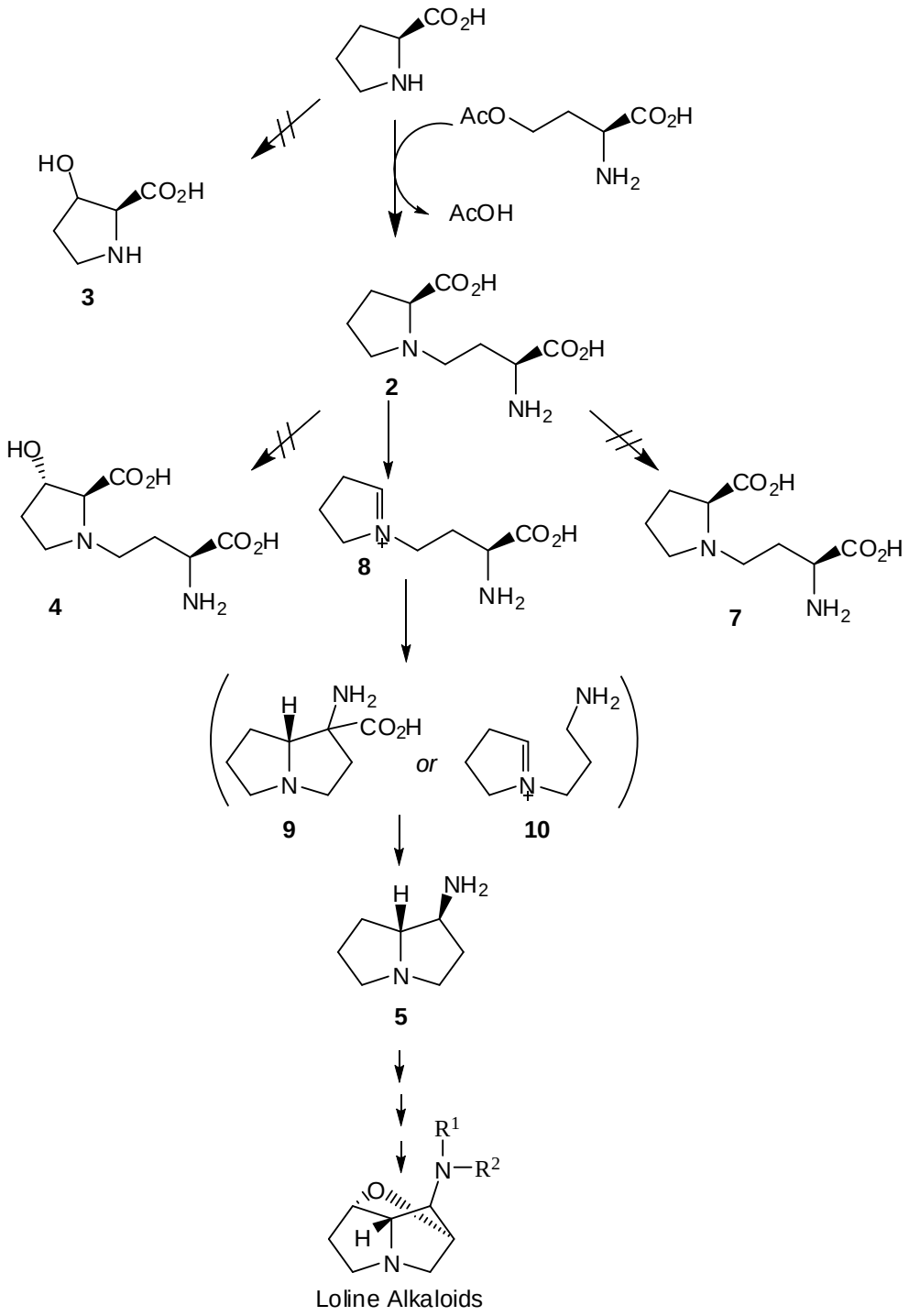


Figure 3.10 Proposed scheme for biosynthesis of loline alkaloids. Black arrows indicate proposed steps in pathway, hashed arrows indicate steps not supported by results of experiments in this chapter.

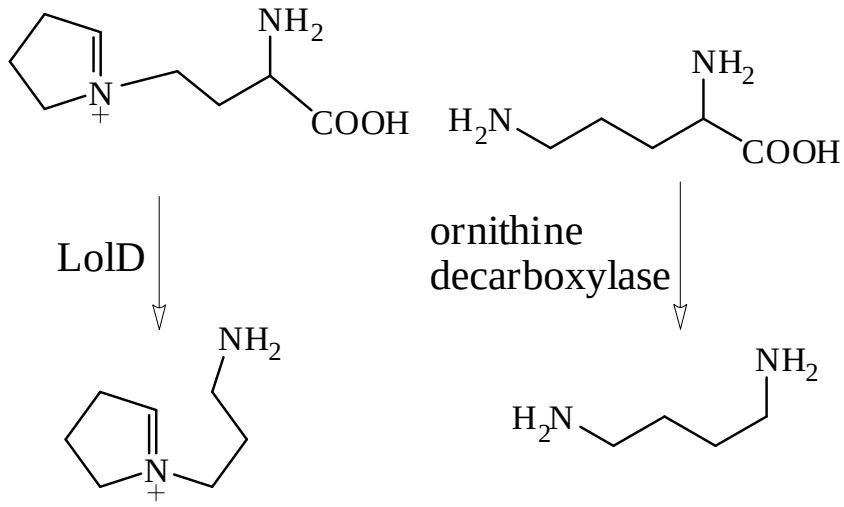


Figure 3.11 Proposed function of LolD and function of ornithine decarboxylase. Ornithine decarboxylase is a PLP enzyme involved in decarboxylation of ornithine to form putrescine. LolD contains a PLP-binding site and has sequence similarity to ornithine decarboxylase.

CHAPTER 4

Investigations into Loline Oxygen Bridge Formation

INTRODUCTION

Loline alkaloids are fungal secondary metabolites that have insecticidal properties (Schardl et al., 2007). Mendelian genetic analysis using parent *Epichloë festucae* strains differing in loline alkaloid production led to identification of the genetic locus (*LOL*) linked to the loline alkaloid production phenotype (Wilkinson et al., 2000). The use of a culture system for loline alkaloid production by *Neotyphodium uncinatum* (Blankenship et al., 2001) led to further characterization of genes at the *LOL* locus. Two genes (*lolC* and *lolA*) were shown to be upregulated during loline alkaloid production and were present in other loline-producing endophytes but not in nonproducers (Spiering et al., 2002). Following the identification of *lolC* and *lolA*, Spiering et al (2005) were able to find seven other genes linked to loline alkaloid production in *N. uncinatum*. The 9-gene *LOL* gene cluster was shown to be present in two copies in *N. uncinatum*, with the same gene order in both copies (Figure 4.1) (Spiering et al., 2005). The same conserved gene order has also been characterized in *Epichloë festucae*, *N. coenophialum*, and *Neotyphodium* sp. PauTG-1 for their respective *LOL* gene clusters (Kutil et al., 2007). Recent genome sequencing and functional assays have indicated two additional genes at one end of the cluster in a loline alkaloid-producing *E. festucae* strain (C.L. Schardl, pers. com.). Thus, the *LOL* gene cluster consists of 11 genes associated with loline production, which are, in order of the cluster, *lolF*, *lolC*, *lolD*, *lolO*, *lolA*, *lolU*, *lolP*, *lolT*, *lolE*, *lolN* and *lolM*.

The sequences for the *LOL* genes have been analyzed and their protein product amino acid sequences have been predicted. Sequence analysis and results of protein motif searches indicate that predicted LolF, LolO, LolP and LolE amino acid sequences have signatures for redox reaction enzymes (Spiering et al., 2005). Predicted amino acid sequences of LolC, LolD, and LolT indicate that each has a binding site for the pyridoxal-5´-phosphate (PLP) cofactor, implying that these enzymes likely act on substrates with primary amines (or, alternatively, with aldehydes or ketones). LolA is predicted to bind amino acids and LolU is a possible transcription factor (Spiering et al., 2005) and shows sequence similarity at low significance to *N*-acyl transferases (M. J. Spiering, pers. com.).

In the previous chapter, probable intermediates of the loline alkaloid pathway were tested by experiments in which deuterated compounds were provided to cultures of *N. uncinatum*. The results implicated proline and homoserine as precursors of loline alkaloids. The *LOL* gene homologies and the two intermediates allow for a working hypothesis of the loline alkaloid biosynthetic pathway, where each *LOL* gene may be involved in the biosynthesis or a regulatory process. The role of *lolP* has been shown to be involved in the conversion of NML to NFL in the later steps of loline alkaloid biosynthesis (Spiering et al., 2008). This provides three possible roles that may be hypothesized for the remaining oxidation enzymes encoded in the *LOL* cluster.

LolF has a signature sequence for FAD binding and significant similarity to cyclopentanone monooxygenase, a FAD-containing oxidation enzyme (Spiering et al., 2005). Cyclopentanone monooxygenase is an oxidoreductase that uses NADPH and cyclopentanone to form 5-valerolactone (Griffin and Trudgill, 1976). LolE has predicted

domain similar to that of phytanoyl-CoA dioxygenase, which is a 2-oxoglutarate Fe^{II} (alpha-ketoglutarate Fe^{II}) dependent enzyme (Spiering et al., 2005). Phytanoyl-CoA dioxygenase converts phytanoyl-CoA into 2-hydroxyphytanoyl-CoA while oxidizing the 2-oxoglutarate cosubstrate to carbon dioxide and succinate (Hausinger, 2004). The conserved domain for LolO is pfam03171 which is the 2-oxoglutarate Fe^{II} dependent superfamily, and LolO is also similar to isopenicillin N synthase, which catalyzes the formation of isopenicillin N from *N*-[(5*S*)-5-amino-5-carboxypentanoyl]-L-cysteinyl-D-valine in penicillin and cephalosporin biosynthesis (Cohen et al., 1990). The 2-oxoglutarate Fe^{II} dependent enzymes have been shown to work in numerous ring closures and secondary metabolism products of plants and animals (Hausinger, 2004).

Prior to my study there was little evidence of the manner in which the oxygen bridge is formed in loline alkaloid biosynthesis. I propose that the oxygen bridge is formed by the action of two oxygenation enzymes, lolE and lolO. In this chapter, I describe my efforts to use a yeast expression system and RNA-interference (RNAi) of *N. uncinatum* gene expression to test the roles of the oxygenation enzymes, and provide evidence that LolO and LolE act on a derivative of *exo*-1-aminopyrrolizidine, namely *N*-acetyl-1-aminopyrrolizidine (NAAP), to catalyze formation of the oxygen bridge characteristic of the loline alkaloids.

MATERIALS AND METHODS

Biological materials

Neotyphodium uncinatum e167 (=CBS102646) used for transformations was isolated from a *Lolium pratense* plant as previously described (Blankenship et al., 2001). Cultures were maintained on PDA plates at 21 °C. Yeast strain YPH499 was used in all yeast-vector cloning experiments. YPH499 is mating type a strain of genotype *ura3-52 lys2-801*^{amber} *ade2-101*^{ochre} *trp1-Δ63 his3-Δ200 leu2-Δ1,* and was supplied by Stratagene with the pESC-LEU vector. DNA cloning used *Escherichia coli* strains XL-1 Blue electrocompetent cells (Bullock et al., 1987) or TOP10 electrocompetent cells (Invitrogen).

Bacterial electroporation and DNA isolation

Bacteria were transformed by electroporation using 1 µL of plasmid or ligation solution along with 40 µL of competent cells. Cells were electroporated at 1.7 kV/cm, 25 µF, and 200 Ω. Immediately following electroporation, 960 µL of SOC medium was added to the cuvette. The cells and SOC mixture were then transferred to a 15 mL tube and incubated at 37 °C, with shaking at 225 RPM for 1 hour. Bacterial cells were spread on LB agar plates with 100 µg/mL of ampicillin and incubated 12-16 hrs at 37 °C. Single colonies were picked and grown overnight in LB agar with 50 µg/mL of ampicillin at 37 °C with shaking at 200 RPM. Plasmid DNA was isolated from the bacterial cells by using the Qiaprep Spin Miniprep kit (Qiagen) according to manufacture's protocol, or plasmid DNA was isolated by the method of Ahn et al (2000).

Fungal DNA extraction

Fungal DNA was extracted as described in Chapter 2. Yeast DNA was extracted using a SDS extraction protocol as described by Akada et al. (2000). Briefly, a small amount of the yeast colony was transferred to a PCR tube using a pipette tip. A solution of 20 µL 0.25 % SDS was added to the yeast cells. The cells were vortexed briefly and heated 90 °C for 3 min in a thermocycler. The solution was centrifuged briefly to pellet the cells, and 0.8 µL of the supernatant was used as the source of template DNA for PCR reactions.

Plasmid for cloning under constitutive fungal promoter expression

A plasmid was created to clone DNA fragments between the *ToxA* gene promoter of *Pyrenophora tritici-repentis* and the nopaline synthase gene (*nos*) terminator of *Agrobacterium tumefaciens*. The plasmid vector pUC18 was used as the backbone for the plasmid. A plasmid was created named pKAES210 from pCT74 (Lorang et al., 2001) and pUC18. The plasmid pCT74 was double-digested with *Eco*RI and *Cla*I to produce a 1.6 kb fragment that was filled in with Klenow fragment (New England Biolabs). The 1.6 kb fragment was ligated to a 2.7 kb fragment of pUC18 that was *Eco*RI digested and filled in with Klenow fragment of DNA polymerase I (New England Biolabs). The newly created 4.3 kb fragment plasmid was named pKAES210. A polylinker containing *Eco*RV, *Mlu*I, *Xho*I and *Spe*I sites was created by annealing oligonucleotide LinkerA with oligonucleotide LinkerB (Table 4.1) by combining the two oligonucleotides in a PCR tube and heating at 70 °C for 5 min, 60 °C 30 sec, 50 °C 30 sec, 40 °C 30 sec, 30°C

30 sec and 25°C 30 sec. The polynucleotide linker was then phosphorylated by the action of T4 polynucleotide kinase (New England Biolabs). The phosphorylated linker was then ligated to the 3.5 kb pKAES210 that was *Nco*I and *Not*I digested, then treated with mung bean nuclease (New England Biolabs). The resulting plasmid was then *Sal*I digested and ligated to the 1.4 kb *Sal*I fragment of pCT74. The resulting plasmid named pKAES215 contained the *TOXA* promoter followed by *Spe*I, *Xho*I, *Mlu*I, and *EcoR*V restrictions sites, the *nos* terminator and the hygromycin B phosphotransferase gene (*hph*) under the *Aspergillus nidulans trpC* promoter (Figure 4.2).

RNAi plasmid constructs

To construct the *lolE* RNAi plasmid, *g*enomic DNA of *N. uncinatum* was used as a template to amplify a 0.8 kb fragment using primers lolE1Fusion and SpeIlolEstart (Table 4.1) by PCR (95 °C 5 min, 35 cycles of 95 °C for 30 sec, 63 °C for 30 sec, and 72 °C for 1 min). The PCR product was digested with *BamH*I and *Spe*I. Also, a 0.8 kb fragment was amplified using primers lolE1intronR and SpeIlolEstart by PCR (95 °C 5 min, 35 cycles of 95 °C for 30 sec, 63 °C for 30 sec, and 72 °C for 1 min), and digested with *BamH*I and *EcoR*V. The two fragments were ligated to *Spe*I- and *EcoR*V-digested pKAES215 to create a 6.3 kb plasmid named pKAES225 (Figure 4.3).

A *lolO* RNAi plasmid was created that contained a segment of *lolO* sequence in both a forward and reverse orientation for RNA silencing (Figure 4.3). An approximately 200 bp PCR product was amplified from *N. uncinatum* DNA using primers lolOrnaiF1 and lolOrnaiR1 (95 °C 5 min, 35 cycles of 95 °C for 30 sec, 61 °C for 30 sec, and 72 °C for 1 min). The PCR product was purified using the PCR purification kit (Qiagen), and

digested with *Eco*RV and *Xba*I, for which the recognition sequences were incorporated into the PCR primers. An approximately 280 bp PCR product was amplified from *N. uncinatum* DNA using primers lolOrnaiF2 and lolOrnaiR2 (95 °C 5 min, 35 cycles of 95 °C for 30 sec, 61 °C for 30 sec, and 72 °C for 1 min). The 280 bp fragment was also purified, and then digested with *Spe*I and *Xba*I for which the recognition sequences were incorporated into the PCR primers. Plasmid pKAES215 was double-digested with *Spe*I and *Eco*RV and the 4.8 kb fragment was purified using the gel extraction kit (Qiagen). The three fragments (0.2 kb, 0.28 kb, and 4.8 kb) were ligated together using the Fast Link DNA ligation kit (Epicentre) in a 2:2:1 molar ratio. The resulting plasmid, named pKAES226 contained the *TOXA* promoter driving exon1 of *lolO* followed by intron 1 of *lolO* and the reverse complement of *lolO* exon1 (Figure 4.3).

Preparation and transformation of protoplasts of *Neotyphodium uncinatum*

Neotyphodium uncinatum was transformed using a protoplast transformation by PEG or electroporation. A 50 mL PDB culture of *N. uncinatum* was grown for 5 to 7 days at 21 °C and 200 RPM. The mycelium was pelletted and resuspended in 10 mL of osmotic medium (1.2 M $MgSO_4$, 10 mM $NaHPO_4$ pH 6.0) with 25 mg bovine serum albumin (Sigma, St Louis, MO), 50 mg vinoflow FCE (Novozymes, Basgvaerd, Denmark), 50 mg driselase (Sigma), 8 mg zymicase (InterSpex Products, San Mateo, CA), and 50 mg lysing enzymes from *Trichoderma harzianum* (Sigma). Protoplasts were isolated as described in Tsai et al (1992).

For electroporation, 5 µg of DNA was mixed with 70 µL protoplast suspension containing between 5×10^6 and 10×10^6 protoplasts. The protoplast DNA mixture was

placed in a cuvette and electroporated at 0.5 kV/cm, 25 µF and 200 Ω, and immediately placed on ice.

In the case of PEG-mediated transformation, the isolated protoplasts were suspended in STC solution (1 M sorbitol, 0.1 M Tris-Cl pH 7.4, 50 mM $CaCl_2$) so that they contained 5 x 10^6 protoplasts in 100 µL. The suspension was combined with a PEG solution (two parts 60% PEG 3350 and one part 1.8 M KCl, 150 mM $CaCl_2$, 150 mM Tris pH 7.4) so that the PEG solution comprised ¼ of the total volume. A 5 µg DNA solution was mixed with 125 µL of the protoplast PEG mixture and incubated on ice for 30 min, then another 20 min at room temperature, after which they were plated out.

Following the electroporation or PEG transformation procedure the transformed protoplasts were mixed with 5 mL of regeneration medium (Panaccione et al., 2001). The suspension was then placed on PDA plates overlain with 5 mL of regeneration medium containing enough hygromycin B to give a hygromycin B concentration of 80 µg/mL over the total volume of the plate (5 mL regeneration medium with protoplasts, 5 mL regeneration medium, 18 mL of PDA with hygromycin B). The protoplasts were incubated at 21 °C for 2 to 3 weeks until colonies were visible. Putative transformant colonies were then moved to PDA plates contain 80 µg/mL hygromycin B and single-spore streaked three times to obtain a pure culture. Transformants were verified by PCR for the *hph* gene and *toxA* promoter.

Yeast expression constructs

RNA was extracted from *N. uncinatum* growing in minimal medium for loline production (Faulkner et al., 2006) and at the loline alkaloid production stage. The RNA

was extracted using the RNEasy RNA extraction kit (Qiagen) according to the manufacture's protocol. The RNA was then treated with the DNA-free DNase removal kit (Ambion) according to manufacture's protocol to remove any DNA contamination. The RNA was then converted to cDNA with Monsterscript cDNA synthesis kit (Epicentre) using the V_3-Oligo$(dT)_{21}$ primer according to manufacture's protocol. The cDNA was stored at -20 °C or immediately used for PCR reactions.

PCR products containing the *lolE*1, *lolF*1 and *lolO*1 complete coding sequences amplified from cDNA were cloned into pCR2.1 using the TOPO PCR cloning kit (Invitrogen) according to manufacture's protocol. The cDNA for *lolE*1 was amplified using primers lolE1fwdcDNA and lolE1revcDNA (95 °C 5 min, 35 cycles of 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 2 min), and cloned into pCR2.1, resulting in plasmid pKAES191. The *lolF*1 cDNA was amplified using primers lolF1fwdcDNA and lolF1revcDNA (95 °C 5 min, 35 cycles of 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 2 min), and cloned into pCR2.1, resulting in plasmid pKAES192. The *lolO*1 cDNA was amplified using primers lolO1fwdcDNA and lolO1revcDNA (95 °C 5 min, 35 cycles of 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 2 min) , and cloned into pCR2.1, resulting in plasmid pKAES193.

Yeast expression vector pESC-LEU was purchased from Stratagene and pESC-HIS was a gift from Dr. Peter Nagy. The two yeast vectors each contain the *GAL*1 and *GAL*10 promoters followed by multiple cloning sites. pKAES191, pKAES192 and pKAES193 were used as source of full-length cDNA coding sequences to create plasmids containing the respective *LOL* genes following the *GAL*1 or *GAL*10 promoters for expression in YPH499 yeast cells.

The cDNA for *lolF* from pKAES192 was cloned into pESC-HIS. pKAES192 was digested with *Eco*RI and the 1.7 kb fragment containing the cDNA of *lolF*1 was gel purified using the gel extraction kit (Qiagen). pESC-HIS was digested with *Eco*RI and then ligated to the 1.7 kb pKAES192 fragment. The ligation products were transformed into XL-1 Blue cells and the colonies were screened by restriction digestion for insertion of the *lolF*1 cDNA behind the *GAL*10 promoter in the correct orientation for expression of the entire coding sequence. The correct orientation plasmid was then sequenced to check for any PCR errors, and the plasmid containing the correct *lolF*1 coding sequence was named pKAES203 (Figure 4.4).

The cDNA for *lolE*1 from pKAES191 was cloned into pESC-LEU. pKAES191 was digested with *Not*I and *Spe*I and the 0.8 kb fragment was gel purified using the gel extraction kit (Qiagen). pESC-LEU was double digested with *Not*I and *Spe*I and then ligated to the 0.8 kb fragment from pKAES191. The ligation products were transformed into XL-1 Blue cells and the colonies were screened by restriction digestion for insertion of the *lolE*1 cDNA behind the *GAL*10 promoter in the correct orientation for expression of the entire coding sequence. The correct orientation plasmid was then sequenced to check for any PCR errors and the plasmid containing the correct *lolE*1 sequence was named pKAES204 (Figure 4.4).

The cDNA for *lolO*1 from pKAES193 was cloned into pESC-HIS. pKAES193 was digested with *Eco*RI and the 1.3 kb fragment was gel purified by using the gel extraction kit (Qiagen). pESC-HIS was digested with *Eco*RI and then ligated to the 1.3 kb pKAES193 fragment. The ligation products were transformed into XL-1 Blue cells and the colonies were screened by restriction digestion for insertion of the *lolO*1 cDNA

behind the *GAL*10 promoter in the correct orientation for expression of the entire coding sequence. The correct orientation plasmid was then sequenced to check for any PCR errors and the plasmid containing the correct *lolO*1 sequence was named pKAES205.

The cDNA for *lolO*1 from pKAES193 was cloned into pESC-LEU. pKAES193 was digested with *Eco*RI and the 1.3 kb fragment was gel purified by using the gel extraction kit (Qiagen). pESC-LEU was digested with *Sal*I and filled in using Klenow fragment (New England Biolabs), then ligated to the 1.3 kb pKAES193 fragment. The ligation products were transformed into XL-1 Blue cells and the colonies were screened by restriction digestion for insertion of the *lolO*1 cDNA behind the *GAL*1 promoter in the correct orientation for expression of the entire coding sequence. The correct orientation plasmid was then sequenced to check for any PCR errors and the plasmid containing the correct *lolO*1 sequence was named pKAES206 (Figure 4.4).

A dual expression vector for simultaneous expression of *lolF* and *lolO* cDNA under galactose induction in yeast cells was constructed from pKAES191 and pKAES203. pKAES193 was *Eco*RI-digested and the 1.3-kb fragment was filled in by the action of Klenow fragment (New England Biolabs) and ligated into pKAES203 that was *Not*I digested and also filled in. The ligation products were transformed into XL-1 Blue cells and the colonies were screened by restriction digestion for insertion of *lolO*1 cDNA following the *GAL*1 promoter in the correct orientation for expression. The plasmid was sequenced to check for the presence of *lolO*1 and *lolF*1 cDNA, and named pKAES207 (Figure 4.4).

A dual expression vector for simultaneous expression of *lolE*1 and *lolF*1 cDNA under galactose induction in yeast cells was constructed from pKAES191 and

pKAES203. The 0.8 kb *Eco*R1 fragment of pKAES191 was filled in using Klenow fragment (New England Biolabs). The 0.8 kb fragment was ligated into pKAES203 that was *Spe*I and *Not*I digested and also filled in. The ligation products were transformed into XL-1 Blue cells and the colonies were screened by restriction digestion for insertion of *lolE*1 cDNA following the *GAL*1 promoter in the correct orientation for expression. The plasmid was sequenced to confirm the orientation of *lolE*1 and named pKAES208.

Yeast transformations

The yeast cell line YPH499 was transformed according to the protocol provided in the pESC Yeast Epitope Tagging Vector manual (Stratagene). Yeast competent cells were prepared by incubating a 5 mL tube of YPAD broth (0.0075 % 1-adenine hemisulfate salt, 1 % yeast extract, 2 % Bacto peptone, 2 % glucose) with YPH499 cells at 31 °C with shaking at 200 RPM overnight. The culture was then diluted approx. 1:20 in 50 mL of YPAD to an OD_{600} of 0.25. The culture was shaken at 31 °C for 4 to 6 hr until it reached an OD_{600} of 1.0. The cells were pelletted at 1000 x g for 5 min and the supernatant removed. The cells were resuspended in 10 mL of LTE buffer (0.1 M LiOAc, 10 mM Tris-HCl pH 7.5, 1 mM EDTA) and again centrifuged at 1000 x g for 5 min. The supernatant was removed and the cells were suspended in 0.5 mL of LTE buffer. The competent cell suspension was immediately used for transformation or stored at 4 °C for a maximum of 3 days. The competent cells were transformed using a PEG-Li acetate protocol. A 50 µL solution of competent cells was mixed with 3 µg of plasmid and 300 µL of transformation mix (40 % polyethylene glycol 3350, 0.1 M LiOAc, 10 mM Tris-HCl pH 7.5, 1 mM EDTA) in a 15 mL tube. The mixture was inverted and

incubated at 30 °C for 30 min then 42 °C for 15 min. The solution was briefly centrifuged to pellet the cells and the supernatant was removed. The cells were resuspended in 350 µL LTE and 100 µL and 250 µL were plated onto SD dropout plates as appropriate for plasmid auxotrophic marker. The transformed yeast cells were screened by PCR for the loline cDNA genes to verify insertion of the plasmids. Vector only transformed yeast cells were screened by PCR for the *his3* or *leu2* gene contained in the vectors.

RESULTS

Expression of *LOL* genes in yeast

Yeast expression vectors pKAES206, pKAES207, and pKAES208 were introduced into the yeast cell line YPH499, and transformants were selected by growing in drop-out media lacking the appropriate amino acids. Each *lolE*, *lolF* and *lolO* expression plasmid was introduced into yeast separately and in pairs as indicated in Table 4.2. The yeast transformants were grown in glucose-containing medium then transferred to galactose-containing medium to induce expression of the cloned *LOL* genes. RNA was extracted from the yeast cells after 12 hours of induction, and assayed by reverse-transcription-PCR (RT-PCR) for gene expression. Results indicated expression of *lolF* and *lolO* in all yeast cell lines containing the respective expression plasmids after galactose induction. RT-PCR indicated that there was no *lolE* mRNA in any of the yeast cells containing the *lolE* cDNA expression plasmid. The *lolE* expression was negative in

all situations including when *lolE* was induced in conjunction with *lolO* and *lolF* (Table 4.3).

Yeast cell lines containing all three galactose-induced *LOL* gene constructs as shown in table 4.2 were all induced for expression in galactose medium or galactose medium containing 1 mM *exo*-1-aminopyrrolizidine for 16 hours. The contents of the induced cell lines were then analyzed by GCMS for loline accumulation. The lyophilized culture and cells did not contain any loline alkaloids after induction of the LOL cluster constructs (Figure 4.5).

RNAi analysis of *lolO* and *lolE* in *N. uncinatum*

Neotyphodium uncinatum strain ernai12 was a transformant with pKAES225, ornai8 was a transformant with pKAES226, and hphl1 was a transformant with the empty vector pKAES215 used to make the RNAi constructs. Three MM cultures were pooled together and analyzed for loline production at 8, 10, 13, 15, 17, 20, and 25 days after inoculation. Loline alkaloid production could be observed by GCMS at 13 days post-inoculation in all three transformant cultures.

In analyzing the GCMS results for loline production in separate ornai8, ernai12 and hphl1 cultures, a peak was identified as accumulating in larger amounts in ornai8 and ernai12 transformants compared to the empty vector control (Figure 4.9). The different sampling time points for loline analysis indicated that this peak was detectable on day 13 in both transformants. The GCMS for the compound was analyzed using the Varian software and no mass spectrum match was found. The peak was also found in the vector

control but the peak height compared to the internal standard was 6 times higher in the RNAi containing cultures (Figure 4.6).

The compound in the novel peak was analyzed in tandem with loline alkaloid, and a mass spectrum of the compound was produced (Figure 4.7). The spectrum was searched online at the spectral database for organic compounds http://riodb01.ibase.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi, and no spectrum matched the compound. A loline analysis sample from ornai8 day 25 was sent to the University of Louisville Center for Regulatory and Environmental Metabolomics Mass Spectrometry Center to be analyzed using the tandem MS-filtered Fourier transform ion cyclotron resonance (FTICR) mass spectrometer with nano-spray electrospray ionization. The facility FTICR reported a molecular ion of 169.13355 m/z for the compound (Figure 4.8). Computer analysis of the molecular ion and fragment ions provided a structure for the compound, determined to be *N*-acetyl-1-aminopyrrolizidine.

DISCUSSION

The significant finding in this chapter was the identification of a likely loline alkaloid biosynthesis intermediate, *N*-acetyl-1-aminopyrrolizidine. *Neotyphodium uncinatum* transformed with constructs designed to cause RNA interference (RNAi) of *lolE* and *lolO* accumulated this novel compound, suggesting that it is an intermediate in the steps from *exo*-1-aminopyrrolizidine to the first loline alkaloid with the oxygen bridge. The results indicate that *exo*-1-aminopyrrolizidine is *N*-acetylated prior to

formation of oxygen bridge, and that formation of the bridge involves LolO and LolE, probably giving *N*-acetylnorloline (NANL) as the first loline alkaloid (Figure 4.9).

The possible loline alkaloid biosynthetic pathway has been proposed based on the results of feeding deuterated and other labeled compounds to *N. uncinatum* loline-producing cultures (Chapter 3, Figure 3.10). The synthesis of deuterated compounds in the laboratory of R. B. Grossman provided means to test possible intermediates in the pathway. Based on results of those studies, and knowledge of the genes involved in loline alkaloid biosynthesis, a complete pathway was proposed with putative gene functions (Figure 4.9).

The use of RNAi-base gene silencing has been successful in linking loline alkaloid production with a *LOL* gene (Spiering et al., 2008). A construct containing inverted repeats of *lolC* DNA sequences has been used to downregulate expression of the target gene. Results of my study on the biosynthesis pathway (Chapter 3) provide evidence that *exo*-1-aminopyrrolizidine is a probable intermediate. Downregulation of the genes involved in the oxygen bridge formation should result in accumulation of one or more precursors to the oxygen bridge formation.

An RNAi approach was used in *N. uncinatum* loline production cultures to determine the function of LolE and LolO. Two constructs were created that provided the DNA elements necessary to create inverted repeats of *lolE* and *lolO* RNA once transformed into *N. uncinatum*. The constructs were then transformed into *N. uncinatum* and in one round of protoplast transformation I was able to obtain multiple tranformants containing the *lolE* and *lolO* constructs. The transformants were able to produce loline alkaloids but also contained noticeable amounts of an unknown compound in the GCMS

profile (Figure 4.6). The unknown compound in the lolORNAi extract was further analyzed by FTICRMS at the University of Louisville, and a molecular ion of 169.13355 was determined for the compound, which was identified as *N*-acetyl-1-aminopyrrolizidine (NAAP, Figure 4.8). Transformants containing the lolO RNAi constructs contained six times the amount of NAAP as vector controls.

The production of NAAP introduces a likely new intermediate in loline alkaloid biosynthesis. It is likely that *exo*-1-aminopyrrolizidine is acetylated by an unknown fungal enzyme to form the NAAP. The acetylation is likely due to the results of feeding deuterated *exo*-1-aminopyrrolizidine to cultures. Cultures supplemented with deuterated *exo*-1-aminopyrrolizidine were analyzed for lolines and the supplemented cultures lacked detectable NAAP in the GCMS analysis (previous Chapter).

The discovery of NAAP as a likely intermediate in loline alkaloid biosynthesis provides an additional step prior to the formation of the oxygen bridge in the loline alkaloid structure. There must be a step that involves acetylation of *exo*-1-aminopyrrolizidine prior to steps catalyzed by LolO and LolE. The results indicate that the acetylated amine is required prior to the addition of the oxygen bridge. Therefore the first loline alkaloid that could be produced would not be norloline but one of the acetylated loline alkaloids. I suggest that the *N*-acetynorloline (NANL) is the likely first loline alkaloid produced, and all other loline alkaloids are derived from NANL. The supporting evidence for this scenario is the findings that some plants contain only NANL in the loline alkaloid profile (Chapter2). The predicted roles of the current loline genes do not contain a gene that would likely acetylate *exo*-1-aminopyrrolizidine. This acetylation step may be performed by a gene that acetylates other primary amines in the

fungal cells or some unknown loline biosynthesis gene. Sequencing of the entire *N. uncinatum* genome could aid in determining if there are any other genes related to loline biosynthesis.

In a separate set of experiments described in this chapter, the *lolE, lolF* and *lolO* genes were transferred into yeast cells in various combinations and induced for expression. My expectation was that a combination of two of the three oxidative enzymes encoded by these genes would catalyze oxygen-bridge formation. Induced yeast cultures were fed *exo*-1-aminiopyrrolizidine, but no loline alkaloid product (and no NAAP) was detected (Figure 4.5). It is important to note that I conducted these experiments before obtaining the results of *lolO* and *lolE* RNAi, and in light of the RNAi results, two reasons for the yeast expression result now seem clear. First, there was no detectable *lolE* transcript in transformed, induced yeast, suggesting that the *lolE* sequence either somehow inhibits transcription in yeast or, more likely, the *lolE* RNA is unstable in yeast (Jacobson and Peltz, 1996). The RNAi results suggests that LolE is involved in oxygen bridge formation, so its lack of detectable expression would have ensured that little or no bridge formation occurred. The second reason is that the RNAi results also suggest that the oxygen bridge is added to the *N*-acetylated form, NAAP, rather than to *exo*-1-aminopyrrolizidine, as originally hypothesized. Therefore, conversion of the latter to a loline alkaloid in yeast would seem to require, at minimum, an *N*-acetyl transferase, LolO and LolE. There is no obvious gene for the *N*-acetyl transferase in the *LOL* cluster, though *lolU* is a possibility (Spiering et al., 2005). This possibility can be tested by heterologous expression of *lolU* in yeast or a *lol*-minus fungus, and feeding *exo*-1-aminopyrrolizidine, then checking for NAAP. In addition, expression of *lolO* and *lolE* in

a fungus that produces or is fed NAAP would help establish the roles of these genes. Gene knockouts and complementation could also be used to test these possibilities, though gene knockouts in *N. uncinatum* are very rare events (Spiering et al., 2008).

Sequence analysis of *lolE* indicates that it is similar to epoA gene of *Penicillium decumbens* (Spiering et al., 2005). The epoA gene codes for epoxidase subunit A which was identified using *P. decumbens* protein sequences (Watanabe et al., 1999). When the *epoA* CDS was sequenced it only contained the N terminus and four peptides from the protein used as the probe for the gene. Therefore, Watanabe et al concluded that *epoA* product was a subunit of a larger protein complex. I believe that *lolE* has a similar role loline biosynthesis. The RNAi results suggest that *lolE* and *lolO* are involved together in their actions. Therefore *lolE* may be involved in a protein complex or may be a subunit that combines with *lolO* or another unnamed loline gene. This could also be a reason for the inability to express *lolE* in yeast cells but further analysis of the *lolE* protein would be needed to determine the activity of the protein.

In summary, this chapter provides evidence for NAAP as an intermediate in loline alkaloids biosynthesis. NAAP is formed after *exo*-1-aminopyrrolizidine and prior to the oxygen bridge formation of the loline alkaloids. The chapter provides evidence that the lolO and lolE genes are involved in acting on NAAP to make NANL the first loline alkaloid to be synthesized. RNAi transformation of *N. uncinatum* with DNA sequence designed to produce dsRNA of *lolO* and *lolE* resulted in accumulation of NAAP in loline production cultures.

Table 4.1 Oligonucleotide names and sequences used in this Chapter.

Name	Sequence
LinkerA	GATATCACGCGTCTCGAGACTAGT
LinkerB	ACTAGTCTCGAGACGCGTGATATC
IolE1Fusion	CGGGATCCACATGATACGTGACCAATCTTACGTAGCGTTTTCCGGAGGGCTGTG
SpelIolEstart	GCACTAGTATGACCGCTGCTTCTTCCCCTCA
IolE1intronR	CGGGATCCAAACATGCAAACATCATCTTTTC
SpelIolEstart	GCACTAGTATGACCGCTGCTTCTTCCCCTCA
IolOrnaiF1	GCGATATCATGACGGTAACAAACAAGCCTG
IolOrnaiR1	CCTCTAGAAATGCAGCCAGGCGAATGCTTACCTCGAGCG
IolOrnaF2	ATTTCTAGAGGCACACAAGATCAATTAGCGATCC
IolOrnaiR2	ACTACTAGTATGACGGTAACAAACAAGCCTG
IolE1fwdcDNA	TCATGACCGCTGCTTCTTCC
IolE1revcDNA	TCAAGTCTGCGCTTCCACTG
IolF1fwdcDNA	TATCGACTCGATATACGACAGAAAGTG
IolF1revcDNA	TTCTGCATATGATTATTTAATTCTTCTTC
IolO1fwdcDNA	TGACCGACTGGCATAAGTGC
IolO1revcDNA	ATATCACGATTCCTCACTCTTTCC

Table 4.2 Yeast cell lines created with galactose inducible lolE, lolF, and lolO cDNA. Plasmids containing lolE, lolF, and lolO cDNA cloned behind galactose inducible promoter were cloned and selected by hisitidine or leucine production. The yeast cells were tested for loline genes by PCR to confirm successful introduction of the plasmids. The table shows all of the possible LOL gene combinations in the yeast cell lines. The + indicates presence of the LOL gene in the yeast cell and – indicates no presence.

Cell Line	*lolE*	*lolF*	*lolO*
yE	+	-	-
yF	-	+	-
yO	-	-	+
yEF	+	+	-
yEO	+	-	+
yFO	-	+	+
yEFO	+	+	+

Table 4.3 Expression of *LOL* genes in yeast cell lines transformed with yeast expression vectors. Each plasmid was introduced separately into a yeast cell and then in pairs such that *lolE*, *lolF* and *lolO* were in each possible combination. Analysis of expression of *LOL* genes were conducted by RT-PCR using RNA from cell lines after galactose induction. RT-PCR showed no expression of *lolE* in any combination with the other *LOL* genes. Additionally no expression was detected when *lolE* was cloned following the *GAL1* promoter or into pESC-HIS (not shown). The + indicates presence and – indicates no presence.

pKAES #	*lolE*	*lolF*	*lolO*
216	-	N/A	N/A
213	N/A	+	N/A
215	N/A	N/A	+
216 + 213	-	+	N/A
216 + 215	-	N/A	+
214	N/A	+	+
216 + 214	-	+	+

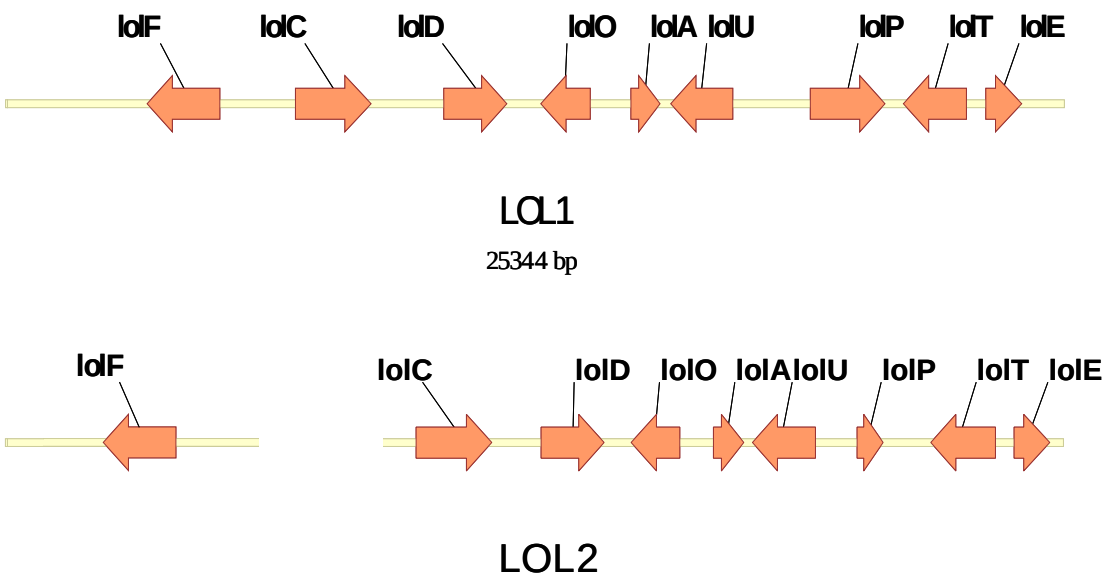


Figure 4.1 Loline biosynthesis (*LOL*) gene clusters of *Neotyphodium uncinatum*. The orange arrows indicate sizes and directions of transcription of the *LOL* genes. The *lolF* and *lolC* genes in the *LOL2* cluster are not known to be connected as they are in *LOL*1. The *LOL*2 cluster has a *lolP* gene with a large deletion in the coding sequence, rendering it nonfunctional.

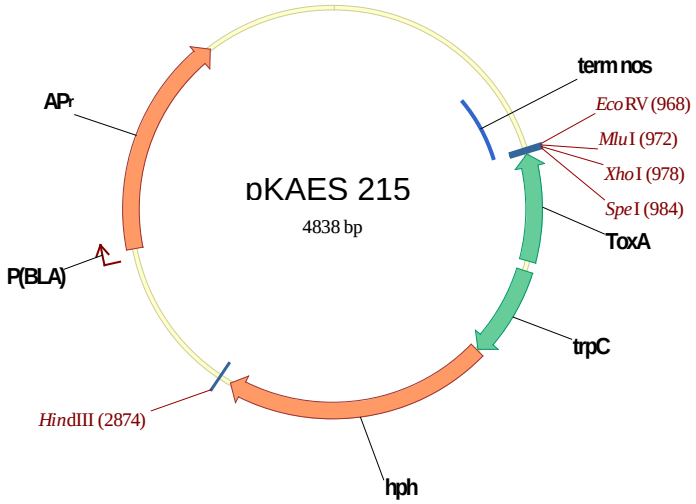


Figure 4.2 Fungal transformation vector with multiple cloning site for constitutive expression. A hygromycin resistance vector (pKAES215) was created containing the *toxA* promoter of *Pyrenophora tritici-repentis* followed by a multiple cloning site and the *nos* terminator. The hygromycin resistance gene is under control of the constitutive *trpC* promoter of *Aspergillus nidulans*. The multiple cloning site allows for directional cloning of DNA elements for expression.

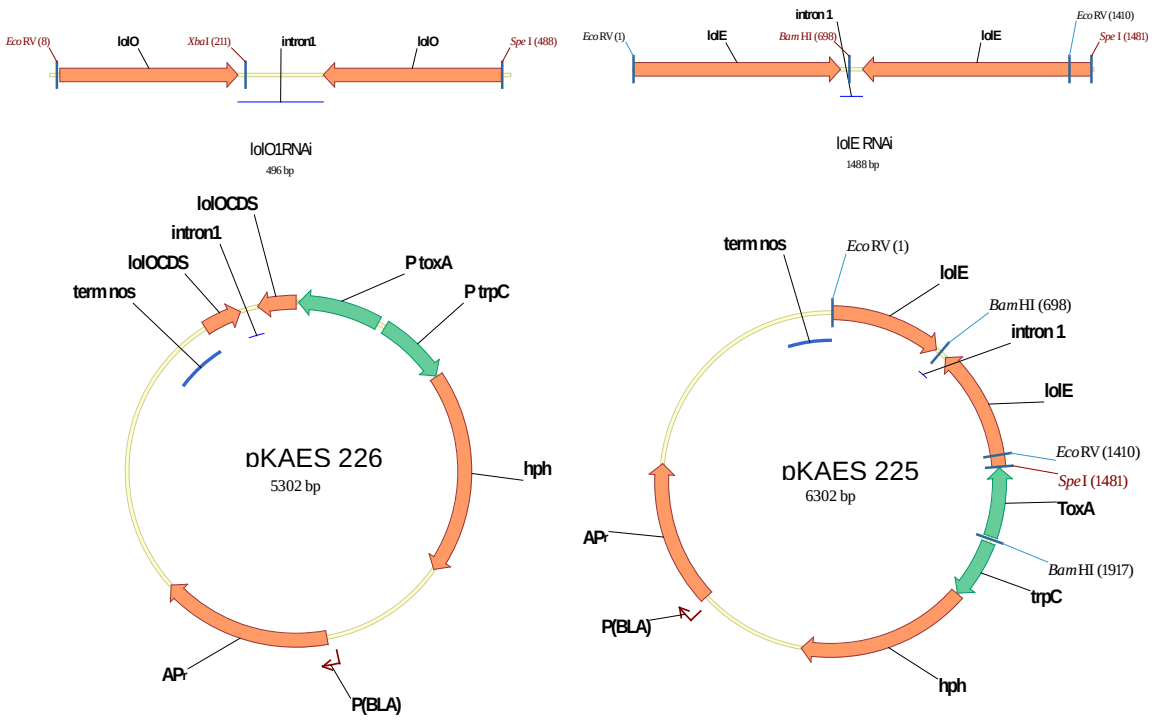


Figure 4.3 Fungal RNAi constructs used to knock down *lolE* and *lolO* in *N. uncinatum*. Using overlapping PCR, two RNAi cassettes were created containing the first exon and intron of *lolE* and *lolO*, respectively, followed by the reverse complement of the first exon. These cassettes were then ligated into pKAES215 using restriction endonuclease sites introduced by PCR primers to obtain RNAi constructs (pKAES225 and pKAES226).

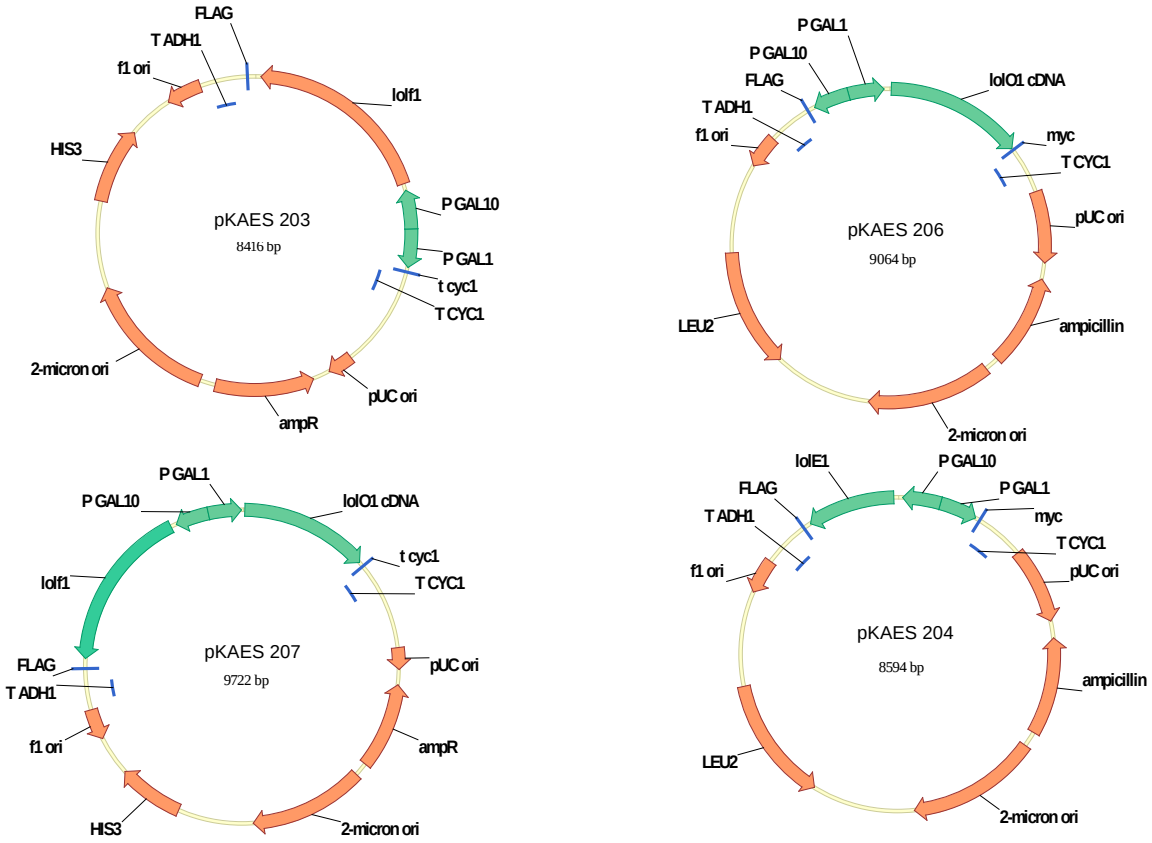


Figure 4.4. Yeast expression vectors for *lol* gene product expression. Using yeast expression vectors pESC-HIS and pESC-LEU cDNA from *lolE, lolO* and *lolF* were cloned behind the respective *GAL1* or *GAL10* promoters. Four constructs were made such that each possible combination of *lolE, lolO* or *lolF* could be introduced into one single yeast cell line and expressed using galactose induction.

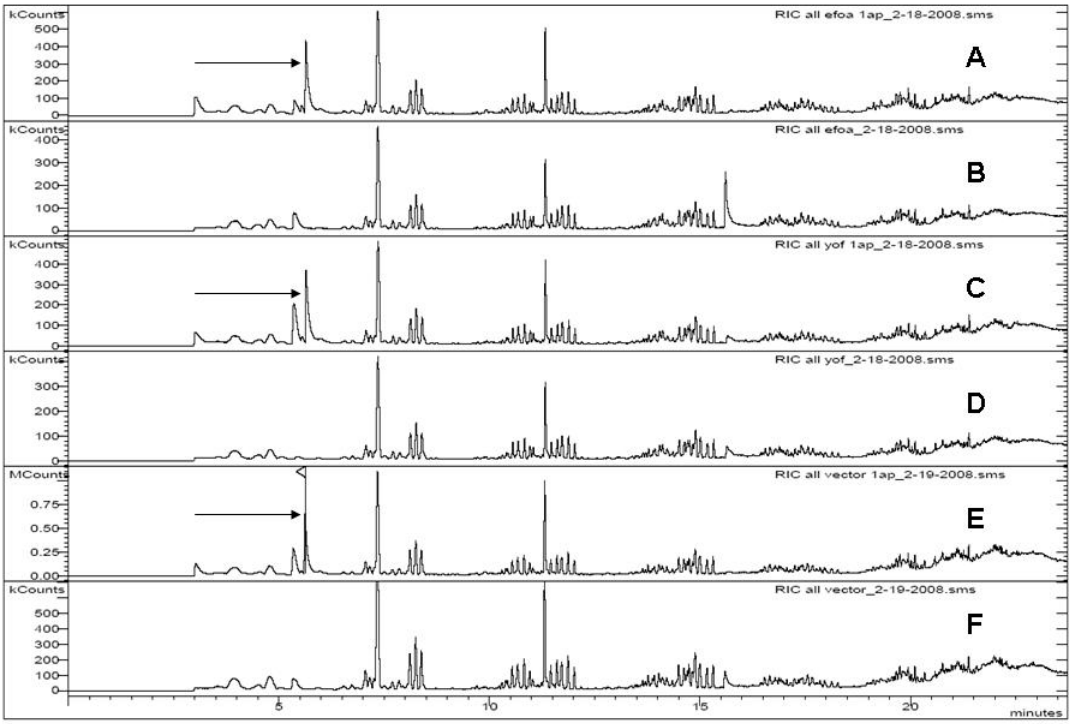


Figure 4.5 GCMS chromatogram of culture supernatants of yeast cell lines containing *LOL* gene cDNAs induced for expression. Analysis was for production of loline alkaloids with or without addition of the *exo*-1-aminopyrrolizidine intermediate. A. Cells containing pKAES216 and pKAES214 supplemented with 2 mM *exo*-1-aminopyrrolizidine. B. Cells containing pKAES216 and pKAES214. C. Cells containing pKAES216 and pKAES215 supplemented with 2 mM *exo*-1-aminopyrrolizidine. D. Cells containing pKAES216 and pKAES215. E. Cells Containing pESC-HIS and pESC-LEU supplemented with 2 mM *exo*-1-aminopyrrolizidine. F. Cells containing pESC-HIS and pESC-LEU. Arrow indicates peak corresponding to *exo*-1-aminopyrrolizidine. No loline alkaloid peaks were observed.

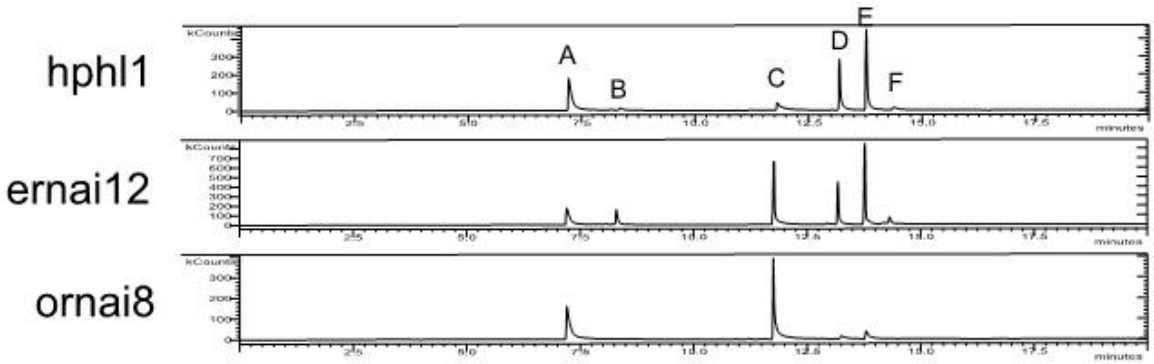


Figure 4.6 GCMS trace of *N. uncinatum* RNAi transformants showing loline alkaloid profiles obtained from day 25 cultures. The transformants are the vector only (hphl1), lolE RNAi (ernai12), and lolO RNAi (ornai8). The peaks for all three chromatograms are labeled A, B, C, D, E and F. The loline peaks are labeled B, D, E and F. Peak A is the internal standard. Peak C was identified as *N*-acetyl-1-aminopyrrolizidine.

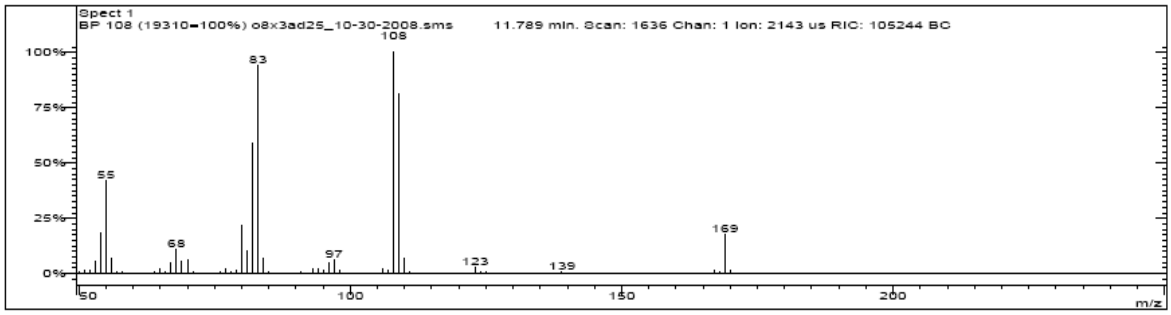


Figure 4.7 Mass spectrum of *N*-acetyl-1-aminopyrrolizidine. Spectrum was obtained from accumulated compound in lolO RNAi transformant.

nanoESI-FTICR MS (25 uL sample + 25 uL 50% MeOH/1% AcOH)

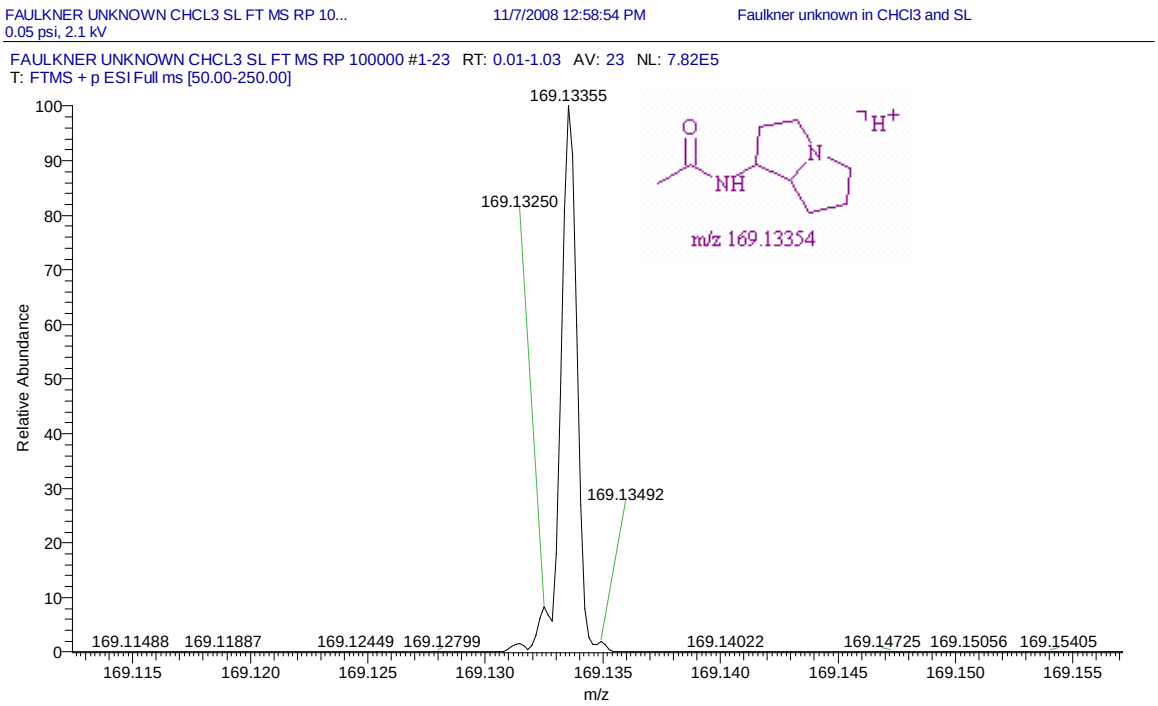


Figure 4.8 Chromatogram and exact mass from nano-electro spray ionization FTICR mass spectrum of unknown compound in obtained in loline samples from RNAi transformants. The inset shows the predicted structure based on the exact mass calculated from the mass spectrum. The predicted compound is *N*-acetyl-1-aminopyrrolizidine.

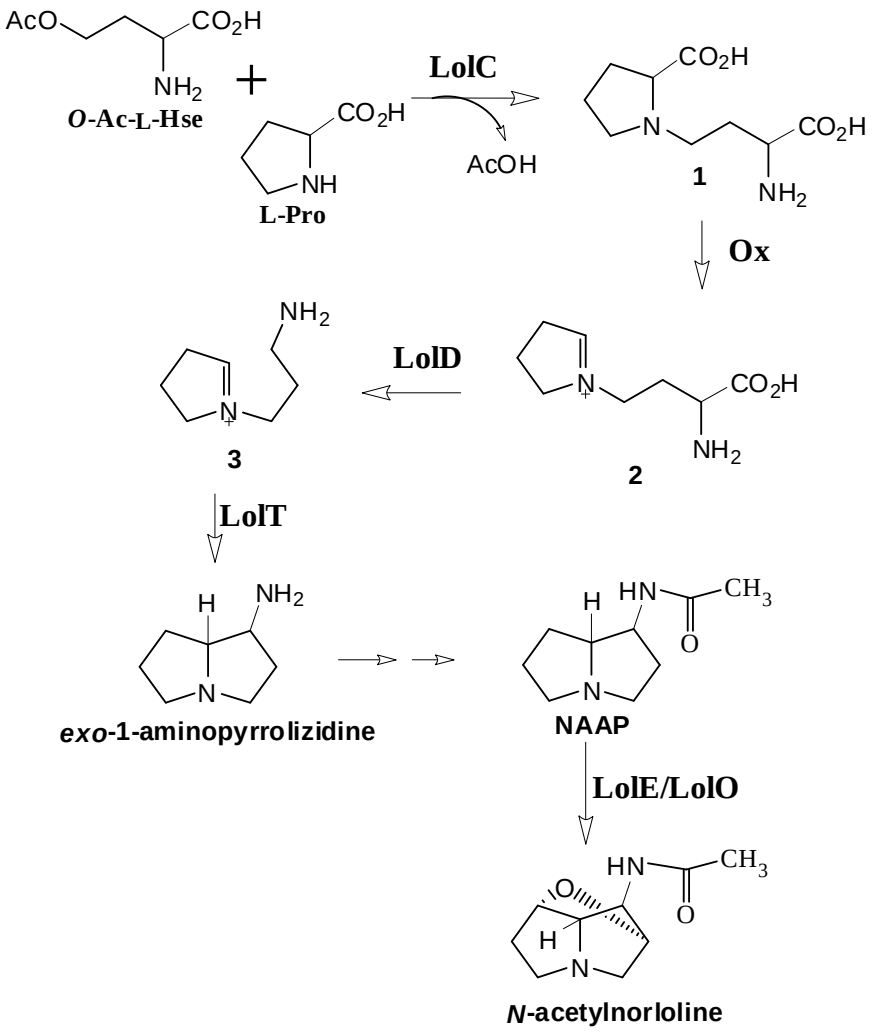


Figure 4.9. Proposed pathway for biosynthesis of *N*-acetylnorloline. L-Pro and O-Ac-L-Hse are combined (involving LolC) to form 1, which is oxidatively decarboxylated to imminium ion 2. Decarboxylation (via LolD) gives 3, and cyclization (involving LolT) gives *exo*-1-aminopyrrolizidine. Subsequent unknown steps involve the formation of NAAP (*N*-acetyl-1-aminopyrrolizidine) from *exo*-1-aminopyrrolizidine. NAAP is then involved in an unknown oxidation/oxygenation (involving LolE and LolO) step to yield N-acetylnorloline the likely first loline produced.

CHAPTER 5

Conclusion and Discussion

Loline alkaloids are insecticidal saturated 1-aminopyrrolizidines with an oxygen bridge. The lolines are derived from amino-acid precursors and their biosynthesis is directed a specific cluster of genes, the *LOL* cluster. This dissertation provides insights into the profiles of lolines in plants and the biosynthesis of lolines in culture. This dissertation provides new evidence of a loline alkaloid profile for plants containing only *N*-acetylnorloline (NANL). Experimental evidence indicated that *N*-aminocarboxypropylproline and *exo*-1-aminopyrrolizidine are intermediates in loline alkaloid biosynthesis. Molecular techniques applied to cultures of the fungus, *Neotyphodium uncinatum,* produced a novel compound in loline alkaloid culture. The compound, identified as *N*-aceto-1-aminopyrrolizidine, is a likely intermediate in the loline-alkaloid pathway (Figure 5.1). The combined results of my dissertation added to knowledge of the loline alkaloid biosynthesis pathway.

The second chapter in this dissertation explored the relationship between loline alkaloid production in plants and the *LOL* cluster genes contained in endophytes. The chapter surveyed many plant species and showed that loline alkaloid profiles in plants provide a better diagnostic for endophyte infection and chemotype than did PCR tests for *lol* genes. The chapter also showed that there may be other loline alkaloid profiles relevant to plant fungal interactions. In particular, I found several symbiota that accumulated mainly NANL, rather than NFL and NAL. Further study should be

conducted to determine if other loline alkaloids are in higher prevalence in any plant fungal symbiosis.

The analysis of plants only having NANL provides a new manner to determine loline alkaloid relevance. The effects of NANL should be analyzed in future experiments to determine its efficacy against grass pests. NANL has not been a commonly researched loline alkaloid and such study may help to determine the mode of actions of the loline alkaloid on herbivorous insects, for example. It is also quite possible that other cool season grasses may contain loline alkaloid profiles that are not readily determined based on current techniques. My study surveyed only a small subset of the biodiversity of grasses and their epichloid symbionts. It would be also be worthwhile to determine the loline alkaloid profiles on plants in their native environments to help distinguish if NANL may benefit the symbiosis.

The third chapter in this dissertation provided experimental evidence in favor of modifying the hypothesis previously put forth for the loline alkaloid biosynthesis pathway (Faulkner et al., 2006). Deuterated proposed intermediates were applied to *N. uncinatum* cultures, and results of analysis for incorporation of the isotope labels in NFL indicated that pyrrolizidine ring formation precedes the oxygen bridge formation in loline alkaloid biosynthesis. The results indicated that N-aminocarboxypropylproline and *exo*-1-aminopyrolizidine are likely intermediates in the loline alkaloid pathway. The deuterated compounds were given to cultures and the end products of loline alkaloid contained deuterium as expected according to their hypothesized roles as intermediates.

This approach with deuterated N-aminocarboxypropylproline and *exo*-1-aminopyrrolizidine demonstrate the utility of this approach and suggest its application to discover additional intermediates in the pathway. The experiments show that loline alkaloid production medium allows *N. uncinatum* to take up and use compounds added to the culture. The *N. uncinatum* culture system could be utilized for other secondary metabolism analysis.

The newly proposed biosynthesis pathway constituted the working hypothesis for Chapter 4. Based on possible functions of the loline alkaloid gene products inferred from bioinformatic analysis, together with results of Chapter 3, RNA interference (RNAi) and yeast cell expression experiments were devised to test the roles of oxidative enzymes encoded by *lolE, lolF,* and *lolO*. The results of the RNAi experiments provided evidence of a previously unknown compound as a pathway intermediate. The RNAi for lolO accumulated *N*-aceto-1-aminopyrrolizidine (NAAP) in large amounts. This exciting finding demonstrated the utility of RNAi for *lol* gene functional analysis. However, as is often the case in other systems, the RNAi system was leaky in *N. uncinatum,* and did not completely inhibit production of the loline alkaloid end products. More definitive tests in future may require gene knockouts, which, however, are very tedious in the *N. uncinatum* system, especially for *lol* genes because of the presence of two expressed *LOL* clusters. The RNAi containing *N. uncinatum* provide additional endophytes to experiment on the loline alkaloid biosynthesis pathway. My RNAi experiments also provided tools such as a

plasmid constructs for RNAi in epichloid fungi, and a set of *lolO* and *lolE* RNAi transformants to be used in future studies to further refine knowledge of the pathway.

The discovery of NAAP as a intermediate in loline biosynthesis provides for a new chemical compound that may be a result of endophyte symbiosis. The ability to assay for this new compound using the same techniques as loline alkaloids creates opportunity to look for possible producers of the compound in nature. It may be possible that there are some endophytes that have previously thought to not produce lolines that may only be missing the final production steps in the pathway. The finding of this compound may provide for new evidence of endophyte infection. At the very least, a survey for NAAP should be performed on the collection of plants that are known to produce loline alkaloids to determine the prevalence of this intermediate in nature. This dissertation shows that it is possible for the endophyte to produce this compound and without deleterious effects to the fungus. The ability of some endophytes to only produce NANL indicates that there may be other endophytes without some of the LOL genes involved in the later steps of loline biosynthesis. Therefore, it is not unlikely that a endophyte may have lost the loline genes after the NAAP step in the pathway.

Prior to this dissertation the loline alkaloid biosynthesis pathway was based on amino acid known precursors and *lol* gene analysis predicted functions. This dissertation provided information to test the loline alkaloid biosynthesis pathway, and created a new framework and a better understanding of how loline alkaloids are produced in epichloid endophytes. This dissertation describes application of a combination of analytical and

molecular techniques to provide insights into the loline alkaloid biosynthesis. The three preceding chapters all describe research that enhances knowledge of the loline alkaloid pathway. The experiments implicated three compounds in the pathway for loline alkaloid biosynthesis: NACPP, *exo*-1-aminopyrrolizidine, and its *N*-acetylated form (NAAP). This dissertation increases the knowledge of the loline alkaloid pathway and creates opportunities for future study.

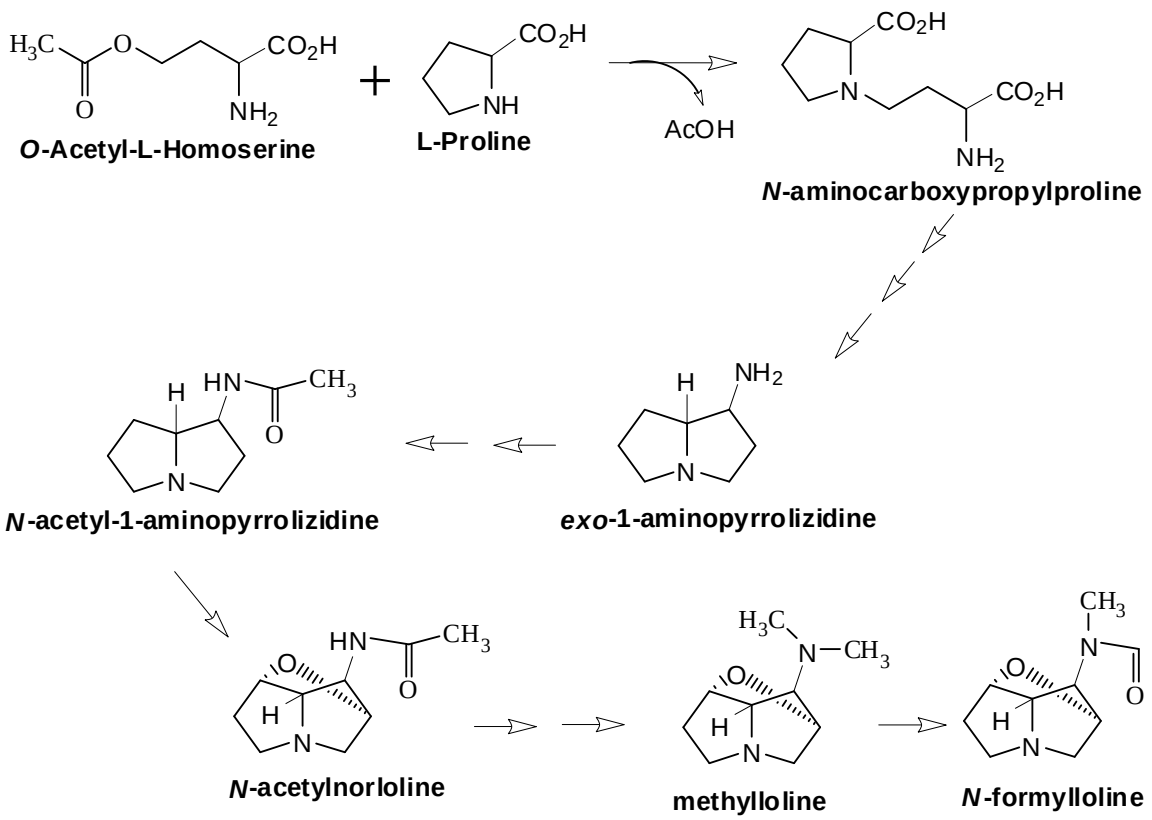


Figure 5.1 Proposed loline alkaloid biosynthesis pathway for *Neotyphodium uncintatum*. The proposed pathway contains experimentally shown intermediates and precursors to loline biosynthesis. L-proline and *O*-acetyl-L-homoserine are combined in the first committed step. The resulting compound is *N*-aminocarboxypropylproline which is then converted over many steps to *exo*-1-aminopyrrolizidine. The *exo*-1-aminopyrolizidine is then converted to *N*-acetyl-1-aminopyrrolizidine. The oxygen bridge formation occurs after *N*-acetyl-1-aminpyrrolizidine to form *N*-acetylnorloline (the first synthesized loline alkaloid). *N*-acetylnorloline is then converted via unknown mechanisms to methylloline which is then converted to *N*-formylloline (the most abundant loline alkaloid in culture).

APPENDIX A

Complementation of the *lolP* knockout

A mutant of *N. uncinatum* with *lolP1* gene knocked out by homologous recombination was complemented as part of the author's work on loline biosynthesis. The complementation was published as part of the study to determine *lolP* function in *N. uncinatum* (Spiering et al, 2008). As part of this the work a Southern hybridization blot of the *lolP* gene was performed. The following figure is the image used to quantify the amount of *lolP* inserted into the complementation endophytes.

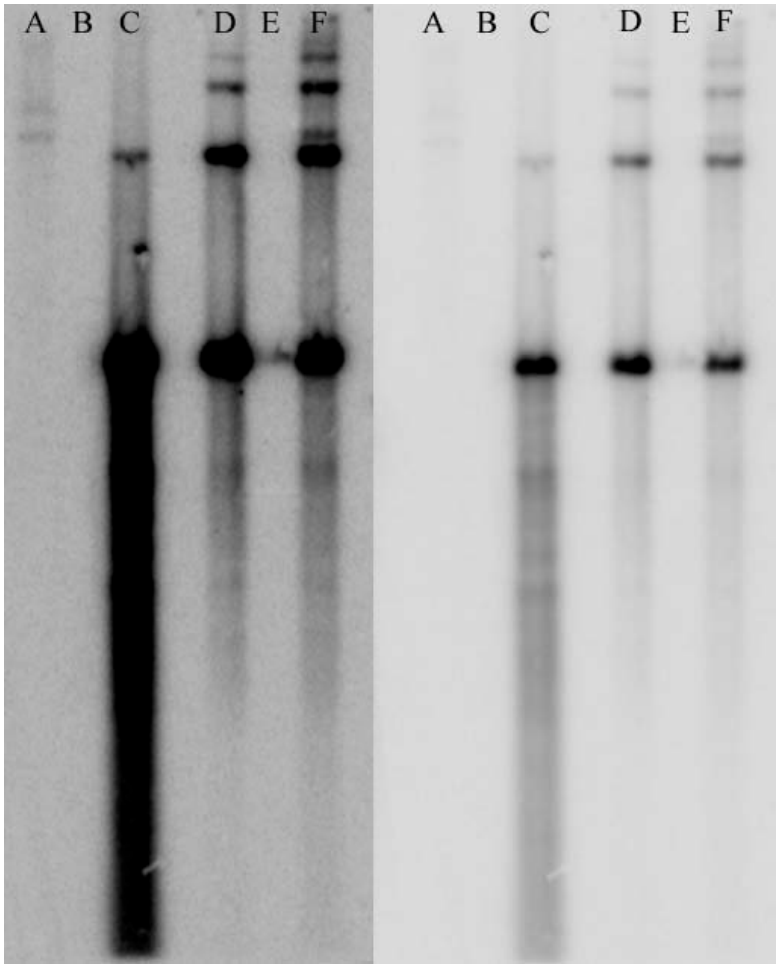


Figure A.1 Presence of *lolP*1 in *N. uncinatum* wild type (WT) and transformed strains. Shown is a Southern hybridization of *Neotyphodium uncinatum Hind*III-digested genomic DNA hybridized with a probe from the portion of *lolP*1 that is not present in *lolP*2. The DNA used in each of the lanes were: A. *Neotyphodium uncinatum* wild type, B. NUMS15-*lolP*1Δ (*lolP* knockout), C. HOPE04 (lolP complement), D. HOPE13 (*lolP* complement), E. pKAES195 (*lolP* complement plasmid), F. PWT1 (*N. uncinatum* wild type transformed with plasmid pKAES195 containing the *lolP*1 complementation construct. *lolP*1 is not detectable in NUMS15-*lolP*1Δ (lane B), while numerous copies of *lolP*1 appeared to have inserted in tandem in the *lolP*1-complemented transformants (lanes C, D, F). The right and left are the same image with the intensity adjusted to show the probe strength.

REFERENCES

Aasen AJ, Culvenor CCJ (1969) Abnormally low vicinal coupling constants for O-CH-CH in a highly strained five-membered-ring ether; the identity of loline and festucine. Australian Journal of Chemistry **22:** 2021-2024

Akramov ST, Yunusov SY (1966) Structure of norloline, loline, and lolinine. Chemistry of Natural Compounds **1:** 203-209

Al-Samarrai TH, Schmid J (2000) A simple method for extraction of fungal genomic DNA. Letters in Applied Microbiology **30:** 53–56

An Z-q, Siegel MR, Hollin W, Tsai H-F, Schmidt D, Schardl CL (1993) Relationships among non-*Acremonium* sp. fungal endophytes in five grass species. Applied and Environmental Microbiology **59:** 1540-1548

Arachevaleta M, Bacon CW, Hoveland CS, Radcliffe DE (1989) Effect of the Tall Fescue Endophyte on Plant-Response to Environmental-Stress. Agronomy Journal **81:** 83-90

Bates RB, Morehead SR (1972) Absolute configurations of pyrrolizidine alkaloids of the loline group. Tetrahedron Letters **13:** 1629-1630

Batirov ÉK, Khamidkhodzhaev SA, Malikov VM, Yunusov SY (1977) An investigation of the alkaloids of *Lolium cuneatum*. Chemistry of Natural Compounds **12:** 50-52

Batirov ÉK, Malikov VM, Yunusov SY (1977) Alkaloids of the seeds of Lolium cuneatum. Chemistry of Natural Compounds **12:** 114-115

Belesky DP, Stringer WC, Hill NS (1989) Influence of Endophyte and Water Regime Upon Tall Fescue Accessions .1. Growth-Characteristics. Annals of Botany **63:** 495-503

Blankenship JD (2004) Loline alkaloid biosynthesis in *Neotyphodium uncinatum*, a fungal endophyte of *Lolium pratense*. Ph.D. Dissertation. University of Kentucky, Lexington

Blankenship JD, Houseknecht JB, Pal S, Bush LP, Grossman RB, Schardl CL (2005) Biosynthetic precursors of fungal pyrrolizidines, the loline alkaloids. Chembiochem **6:** 1016-1022

Blankenship JD, Spiering MJ, Wilkinson HH, Fannin FF, Bush LP, Schardl CL (2001) Production of loline alkaloids by the grass endophyte, Neotyphodium uncinatum, in defined media. Phytochemistry **58:** 395–401

Boettcher F, Adolph RD, Hartmann T (1993) Homospermidine synthase, the first pathway-specific enzyme in pyrrolizidine alkaloid biosynthesis. Phytochemistry **32:** 679-689

Brem D, Leuchtmann A (1999) High prevalence of horizontal transmission of the fungal endophyte Epichloe sylvatica. Bulletin of the Geobotanical Institute ETH **64:** 3 - 12

Bullock WO, Fernandez JM, Short JM (1987) XL1-Blue - A high-efficiency plasmid transforming RecA *Escherichia-coli* strin with beta-galactosidase selection. BioTechniques **5:** 376-378

Bultman TL, Bell G, Martin WD (2004) A fungal endophyte mediates reversal of wound-induced resistance and constrains tolerance in a grass. Ecology **85:** 679-685

Bultman TL, Bell GD (2003) Interaction between fungal endophytes and environmental stressors influences plant resistance to insects. Oikos **103:** 182-190

Bultman TL, Borowicz KL, Schneble RM, Coudron TA, Bush LP (1997) Effect of a fungal endophyte on the growth and survival of two Euplectrus parasitoids. Oikos **78:** 170-176

Bush LP, Cornelius PL, Buckner RC, Varney DR, Chapman RA, Burrus PB, Kennedy CW, Jones TA, Saunders MJ (1982) Association of N-Acetyl Loline and N-Formyl Loline with Epichloe-Typhina in Tall Fescue. Crop Science **22:** 941-943

Bush LP, Fannin FF, Siegel MR, Dahlman DL, Burton HR (1993) Chemistry, occurrence and biological effects of saturated pyrrolizidine alkaloids associated with endophyte-grass interactions. Agriculture, Ecosystems & Environment **44:** 81-102

Bush LP, Fannin FF, Siegel MR, Dahlman DL, Burton HR (1993) Chemistry, Occurrence and Biological Effects of Saturated Pyrrolizidine Alkaloids Associated with Endophyte Grass Interactions. Agriculture Ecosystems & Environment **44:** 81-102

Bush LP, Wilkinson HH, Schardl CL (1997) Bioprotective alkaloids of grass-fungal endophyte symbioses. Plant Physiology **114:** 1-7

Casabuono AC, Pomilio AB (1997) Alkaloids from endophyte-infected *Festuca argentina*. Journal of Ethnopharmacology **57:** 1-9

Christensen MJ (1995) Variation in the Ability of Acremonium Endophytes of Lolium-Perenne, Festuca-Arundinacea and F-Pratensis to Form Compatible Associations in the 3 Grasses. Mycological Research **99:** 466-470

Chung KR, Hollin W, Siegel MR, Schardl CL (1997) Genetics of Host Specificity in Epichloe typhina. Phytopathology **87:** 599-605

Cohen G, Shiffman D, Mevarech M, Aharonowitz Y (1990) Microbial Isopenicillin-N Synthase Genes - Structure, Function, Diversity and Evolution. Trends in Biotechnology **8:** 105-111

Dannhardt G, Steindl L (1985) Alkaloids of Lolium-Temulentum - Isolation, Identification and Pharmacological Activity. Planta Medica**:** 212-214

Faulkner JR, Hussaini SR, Blankenship JD, Pal S, Branan BM, Grossman RB, Schardl CL (2006) On the sequence of bond formation in loline alkaloid biosynthesis. Chembiochem: A European Journal Of Chemical Biology **7:** 1078-1088

Gonthier DJ, Sullivan TJ, Brown KL, Wurtzel B, Lawal R, VandenOever K, Buchan Z, Bultman TL (2008) Stroma-forming endophyte Epichloe glyceriae provides wound-inducible herbivore resistance to its grass host. Oikos **117:** 629-633

Griffin M, Trudgill PW (1976) Purification and Properties of Cyclopentanone Oxygenase of Pseudomonas Ncib-9872. European Journal of Biochemistry **63:** 199-209

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology **52:** 696-704

Gwinn KD, Collinsshepard MH, Reddick BB (1991) Tissue Print-Immunoblot, an Accurate Method for the Detection of Acremonium-Coenophialium in Tall Fescue. Phytopathology **81:** 747-748

Hausinger RP (2004) Fe(II)/alpha-ketoglutarate-dependent hydroxylases and related enzymes. Critical Reviews in Biochemistry and Molecular Biology **39:** 21-68

Hill NS, Belesky DP, Stringer WC (1991) Competitiveness of Tall Fescue as Influenced by Acremonium-Coenophialum. Crop Science **31:** 185-190

Jacobson A, Peltz SW (1996) Interrelationships of the pathways of mRNA decay and translation in eukaryotic cells. Annu Rev Biochem **65:** 693-739

Koulman A, Lane GA, Christensen MJ, Fraser K, Tapper BA (2007) Peramine and other fungal alkaloids are exuded in the guttation fluid of endophyte-infected grasses. Phytochemistry **68:** 355-360

Kutil BL, Greenwald C, Liu G, Spiering MJ, Schardl CL, Wilkinson HH (2007) Comparison of loline alkaloid gene clusters across fungal endophytes: predicting the co-regulatory sequence motifs and the evolutionary history. Fungal Genet Biol **44:** 1002-1010

Kutil BL, Greenwald C, Liu G, Spiering MJ, Schardl CL, Wilkinson HH (2007) Comparison of loline alkaloid gene clusters across fungal endophytes: Predicting the co-regulatory sequence motifs and the evolutionary history. Fungal Genetics and Biology **44:** 1002-1010

Lehtonen P, Helander M, Wink M, Sporer F, Saikkonen K (2005) Transfer of endophyte-origin defensive alkaloids from a grass to a hemiparasitic plant. Ecology Letters **8:** 1256-1263

Leuchtmann A, Schmidt D, Bush LP (2000) Different levels of protective alkaloids in grasses with stroma-forming and seed-transmitted *Epichloë/Neotyphodium* endophytes. Journal of Chemical Ecology **26:** 1025-1036

Lorang JM, Tuori RP, Martinez JP, Sawyer TL, Redman RS, Rollins JA, Wolpert TJ, Johnson KB, Rodriguez RJ, Dickman MB, Ciuffetti LM (2001) Green fluorescent protein is lighting up fungal biology. Appl Environ Microbiol **67:** 1987-1994

Mauger AB (1996) Naturally Occurring Proline Analogues. Journal of Natural Products **59:** 1205-1211

Moon CD, Miles CO, Jarlfors U, Schardl CL (2002) The evolutionary origins of three new Neotyphodium endophyte species from grasses indigenous to the Southern Hemisphere. Mycologia **94:** 694-711

Panaccione DG, Johnson RD, Wang J, Young CA, Damrongkool P, Scott B, Schardl CL (2001) Elimination of ergovaline from a grass-Neotyphodium endophyte symbiosis by genetic modification of the endophyte. Proc Natl Acad Sci U S A **98:** 12820-12825

Patterson CG, Potter DA, Fannin FF (1991) Feeding Deterrency of Alkaloids from Endophyte-Infected Grasses to Japanese-Beetle Grubs. Entomologia Experimentalis Et Applicata **61:** 285-289

Petroski RJ, Yates SG, Weisleder D, Powell RG (1989) Isolation, Semi-Synthesis, and Nmr Spectral Studies of Loline Alkaloids. Journal of Natural Products **52:** 810-817

Powell RG, Petroski RJ (1992) The loline group of pyrrolizidine alkaloids. Alkaloids, chemical and biological perspectives **8:** 320-338

Riedell WE, Kieckhefer RE, Petroski RJ, Powell RG (1991) Naturally-Occurring and Synthetic Loline Alkaloid Derivatives - Insect Feeding-Behavior Modification and Toxicity. Journal of Entomological Science **26:** 122-129

Robbins JD, Wilkinso.R, Sweeny JG, Burdick D (1972) Volatile Alkaloids of Kentucky 31 Tall Fescue Seed (Festuca-Arundinancea Schreb. Journal of Agricultural and Food Chemistry **20:** 1040-&

Rowan DD, Gaynor DL (1986) Isolation of feeding deterrents against Argentine stem weevil from ryegrass infected with the endophyte *Acremonium loliae*. Journal of Chemical Ecology **12:** 647-658

Rowan DD, Hunt MB, Gaynor DL (1986) Peramine, a Novel Insect Feeding Deterrent from Ryegrass Infected with the Endophyte Acremonium-Ioliae. Journal of the Chemical Society-Chemical Communications**:** 935-936

Schardl CL (1996) Epichloe species: Fungal symbionts of grasses. Annual Review of Phytopathology **34:** 109-130

Schardl CL (1996) EPICHLOE SPECIES: fungal symbionts of grasses. Annu Rev Phytopathol **34:** 109-130

Schardl CL (2001) Epichloe festucae and related mutualistic symbionts of grasses. Fungal Genet Biol **33:** 69-82

Schardl CL, Grossman RB, Nagabhyru P, Faulkner JR, Mallik UP (2007) Loline alkaloids: Currencies of mutualism. Phytochemistry **68:** 980-996

Schardl CL, Leuchtmann A, Spiering MJ (2004) Symbioses of grasses with seedborne fungal endophytes. Annu Rev Plant Biol **55:** 315-340

Shiba T, Sugawara K (2008) Fungal loline alkaloids in grass-endophyte associations confer resistance to the rice leaf bug, *Trigonotylus caelestialium*. Entomologia Experimentalis et Applicata

Shiba T, Sugawara K (2009) Fungal loline alkaloids in grass–endophyte associations confer resistance to the rice leaf bug, *Trigonotylus caelestialium*. Entomologia Experimentalis et Applicata **130:** 55-62

Siegel MR, Latch GCM, Bush LP, Fannin FF, Rowan DD, Tapper BA, Bacon CW, Johnson MC (1990) Fungal endophyte-infected grasses: alkaloid accumulation and aphid response. Journal of Chemical Ecology **16:** 3301-3315

Sienko M, Topczewski J, Paszewski A (1998) Structure and regulation of cysD, the homocysteine synthase gene of Aspergillus nidulans. Current Genetics **33:** 136-144

Spiering MJ, Faulkner JR, Zhang D-X, Machado C, Grossman RB, Schardl CL (2008) Role of the LolP cytochrome P450 monooxygenase in loline alkaloid biosynthesis. Fungal Genetics and Biology **45:** 1307-1314

Spiering MJ, Faulkner JR, Zhang DX, Machado C, Grossman RB, Schardl CL (2008) Role of the LolP cytochrome P450 monooxygenase in loline alkaloid biosynthesis. Fungal Genet Biol **45:** 1307-1314

Spiering MJ, Moon CD, Wilkinson HH, Schardl CL (2005) Gene clusters for insecticidal loline alkaloids in the grass-endophytic fungus *Neotyphodium uncinatum*. Genetics **169:** 1403-1414

Spiering MJ, Moon CD, Wilkinson HH, Schardl CL (2005) Gene clusters for insecticidal loline alkaloids in the grass-endophytic fungus *Neotyphodium uncinatum*. Genetics **169:** 1403-1414

Spiering MJ, Wilkinson HH, Blankenship JD, Schardl CL (2002) Expressed sequence tags and genes associated with loline alkaloid expression by the fungal endophyte *Neotyphodium uncinatum*. Fungal Genetics and Biology **36:** 242-254

Spiering MJ, Wilkinson HH, Blankenship JD, Schardl CL (2002) Expressed sequence tags and genes associated with loline alkaloid production in the grass endophyte *Neotyphodium uncinatum*. Fungal Genetics and Biology **36:** 242-254

Steiner U, Ahimsa-Muller MA, Markert A, Kucht S, Gross J, Kauf N, Kuzma M, Zych M, Lamshoft M, Furmanowa M, Knoop V, Drewke C, Leistner E (2006) Molecular characterization of a seed transmitted clavicipitaceous fungus occurring on dicotyledoneous plants (Convolvulaceae). Planta **224:** 533-544

Tanaka A, Tapper BA, Popay A, Parker EJ, Scott B (2005) A symbiosis expressed non-ribosomal peptide synthetase from a mutualistic fungal endophyte of perennial ryegrass confers protection to the symbiotum from insect herbivory. Molecular Microbiology **57:** 1036-1050

TePaske MR, Powell RG, Clement SL (1993) Analyses of selected endophyte-infected grasses for the presence of loline-type and ergot-type alkaloids. Journal of Agricultural and Food Chemistry **41:** 2299-2303

Thompson FN, Stuedemann JA (1993) Pathophysiology of Fescue Toxicosis. Agriculture Ecosystems & Environment **44:** 263-281

Tofern B, Kaloga M, Witte L, Hartmann T, Eich E (1999) Phytochemistry and chemotaxonomy of the Convolvulaceae part 8 - Occurrence of loline alkaloids in Argyreia mollis (Convolvulaceae). Phytochemistry **51:** 1177-1180

Watanabe M, Sumida N, Murakami S, Anzai H, Thompson CJ, Tateno Y, Murakami T (1999) A Phosphonate-Induced Gene Which Promotes Penicillium-Mediated Bioconversion of cis-Propenylphosphonic Acid to Fosfomycin. Applied and Environmental Microbiology **65:** 1036-1044

Wilkinson HH, Siegel MR, Blankenship JD, Mallory AC, Bush LP, Schardl CL (2000) Contribution of fungal loline alkaloids to protection from aphids in a grass-endophyte mutualism. Molecular Plant-Microbe Interactions **13:** 1027-1033

Yates SG, Fenster JC, Bartelt RJ (1989) Assay of Tall Fescue Seed Extracts, Fractions, and Alkaloids Using the Large Milkweed Bug. Journal of Agricultural and Food Chemistry **37:** 354-357

Yates SG, Petroski RJ, Powell RG (1990) Analysis of Loline Alkaloids in Endophyte-Infected Tall Fescue by Capillary Gas-Chromatography. Journal of Agricultural and Food Chemistry **38:** 182-185

Yunusov SY, Akramov ST (1960) Investigation of alkaloids of *Lolium cuneatum* II. Zhurnal Obshchei Khimii **30:** 677-682

Yunusov SY, Akramov ST (1960) Investigation of alkaloids of *Lolium cuneatum* III. Zhurnal Obshchei Khimii **30:** 683-689

Yunusov SY, Akramov ST (1960) Structure of norloline, loline and lolinine IV. Zhurnal Obshchei Khimii **30:** 3132-3137

Zhang DX, Nagabhyru P, Blankenship JD, Schardl CL (2010) Are loline alkaloid levels regulated in grass endophytes by gene expression or substrate availability? Plant Signaling and Behavior **5**

Zhang DX, Nagabhyru P, Schardl CL (2009) Regulation of a chemical defense against herbivory produced by symbiotic fungi in grass plants. Plant Physiol **150:** 1072-1082

Zhang DX, Stromberg AJ, Spiering MJ, Schardl CL (2009) Coregulated expression of loline alkaloid-biosynthesis genes in Neotyphodium uncinatum cultures. Fungal Genet Biol **46:** 517-530

VITA

Jerome Ralph Faulkner

Born July 21, 1978 in Princeton, Kentucky

Education	1996 to 2001	Kentucky State University	Frankfort, KY
	B.S. Biology and B.S. Chemistry		
Professional experience	1999 to 2001	Kentucky State University	Frankfort, KY
	Student Laboratory Assistant		
	2001 to 2003	United States Department of Agriculture Natural Resources Conservation Service (USDA NRCS)	Hickman, KY
	Biologist		
	2001 to 2010	USDA NRCS	Lexington, KY
	Biologist		
	2010 to Present	USDA NRCS	Lexington, KY
	Resource Conservationist for Planning		

Publications and Presentations

Jerome R. Faulkner (2004) Roles and evolution of loline alkaloids produced by plant-symbiotic fungi. Oral presentation at Center for Ecology, Evolution and Behavior Spring Research Symposium at University of Kentucky.

Jerome R. Faulkner, Syed R. Hussaini, Jimmy D. Blankenship, Sitaram Pal, Bruce M. Branan, Robert B. Grossman, and Christopher L. Schardl. "On the Sequence of Bond Formation in Loline Alkaloid Biosynthesis." *ChemBioChem*, 2006, 7, 1078-1088.

Christopher L. Schardl, Robert B. Grossman, Padmaja Nagabhyru, Jerome R. Faulkner, and Uma P. Malik. "Loline alkaloids: Currencies of mutualism." *Phytochemistry*, 2007, 68, 980-996.

Martin J. Spiering, Jerome R. Faulkner, Dong-Xiu Zhang, Caroline Machado, Robert B. Grossman, Christopher L. Schardl. "Role of the LolP cytochrome P450 monooxygenase in loline alkaloid biosynthesis." *Fungal Genetics and Biology*, 2008, 45, 1307-1314.

Jerome R. Faulkner, Martin J. Spiering, Robert B. Grossman, Christopher L. Schardl. (2008) Biosynthesis of loline alkaloids in fungal endophytes. Poster and Oral Presentation at the American Phytopathological Society Centennial Meeting in Minneapolis, Minnesota.

Honors

USDA 1890 Scholar
Lyman T Johnson Fellowship
Gamma Sigma Delta Agriculture Honor Society