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Ether bridge formation in loline alkaloid biosynthesis

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ABSTRACT

Lolines are potent insecticidal agents produced by endophytic fungi of cool-season grasses. These alkaloids are composed of a pyrrolizidine ring system and an uncommon ether bridge linking carbons 2 and 7. Previous results indicated that 1-aminopyrrolizidine was a pathway intermediate. We used RNA interference to knock down expression of *lolO*, resulting in the accumulation of an alkaloid identified as *exo*-1-acetamidopyrrolizidine based on high-resolution MS and NMR. Genomes of endophytes differing in alkaloid profiles were sequenced, revealing that those with mutated *lolO* accumulated *exo*-1-acetamidopyrrolizidine but no lolines. Heterologous expression of wild-type *lolO* complemented a *lolO* mutant, resulting in the production of *N*-acetylnorloline. These results indicated that the non-heme iron oxygenase, LolO, is required for ether bridge formation, probably through oxidation of *exo*-1-acetamidopyrrolizidine.

Introduction

The *Epichloë* species are fungi in the family Clavicipitaceae that grow as symbionts of cool-season grasses (Poaceae, subfamily Pooideae). Often *Epichloë* species confer to their hosts a range of fitness benefits, including enhanced resistance to biotic and abiotic stresses (Schardl et al., 2004). One such benefit is defense against plant herbivores that is attributable to the various alkaloids produced by these fungi. For example, tall fescue (*Lolium arundinaceum* (Schreb.) Darbysh.) cv. Kentucky 31 infected with *Epichloë coenophiala* C.W. Bacon & Schardl (Morgan-Jones et W. Gams) [=*Neotyphodium coenophialum* (Morgan-Jones et W. Gams) Glenn, C.W. Bacon & Hanlin] possesses three classes of protective alkaloids: ergot alkaloids, peramine, and lolines. Ergot alkaloids from this grass are notorious for causing fescue toxicosis to livestock, resulting in hundreds of millions of dollars in annual losses to the U.S. cattle industry (Hoveland, 1993; Schardl, 2006). Loline alkaloids, also produced by the symbionts of other forage grasses such as Italian ryegrass (*Lolium multiflorum* Lam.) and meadow fescue (*Lolium pratense* (Huds.) Darbysh.), as well as many wild grasses (Schardl et al., 2012), appear to have no adverse effects on livestock and vertebrate wildlife (Schardl et al., 2007). However, lolines are potently active against a broad spectrum of insect species (Bultman et al., 2004; Wilkinson et al., 2000; Yates et al., 1989) and may also help protect against nematodes (Bacetty et al., 2009). This spectrum of biological activity makes the lolines particularly attractive for *Epichloë* species that could provide bioprotection to forage grasses and thereby contribute to sustainable agriculture.

The lolines (Fig. 1) are saturated *exo*-1-aminopyrrolizidines with an oxygen bridge between carbons 2 (C2) and 7 (C7), causing the pyrrolizidine ring to be strained. Such an ether linkage is a characteristic rarely found in natural metabolites. Through isotopic enrichment experiments, we have identified L-proline (Pro) and L-homoserine as precursors in a loline-forming biosynthetic pathway that proceeds via *N*-(3-amino-3-carboxypropyl)proline and *exo*-1-aminopyrrolizidine (**1a**) (Blankenship et al., 2005; Faulkner et al., 2006). These findings indicate that the ether bridge forms after the completion of the pyrrolizidine ring system, which, in turn, excludes many common routes of ether formation in natural products, such as reduction of acetals or hemiacetals (Dominguez de Maria et al., 2010).

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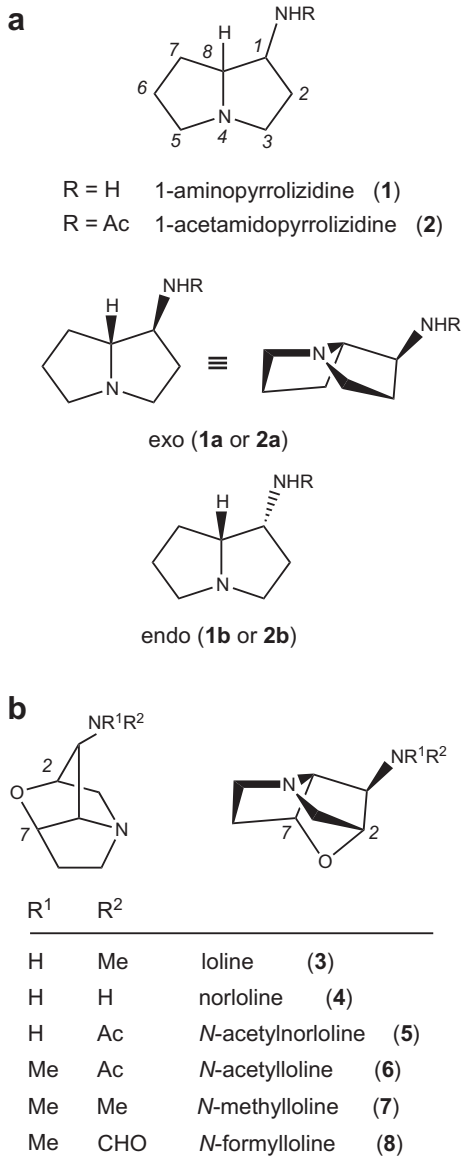

Fig. 1. Perspective illustrations of loline alkaloids. (a) 1-Aminopyrrolizidines. (b) Loline alkaloids, which possess a heterotricyclic core including an ether bridge linking C2 and C7. Substitutions on the nitrogen at C1 differentiate the lolines.

In several *Epichloë* species, a gene cluster, designated *LOL*, has been identified with up to 11 genes, in the sequence *lolF, lolC, lolD, lolO, lolA*, *lolU, lolP, lolT*, *lolE*, *lolN*, and *lolM*, and is strictly associated with the biosynthesis of lolines (Kutil et al., 2007; Schardl et al., 2013; Spiering et al., 2005). The predicted products of *LOL* genes include three pyridoxal-phosphate (PLP)-containing enzymes (LolC, LolD, and LolT) and four enzymes involved in oxidation/oxygenation reactions (LolF, LolO, LolP, and LolE). Among the potential oxidizing enzymes, LolP has been functionally characterized previously to catalyze the oxidation of *N*-methylloline (**7**) to *N*-formylloline (**8**) (one of the most abundant loline alkaloids found in grasses) and is not required for earlier steps (Spiering et al., 2008). LolF is likely to be involved in pyrrolizidine formation (Schardl et al., 2007), and, as an FAD-containing monooxygenase, it probably would not provide the oxidative potential for formation of the ether bridge. Hence LolO and LolE, predicted to be non-heme iron α-ketoacid-dependent dioxygenases, are the most likely candidate enzymes for catalyzing ether bridge formation. Here, we demonstrate that LolO is required to form the ether bridge, and we identify a new pathway intermediate, *exo*-1-acetamidopyrrolizidine (**2a**) (Fig. 1a), and hypothesize it to be the direct biosynthetic precursor of the loline alkaloids.

Results

Identification of exo-1-acetamidopyrrolizidine (2a)

Expression of the *lolO* RNAi construct in transformed *Epichloë uncinata* (W. Gams, Petrini & D. Schmidt) Leuchtm. & Schardl [=*Neotyphodium uncinatum* (W. Gams, Petrini & D. Schmidt) Glenn, C.W. Bacon & Hanlin] altered the loline alkaloid profile, giving a major peak of a previously unknown compound with a 12.0 min retention time in the gas chromatogram (Fig. 2). Although the same peak was also observed in the vector-only and wild-type controls, the area of this peak relative to **8** and *N*-acetylnorloline (**5**) was much greater in extracts from the RNAi strain cultures compared to the controls. The mass spectrum of the compound had no match when searched against the organic spectral database at http://riodb01.ibase.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi. Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) determined the mass to be 169.13355 amu, which is within 0.059 ppm of the theoretical mass of protonated 1-acetamidopyrrolizidine (**2**), an aminopyrrolizidine alkaloid related to the lolines, but lacking the ether bridge (Fig. 1). The newly discovered alkaloid **2** was also identified as the only loline-related metabolite produced in *Brachyelytrum erectum* (Schreb.) P.Beauv. symbiotic with *Epichloë brachyelytri* Schardl & Leuchtm. strain E4804, and in *Elymus canadensis* L. plant 4814, symbiotic with a strain (designated e4814) of *Epichloë canadensis* N.D. Charlton & C.A. Young.

In order to determine the relative configuration (**2a** or **2b**) with certainty, compound **2** was purified from tillers collected from plant 4814 and compared to synthetic (±)-*exo*-1-acetamidopyrrolizidine ((±)-**2a**). We initiated the synthesis of **2a** (Fig. 3) by reducing (±)-1-oximinopyrrolizidine with Raney nickel in tetrahydrofuran (THF) until starting material had been consumed. At this point, we observed one major and one minor spot by TLC, consistent with the formation of diastereomers (±)-**1a** and (±)-**1b**, respectively, as previously reported for the reduction of the oxime in isopropanol (Christine et al., 2000; Faulkner et al., 2006). We then added Ac_2O and 4-dimethylaminopyridine (DMAP) to the reaction mixture. After 2 h, the minor diastereomer of **1** remained unchanged, but the major diastereomer was replaced by a product that we identified as **2**. The reaction did not proceed further after

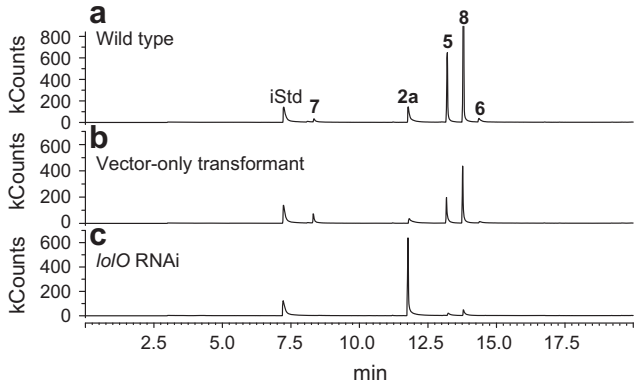

Fig. 2. GC–MS total ion traces of *E. uncinata* RNAi transformant and controls showing loline-alkaloid profiles from 25 day-old cultures. Bold numbers indicate the peaks expected for compounds listed in Fig. 1. The internal standard (iStd), quinoline, was used for quantification. (a and b) Chromatograms of products from wild-type *E. uncinata* e167 and the vector-only transformant, respectively; (c) chromatogram of products from a *lolO* RNAi transformant.

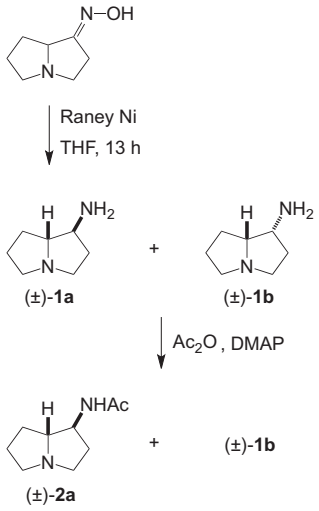

Fig. 3. Scheme for the synthesis of (±)-*exo*-1-acetamidopyrrolizidine **2a**.

overnight stirring. Because only the major diastereomer of **1**, which should be **1a**, reacted with Ac_2O to give **2**, we concluded the relative configuration of the new compound to be exo, or **2a**. Comparison of the synthetic (±)-**2a** to the natural **2** established the structure of the latter to be **2a** as well (see Discussion).

*Accumulation of compound **2a** is associated with lolO mutations*

Different loline alkaloid profiles were found to be associated with grasses symbiotic with various fungi including strains of *Epichloë amarillans* J.F. White in *Agrostis hyemalis* (Walter) Britton, Sterns & Poggenb., *E. amarillans* in *Sphenopholis obtusata* (Michx.) Scribn., *Epichloë festucae* Leuchtm. Schardl et M.R. Siegel in *L. pratense*, and the related fungus, *Atkinsonella hypoxylon* (Pk.) Diehl, in *Danthonia spicata* (L.) P. Beauv. ex Roem. & Schult. Three distinct loline profiles were observed. Plants with *E. festucae* E2368, like plants with *E. uncinata* e167 and *E. coenophiala* e19, accumulated loline (**3**), **5**, *N*-acetylloline (**6**), **7**, and **8.** Plants with *E. amarillans* E57 and *E. canadensis* e4815 had **5**, but no other fully-cyclized lolines, and plants with *E. amarillans* strains E721, E722 and E862, *E. brachyelytri* E4804, *E. canadensis* e4814, and *A. hypoxylon* B4728 had **2**, but none of the lolines.

Genomes of several symbionts were sequenced to identify and characterize all *LOL*-cluster genes in each (Fig. 4). Strains from plants with **2a**, but without lolines, had mutated *lolO* but apparently functional genes *lolF, lolC, lolD, lolA, lolU, lolT* and *lolE* in the *LOL* clusters. The genome sequences, as well as sequences of PCR products from additional strains, indicated that each strain that produced several lolines or only **5** had intact *lolO* genes, whereas those producing only **2a** all had mutant *lolO* genes (Fig. 5). For example, the *lolO* gene of *E. brachyelytri* E4804 had a frame-shift mutation in the first exon and a deletion that extended into the second exon. Likewise, in *A. hypoxylon* B4728, *lolO* had a large deletion extending through the first exon and part of the second, and a frame-shift mutation in the second exon. The observation that defective *lolO* genes in four strains differed in positions of insertions and deletions implied independent origins of *lolO*-inactivating mutations associated with the accumulation of **2a** and absence of lolines.

Heterologous expression of wild-type lolO complemented a natural lolO mutant

A genetic complementation experiment was conducted to test the hypothesis that *lolO* mutations caused the loline alkaloid pathway to terminate at **2a**. *Epichloë canadensis* e4814, possessing a mutated *lolO*, was transformed with pKAES309, which contains wild-type *lolO* with its own promoter cloned from *E. festucae* E2368. Three independent transformants were obtained and introduced back into endophyte-free *El. canadensis* plants. Expression of *lolO in symbio* was checked by RT-PCR with primers targeting the region that included the deletion in e4814. Sequences of the RT-PCR products indicated that the pKAES309 transformants transcribed both the endogenous mutated *lolO* and the introduced wild-type *lolO*, whereas only the mutated *lolO* was transcribed in the wild-type and vector-only controls. Furthermore, plants

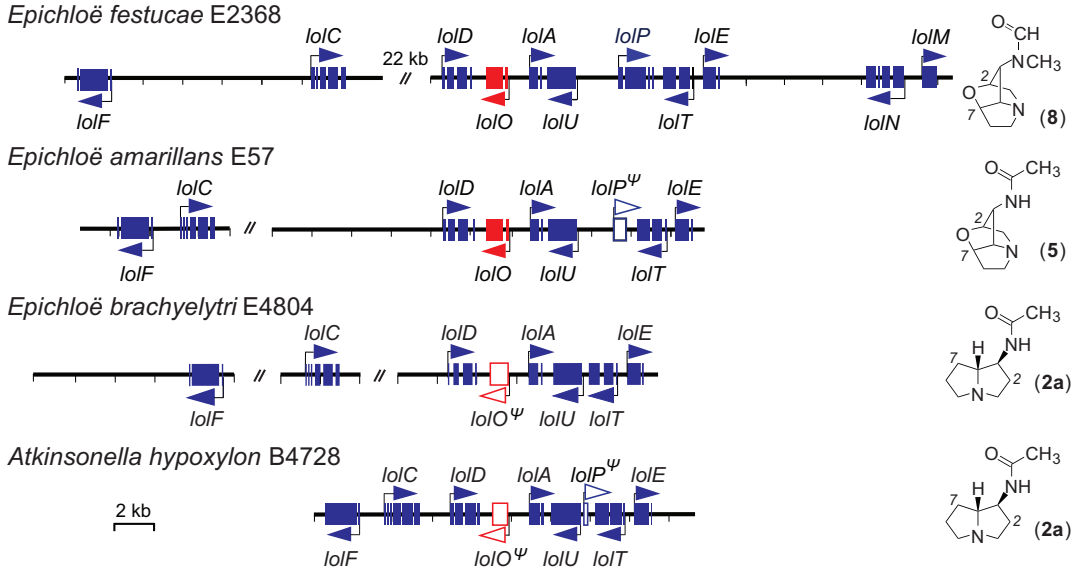

Fig. 4. Comparison of *LOL* clusters in four fungal species with different loline alkaloid profiles. The *LOL* genes are drawn to scale, with filled boxes representing the exons, and gaps between boxes representing introns. Arrows indicate directions of transcription. Empty boxes indicate pseudogenes, and *lolO* is depicted in red. The chemical structure shown beside each cluster indicates the pathway end product found in each strain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

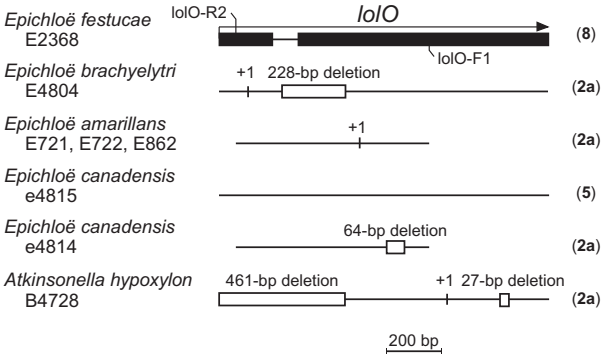

Fig. 5. Schematic representation of *lolO* from species that differ in alkaloid profiles. The coding region of *lolO* from *E. festucae* E2368 is represented by filled boxes. Binding positions of the primers used for amplification of *lolO* fragments (for E721, E722, E862 and e4814) are indicated as lolO-F1 and lolO-R2. Sequence coverage of the *lolO* gene in each strain is indicated by a black bar. Sequence variations are shown as +1 to indicate a frameshift, and open boxes as deletions with the size indicated above each box. The compound listed to the right of each map indicates the pathway end product detected in plants infected with the respective strain.

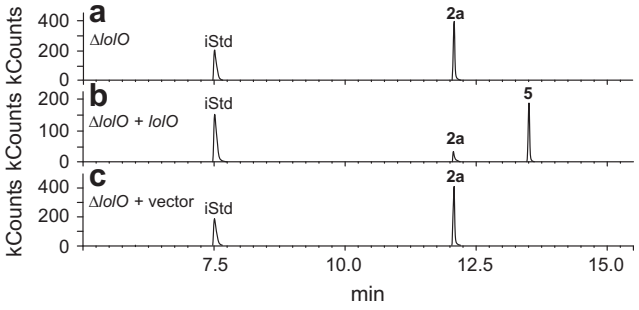

Fig. 6. Complementation of *E. canadensis* e4814 with wild-type *lolO*. Shown are GC–MS total ion chromatograms of loline alkaloids from the host grass *El. canadensis*, when symbiotic with: (a) e4814, which has a mutated *lolO* (Δ*lolO*); (b) e4814 transformed with a copy of wild-type *lolO*; (c) e4814 transformed with the vector without *lolO*.

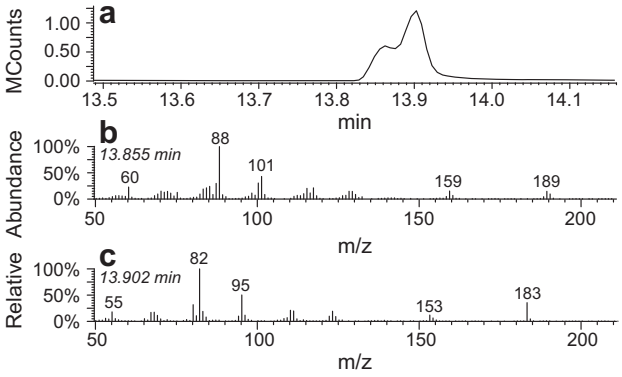

Fig. 8. GC–MS total ion chromatogram and mass spectrum of **8** extracted from L-[U-$^2H_7$]Pro feeding. The enrichment of deuterated **8** is observed as a shoulder peak separated from its non-deuterated form. (a) Chromatogram of **8** extracted from L-[U-$^2H_7$]Pro feeding. (b) Mass spectrum of the peak at 13.905 min. (c) Mass spectrum of the peak at 13.855 min.

inoculated with pKAES309 transformants showed accumulation of both **2a** and **5** (Fig. 6), supporting the hypothesized role of LolO.

Labeling from L-[U-$^2H_7$]Pro showed retention of the deuterium atoms in lolines

In order to determine if exchange of hydrogen on the Pro-derived ring occurs during loline alkaloid biosynthesis, L-[U-$^2H_7$]Pro was applied to *El. canadensis* plant 4814. Loline alkaloids were extracted and analyzed by GC–MS. Heptadeuterated **2a** was observed at the front edge of the GC peak of **2a** (Fig. 7). A +7 *m*/*z* enrichment was observed in the parent ion (*m*/*z* = 169–176), and in the major fragment ion, which contains the Pro-derived ring (*m*/*z* = 83–90). Similarly, L-[U-$^2H_7$]Pro was applied to *E. uncinata* e167 cultures, and loline alkaloids were analyzed by GC–MS. Extracts from the treated culture (Fig. 8), but not from culture lacking labeled Pro (not shown), gave a GC peak adjacent to the peak corresponding to **8**. This novel peak was determined by MS to contain mainly hexadeuterated **8**. A +6 *m*/*z* enrichment was observed in the parent

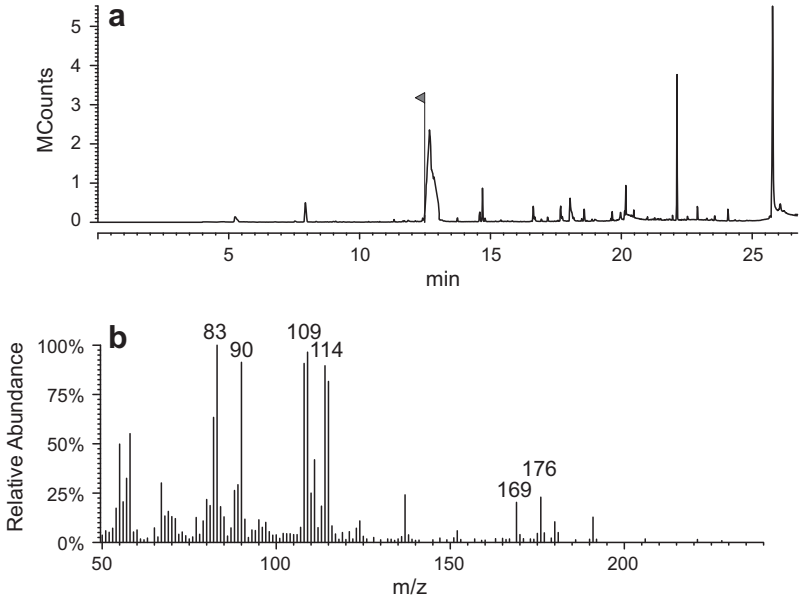

Fig. 7. Enrichment of deuterated **2a** from application of L-[U-$^2H_7$]Pro. Shown are mass spectrum (a) and GC–MS total ion chromatogram (b) of compound **2a** peak front edge (flagged).

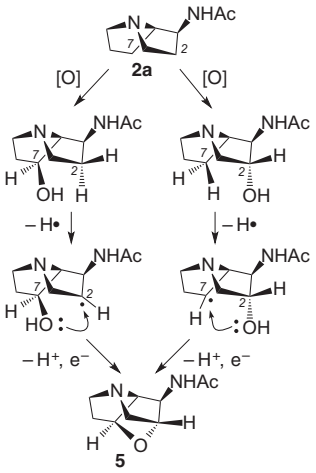

Fig. 9. A possible pathway for ether bridge formation in loline alkaloid biosynthesis. Other possibilities can also be envisioned.

ion (m/z = 183–189), its likely deformylated derivative (m/z = 153–159), the major fragment ion (m/z = 82–88), and other fragment ions predicted to contain all carbon atoms from the proline ring (Schardl et al., 2007). These results imply that the loline alkaloid biosynthetic pathway, including ether bridge formation, never involves desaturation or epoxide formation of the Pro-derived ring.

Discussion

Loline alkaloids produced by endophytic fungi protect host grasses by affecting a large range of insects, so elucidation of their biosynthetic pathway aids the application of lolines in insect control in forage grasses. Our evidence supports the hypothesis that LolO is required for ether bridge formation in the biosynthesis of loline alkaloids. The lack of a functional *lolO* gene consistently correlated with accumulation of **2a** and the apparent absence of any loline alkaloids. Independent mutations in *lolO* were evident in *E. brachyelytri* E4804, *E. canadensis* e4814, *E. amarillans* E722, E721 and E862, and *A. hypoxylon* B4728, all of which accumulated **2a**. In the *lolO* RNAi experiment, **2a** consistently accumulated to high levels relative to the lolines. Furthermore, a copy of wild-type *lolO* complemented the natural *lolO* mutation in e4814, giving substantial levels of **5**. All of these findings indicate that LolO, and possibly additional enzymes (discussed later), catalyze ether formation.

Lolines have an unusual ether linkage of two bridgehead carbons. Previous finding that **1a** forms prior to the ether bridge (Faulkner et al., 2006), along with the current observation that six deuterium atoms from L-[U-$^2H_7$]Pro are retained in lolines, eliminates several possible mechanisms commonly responsible for biological ether formation, including addition to double bonds and opening of epoxides (Dominguez de Maria et al., 2010), suggesting an unusual mechanism for loline ether formation.

We propose a possible mechanism of ether bridge formation in lolines, involving hydroxylation of one of the bridgehead carbons, abstraction of a hydrogen atom from the other, and ether bridge closure (Fig. 9), although other mechanisms are possible. Of the other three oxidative enzymes encoded in the *LOL* cluster, a gene knockout demonstrated that LolP is not required for this step (Spiering et al., 2008), results of a similar test suggest that LolE may not be required (J.P., unpublished data), and LolF, which is predicted to be an FAD-containing monooxygenase, probably does not provide sufficient oxidative potential to catalyze oxidation at C2 or C7. A possible function of LolF is oxidative decarboxylation of the proline-derived ring to give an iminium ion intermediate (Faulkner et al., 2006; Zhang et al., 2009), but so far we cannot rule out the possibility of its involvement in conjunction with LolO. Alternatively, LolO could be the only enzyme required to form the ether linkage. An example of a single enzyme that catalyzes several oxidizing steps in a pathway is clavaminic acid synthase, which is also involved in generating an ether linkage (Zhang et al., 2000).

Since *lolO* RNAi and *lolO* mutant strains all showed accumulation of **2a**, it is likely that **2a** is the first substrate for the series of steps involved in ether bridge formation, and that LolO catalyzes

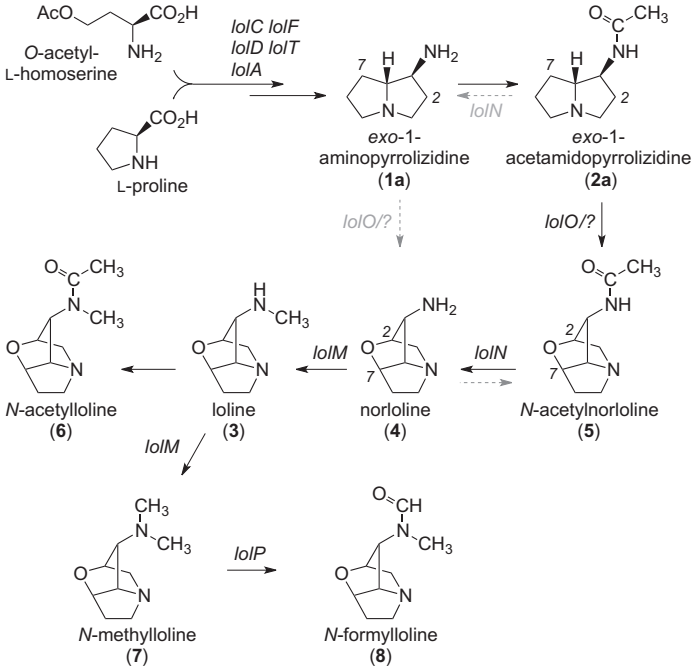

Fig. 10. Proposed loline alkaloid biosynthetic pathway. Compounds shown are the known precursors, proposed intermediates, and known loline alkaloids produced *in symbio*. *LOL* genes with predicted functions are shown with arrows at hypothesized steps. LolO, possibly with another enzyme so far uncharacterized, is proposed to catalyze ether-bridge formation. Gray dashed arrows indicate an alternative hypothesis discussed in the text.

Table 1
Oligonucleotides used in this study.

Primer name	Sequence[a]
lolOrnaiF1	GCGATATCATGACGGTAACAAACAAGCCTG
lolOrnaiR1	CCTCTAGAAATGCAGCCAGGCGAATGCTTACCTCGAGCG
lolOrnaiF2	ATTTCTAGAGGCACACAAGATCAATTAGCGATCC
lolOrnaiR2	ACTACTAGTATGACGGTAACAAACAAGCCTG
lolAkoupf	GCGCGGCCGCGAGCTAACCATGCATGGTGT
lolAkoupr	GCTCTAGATTCATCGAGCATCGTTAGAATA
lolOs1	ACCTGCCTCTGGCGGTCAAG
lolOr	CTTGCGCTCATACTCAAGAGC
lolO cDNA 3-5	CTCCGCCATCTGCCGTTG
lolO-F1	GTGAACTGGCAGTAGTCCGTATG
lolO-R2	AATCCATGCCAGTGTCGGGAATG

[a] Underlined segments indicate restriction-endonuclease cleavage sites incoporated in the primers to facilitate cloning.

at least the first step. The *LOL* gene clusters of strains that produce **5** (E57 and e4815), and those that accumulate **2a** (e4814, E722, E4804, and B4728), have the same complement of potentially functional genes in the *LOL* cluster, with the exception of the mutated *lolO* genes in the latter. Hence, it is reasonable to hypothesize that **2a** is the intermediate that is converted to the first loline alkaloid, **5**. Considering the previous evidence that **1a** is a pathway intermediate, predict that **2a** is generated by acetylation of **1a** (Fig. 10).

Based on the observation that the lolines **3**, **4**, **5**, **7**, and **8** are found in many grasses, and the predicted functions of *LOL* genes in the cluster, we hypothesize that, after formation of **2a**, the loline biosynthetic pathway proceeds through deacetylation of **5** to form **4** by LolN, the predicted acetamidase (Fig. 10). Serial methylations of **4** by LolM, the predicted methyltransferase, then produce **3** and **7**, the latter of which is oxidized to **8** by the cytochrome P450 enzyme, LolP (Spiering et al., 2008). We are also considering an alternative but more complex hypothesis, namely, that **1a** is the actual substrate on which the ether is formed, whereas **2a** is a side product. If that were the case, the likely role of LolN would be to deacetylate **1a** to **2a**, and **4** would be the first loline (Fig. 10). However, in this alternative pathway, LolN would appear to be either unnecessary or involved in a futile cycle between **4** and **5**. Hence, it is more plausible that **2a** is the actual intermediate in loline biosynthesis that is oxidized to **5**. We plan further experiments to test this hypothesis.

Neither alkaloid **2a** nor **2b** has been previously reported. We were unable to establish the relative configuration (exo, **2a**, or endo, **2b**) of isolated **2** by analysis of its NOESY spectrum, so we prepared synthetic (±)-**2a** as a comparison material. The NMR spectra (^{1}H, ^{13}C, COSY, NOESY, and HSQC) and GC–MS of the synthetic (±)-**2a** and the isolated **2** were very similar, and the GC and the ^{1}H and ^{13}C NMR spectra of an equimolar mixture of synthetic and isolated material (Supplementary data) showed only a single compound, establishing with high confidence that the isolated alkaloid was also **2a**. This assignment was consistent with both the known configuration of the loline alkaloids and our previous work showing that **1a** was an intermediate in the biosynthetic pathway of lolines (Faulkner et al., 2006).

The lolines are comparable to nicotine in insecticidal activity (Riedell et al., 1991), so they are probably a major factor in the protective effects of certain endophytes. For example, the loline-producing endophytes, *Epichloë occultans* (C.D. Moon, B. Scott & M.J. Chr.) Schardl in *L. multiflorum* and *E. coenophiala* in tall fescue, have documented activity on herbivorous insects as well as their parasitoids, playing an important role in arthropod diversity and food-web structures (Omacini et al., 2001; Rudgers and Clay, 2008). Notably, different lolines vary in specificity and impact on different insects (Jensen et al., 2009; Popay et al., 2009; Riedell et al., 1991). Considering that **2a** is the pathway end-product that originated independently in several natural species of grass endophytes, it is possible that this alkaloid is specifically selected in some environments, perhaps by variation of the dominant herbivore species. Hence, the biological activity of **2a** merits further study.

Concluding remarks

Through genome sequencing and molecular genetic methods, LolO, a non-heme iron oxygenase, was revealed to be required for loline ether bridge formation. The mechanism of the reaction is not yet determined, but the finding that six deuterium atoms from L-[U-$^2H_7$]Pro were retained in **8**, together with the finding that the pyrrolizidine rings form before the ether bridge, rules out several possible common mechanisms, such as epoxide formation and opening. Hence, it is likely that formation of the ether bridge of lolines occurs through an unusual route, possibly through a free radical at C2 or C7. A novel natural product described here, *exo*-1-acetamidopyrrolizidine (**2a**), is a likely pathway intermediate that is oxidized at C2 and C7 to form the ether bridge, giving rise to **5**, the first loline alkaloid in the pathway.

Experimental

General experimental procedures

GC–MS was conducted using a Varian CP-3800 GC (Agilent Technologies, Santa Clara, CA, USA) and a Varian Saturn 2200 MS (Agilent Technologies). The GC was equipped with an Agilent J&W DB-5MS capillary column (30 m, 0.25 mm i.d., 0.25 μm thickness). Helium at 1 ml/min was used as the carrier gas with an injection temperature of 250 °C. Column temperature was initially 75 °C, increased to 225 °C at 8 °C/min, then increased to 300 °C at 25 °C/min, and held for 5 min for a total run time of 27 min. For GC–MS detection, an electron ionization system was used with

Table 2
Origins and source information for fungal strains used in this study.

Fungus	Isolates	Host	Origin
Atkinsonella hypoxylon	B4728	*Danthonia spicata*	Lexington, North Carolina, USA
Epichloë amarillans	E57	*Agrostis hyemalis*	Brazoria Co., Texas, USA
Epichloë amarillans	E721, E722, E862	*Sphenopholis obtusata*	Georgia, USA
Epichloë brachyelytri	E4804	*Brachyelytrum erectum*	Edmonson Co., Kentucky, USA
Epichloë canadensis	e4814[a]	*Elymus canadensis*	Nuevo León State, Mexico
Epichloë canadensis	e4815[a]	*Elymus canadensis*	Throckmorton Co., Texas, USA
Epichloë coenophiala	e19	*Lolium arundinaceum*	Lexington, Kentucky, USA
Epichloë festucae	E2368	*Lolium pratense*	Lexington, Kentucky, USA
Epichloë uncinata	e167	*Lolium pratense*	Nyon, Switzerland

[a] Strains e4815 and e4814 are the equivalent of CWR 5 and CWR 34/36 in Charlton et al. (2012).

ionization energy of 70 eV. Mass range was set at 50–250 *m*/*z* with a filament delay of 4 min.

Fourier transform ion cyclotron resonance (FTICR) mass spectrometry was performed using a Thermo Electron LTQ-FT with Advion Nanomate nanoelectrospray source operating in the positive ion mode. Fragmentation in the ICR cell used infrared multiphoton dissociation (IRMPD) with 80 msec pulse following a 2 msec delay. Spectra were acquired using a resolving power of 100,000 @ 400 *m*/*z*, resulting in resolution of much better than 1 ppm across the measured *m*/*z* range. The instrument was externally calibrated to protonated reserpine.

DNA cloning was carried out by standard methods, and plasmids were grown in *Escherichia coli* XL1-Blue (Agilent Technologies). All plasmid DNA was isolated from bacterial cultures (LB medium at 37 °C for 20 h on a rotary shaker at 200 rpm) with ZR Plasmid Miniprep-Classic kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. Fungal genomic DNA was isolated with ZR Fungal/Bacterial DNA MiniPrep (Zymo Research) following the manufacturer's instructions. Gene screenings by polymerase chain reaction (PCR) were conducted with AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) in manufacturer-provided PCR buffer with 1.5 mM $MgCl_2$. For gene cloning, PCR was performed with Phusion Hot Start High-Fidelity DNA Polymerase (Thermo Scientific, Ratastie, Vantaa, Finland) with provided HF buffer with 1.5 mM $MgCl_2$. All oligonucleotide primers were ordered from Integrated DNA Technologies (Coralville, Iowa, USA), and are listed in Table 1.

Biological materials

Fungal isolates were cultured from infected plants as previously described (Blankenship et al., 2001), and are listed in Table 2. The strain designations refer to the plant numbers from which they were isolated, with prefix of "E" and "e" for sexual and asexual *Epichloë* species, respectively, and "B" for *Atkinsonella hypoxylon* (*Balansia hypoxylon* Diehl).

Loline alkaloid analysis

Grass materials used in this study were all clipped from the crown, lyophilized and ground. For GC–MS analysis, ground plant material (100 mg) was extracted with NaOH (100 µl, 1 M) and loline-alkaloid extraction solution (1 ml, 99% $CHCl_3$ with 1% quinoline as internal standard). The mixture was agitated for 1 h at room temperature, then centrifuged at $16 \times 10^3 g$ for 10–15 min. The organic phase was transferred to a capped glass vial for GC–MS analysis as previously described (Faulkner et al., 2006).

Loline alkaloid production by *E. uncinata* e167 and the transformed strains was induced in minimum medium (MM) as described in (Blankenship et al., 2001; Chung and Schardl, 1997), with 15 mM urea as the nitrogen source and 20 mM sucrose as the carbon source. For isotopic labeling, 4 mM L-[U-$^2H_7$]Pro was included in the medium. Fungal isolates were ground in sterile water and inoculated to 30 ml of MM in 100 × 25 mm polystyrene Petri dishes. After 13–20 days shaking at 21 °C, the medium was sampled for loline alkaloids analysis by GC–MS, as previously described (Blankenship et al., 2005).

Plasmid construction

For lolO RNAi, a 200 bp fragment was amplified from *E. uncinata* e167 using primers lolOrnaiF1 and lolOrnaiR1 (95 °C for 5 min, 35 cycles of 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 1 min). The PCR product was purified and digested with *Eco*RV and *Xba*I, for which sites were incorporated into the primers. Similarly, a 280 bp fragment of *lolO* was amplified from *E. uncinata* e167 by PCR with primers lolOrnaiF2 and lolOrnaiR2 (95 °C for 5 min, 35 cycles of 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 1 min), purified and digested with *Spe*I and *Xba*I. Plasmid pKAES215 was digested with *Spe*I and *Eco*RV. The 4.8 kb fragments of the digested plasmid and digested PCR products were gel purified by QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). The three fragments of sizes 0.2, 0.28, and 4.8 kb were mixed in a molar ratio of 2:2:1 and ligated using the Fast-Link DNA ligation kit (Epicentre, Madison, WI, USA). The resulting plasmid, designated pKAES226, contained the *TOXA* promoter from *Pyrenophora tritici-repentis* (Died.) Drechsler (Andrie et al., 2005) driving the first exon and intron of *lolO*, followed by the reverse complement of the first exon of *lolO*.

For *lolO* complementation, a DNA segment including *lolO* and the entire flanking intergenic regions was amplified from DNA of *E. festucae* E2368, by PCR with primers lolAkoupf and lolAkoupr (98 °C for 5 min, 35 cycles of 98 °C for 30 s, 57 °C for 30 s, and 72 °C for 2 min). The PCR product was purified and digested with *Xba*I, the site for which was incorporated in the primer lolAkoupr. Plasmid pKAES215 was digested with *Xba*I and *Sma*I. The two fragments were then gel purified and ligated to produce plasmid pKAES309, which has *lolO* of *E. festucae* E2368 under its own promoter.

Fungal transformation

Epichloë canadensis e4814 was grown in potato dextrose broth (50 ml) for 5 days at 22 °C with rotary shaking (200 rpm). Fungal mycelium was harvested by centrifugation at 4885*g*, resuspended in 3.75 mg/ml vinoflow (Novozymes, Bagsvaerd, Denmark), 5 mg/ml driselase (InterSpex products, San Mateo, CA), 0.7 mg/ml zymicase I (InterSpex Products), 5 mg/ml lysing enzymes (Sigma, St. Louis, MO), and 2.5 mg/ml bovine serum albumin (Sigma) in osmotic solution (1.2 M $MgSO_4$, 50 mM sodium citrate, pH 6.0), and gently agitated 3 h at 30 °C. Protoplast isolation and electroporation were performed as described by Tsai et al. (1992).

After electroporation, the protoplasts were mixed with regeneration medium (5 ml) (Panaccione et al., 2001) and plated onto potato dextrose agar (PDA) (20 ml) with 125 µg/ml hygromycin B, so the final concentration of hygromycin B over the whole plate (25 ml) was 100 µg/ml. Plates were kept at 22 °C and checked after ca. 2 wk. Fungal colonies were transferred to PDA plates with 100 µg/ml hygromycin B and then single-spore isolated two times before further analysis.

Transformants were introduced into host plants by the method of Latch and Christensen (1985), by inoculation of seedlings from endophyte-free seed lots. When the plants had at least three tillers, a tiller from each was sacrificed for tissue-print immunoblot as previously described (An et al., 1993).

*Purification of exo-1-acetamidopyrrolizidine (**2a**)*

Approximately 10 g lyophilized *El. canadensis* plant 4814 tillers were used to extract **2a** with the loline extraction method described in part 5.3. The extract was subjected twice to silica gel chromatography, using 1 mm thick silica gel prep. TLC plates (EMD Chemicals, Darmstadt, Germany) (Blankenship et al., 2005). Purified **2a** was recovered in $CDCl_3$ and analyzed by NMR spectroscopy. Compound **2a** was named *N*-[(1*S*,7a*R*)-hexahydro-1*H*-pyrrolizin-1-yl]acetamide according to IUPAC nomenclature and was given the common name, *exo*-1-acetamidopyrrolizidine. Purified **2a** showed ^1H NMR (600 MHz, $CDCl_3$, 0.10 M): δ 1.59 (dq, J_d = 12.6 Hz, J_q = 7.4 Hz, 1H), 1.72 (dq, J_d = 13.8 Hz, J_q = 7.1 Hz, 1H), 1.73 (dq, J_d = 13.8 Hz, J_q = 7.1 Hz, 1H), 1.83 (m, 1H), 1.96 (s, 3H), 1.95–2.01 (m, 1H), 2.17 (dq, J_d = 12.5 Hz, J_q = 6.2 Hz, 1H), 2.61 (dq, J_d = 11.0 Hz, J_q = 6.7 Hz, 2H), 3.03 (dt, J_d = 10.6 Hz, J_t = 6.4 Hz, 1H), 3.23 (dt, J_d = 11.0 Hz, J_t = 6.0 Hz, 1H), 3.27 (∼q, ∼6.4 Hz, 1H),

4.12 (~quintet, 6.5 Hz, 1H), 6.10 (bs, 1H). $^{13}C\{H\}$ NMR (100 MHz, $CDCl_3$, 0.10 M): δ 23.6, 25.5, 30.7, 32.7, 53.3, 55.1, 55.3, 70.8, 170.2. EI-MS (70 eV) *m*/*z* (rel. int.) M^+ 169 (12.5), 109 (77.5), 108 (100), 83 (97), 82 (52.5), 55 (42.5).

*Synthesis of (±)-exo-1-acetamidopyrrolizidine ((±)-**2a**)*

Raney Ni (excess) was added to a solution of (±)-1-oximinopyrrolizidine (292 mg, 2.08 mmol) (Christine et al., 2000) in THF (75 mL). The mixture was stirred for 13 h at rt. Ac_2O (0.196 ml, 2.08 mmol) was added to the mixture, which was stirred at rt for a further 2 h. It was then filtered through Celite. The filtrate was diluted with saturated aq. $NaHCO_3$ and brine. The aqueous layer was brought to pH 12 with 1 M NaOH, and $CHCl_3$ was added. The phases were allowed to separate for 30 min at RT. The aqueous layer was extracted with $CHCl_3$ twice, and the combined organic layers were dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash chromatography (silica gel, CH_2Cl_2/MeOH/NH_4OH, 6:4:1.5, R_f 0.25), affording (±)-**2a** (29 mg, 0.17 mmol, 8% yield) as a white solid, mp 72–74 °C. ^1H NMR (400 MHz, $CDCl_3$, 0.17 M): δ 1.63 (dq, J_d = 12.4 Hz, J_q = 7.0 Hz, 1H), 1.75 (m, 2H), 1.83 (m, 1H), 1.93 (s, 3H), 1.99 (dq, J_d = 12.9 Hz, J_q = 7.0 Hz, 1H), 2.16 (dq, J_d = 12.6 Hz, J_q = 6.5 Hz, 1H), 2.62 (dq, J_d = 11.0 Hz, J_q = 7.0 Hz, 2H), 3.07 (dt, J_d = 10.8 Hz, J_t = 6.5 Hz, 1H), 3.29 (dt, J_d = 10.7 Hz, J_t = 6.5 Hz, 1H), 3.35 (~q, 6.5 Hz, 1H), 4.12 (~quintet, 6.5 Hz, 1H), 6.64 (bd, 5.9 Hz, 1H). $^{13}C\{H\}$ NMR (100 MHz, $CDCl_3$, 0.17 M): δ 23.6, 25.6, 30.8, 33.0, 53.5, 55.4, 55.5, 71.0, 170.1. IR (ATR): 3272, 1649, 1554. HRMS: *m*/*z* calcd for $C_9H_{17}N_2O$ (M+H): 169.1341; found: 169.1336.

Genome sequencing

All genome sequencing and assembly was conducted at the University of Kentucky Advanced Genetic Technologies Center. Genome assemblies of *E. festucae* E2368, *E. amarillans* E57, and *E. brachyelytri* E4804 were described previously (Schardl et al., 2013). The genome of *A. hypoxylon* B4728 was sequenced on a Roche/454 Titanium pyrosequencer configured for extended reads (average read length 722 nt; 32-fold coverage). Assembly was as described previously (Schardl et al., 2013). Assemblies were uploaded to the National Center for Biotechnology Information (NCBI) (Bioproject identifiers: PRJNA42133 for E2368, PRJNA67245 for E4804, and PRJNA67301 for E57, and PRJNA221544 for B4728), and are provided with annotations on GBrowse web sites (www.endophyte.uky.edu). Loline gene sequences are deposited in NCBI (GenBank ID: JF830812, JF830813, JF800659, JF800661, JF800660, JF830815, JF830814, JF830816, and KF056806).

Analysis of lolO gene

DNA was purified from endophytes and a fragment of *lolO* was amplified by PCR with primer pairs: lolO-F1 and lolO-R2 (Table 2). PCR was performed at 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min. The products were sequenced with the PCR primers.

RNA extraction and RT-PCR

RNA from plant materials were extracted with RNeasy Plant Minikit (Qiagen), removed contaminating DNA using DNA-free™ kit (Applied Biosystems), and reverse transcribed with high-capacity cDNA Reverse Transcription kit (Applied Biosystems). The resulting cDNA was used as templates to amplify a *lolO* fragment by PCR with primers lolOs1 and lolOr. The PCR products were then sequenced with primers lolOs1 and lolO cDNA 3-5.

Application of L-[U-$^2H_7$]Pro to plants

A single tiller of *El. canadensis* plant 4814, cut above the first node, was placed in a test tube with Murashige and Skoog Medium (MS medium) (600 µl) (MP Biomedicals, Solon, OH, USA), pH 7.4 with 4 mM L-[U-$^2H_7$]Pro in a 1.7 ml microcentrifuge tube, and maintained at 25 °C, 16 h light, until all the medium was consumed. A total of 10 tillers were used for both L-[U-$^2H_7$]Pro and control (Pro) feeding. The tillers from each experiment were pooled at the end of the feeding period and checked for loline alkaloids following the standard procedure described in Section 'Loine alkaloid analysis'.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2013.11.015.

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