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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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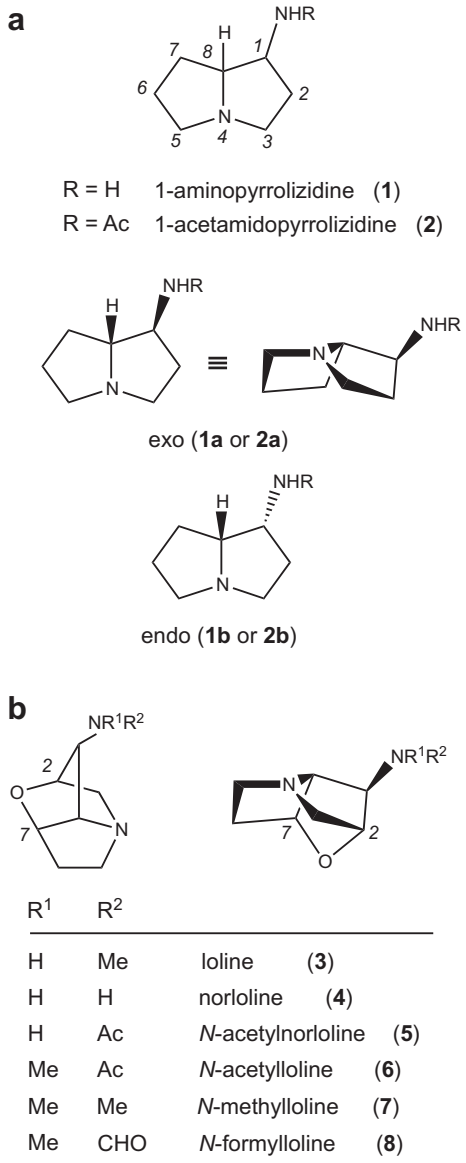

Fig. 1. Perspective illustrations of loline alkaloids. (a) 1-Aminopyrrolizidines. (b) Loline alkaloids, which possess a heterotricyclic core including an ether bridge linking C2 and C7. Substitutions on the nitrogen at C1 differentiate the lolines.

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

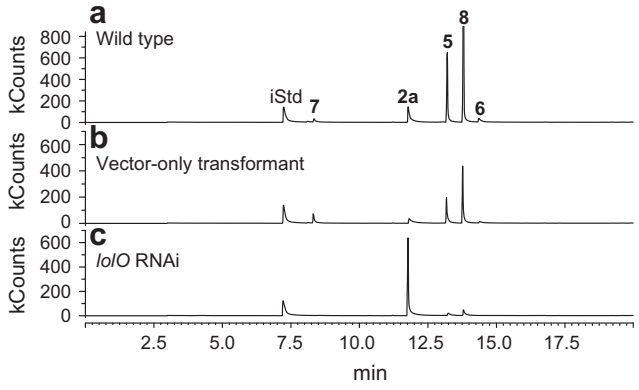

Fig. 2. GC–MS total ion traces of *E. uncinata* RNAi transformant and controls showing loline-alkaloid profiles from 25 day-old cultures. Bold numbers indicate the peaks expected for compounds listed in Fig. 1. The internal standard (iStd), quinoline, was used for quantification. (a and b) Chromatograms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