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# Biochemical Outcome of Blocking the Ergot Alkaloid Pathway of a Grass Endophyte

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*Neotyphodium* sp. Lp1, an endophytic fungus from perennial ryegrass (*Lolium perenne*), produces the mycotoxin ergovaline in infected grasses, whereas a mutant in which a particular peptide synthetase gene is knocked out does not. We examined the impact of this knockout on other constituents of the ergot alkaloid pathway. Two simple lysergic acid amides, ergine and a previously undescribed amide, were eliminated by the knockout. Lysergic acid accumulated in the knockout endophyte, but quantities were only 13% of the total lysergic acid derivatives accumulated in the wild type. Concentrations of several clavines were not substantially affected. However, a novel clavine accumulated to higher concentrations in perennial ryegrass containing the knockout strain. The results indicate that production of simple lysergic acid amides requires the activity or products of the ergovaline-associated peptide synthetase and that the regulation of ergot alkaloid production is modified in response to the relatively late block in the pathway.

**KEYWORDS:** ***Neotyphodium*; endophyte; mycotoxins; ergot alkaloids; clavines; ergovaline**

## INTRODUCTION

*Neotyphodium* spp. grow as mutualistic endophytes within several important forage and turf grasses. These seed-transmitted, endophytic fungi confer numerous agronomic benefits upon their hosts, including increased vigor and reproduction and greater tolerance to biotic and abiotic stresses (*1*−*3*). However, endophytes often are associated with livestock toxicosis (*4*−*7*). Collectively, *Neotyphodium* endophytes produce up to four classes of bioactive alkaloids that are hypothesized to contribute, to varying degrees, to the mammalian toxicity of the endophyte−grass associations and/or to the various agronomic benefits conferred upon the grasses in which they live (*8*−*10*). The four classes are the ergot alkaloids, the lolines, the lolitrems, and peramine. Most endophytes produce some subset of these four classes. For example, *Neotyphodium coenophialum* (Morgan-Jones and Gams) Glenn, Bacon, and Hanlin associated with tall fescue (*Festuca arundinacea* Schreb.) typically produces ergot alkaloids, lolines, and peramine, whereas *Neotyphodium lolii* (Latch, Christensen, and Samuels) Glenn, Bacon, and Hanlin associated with perennial ryegrass (*Lolium perenne* L.) typically produces lolitrems along with peramine and the ergot alkaloid ergovaline. *Neotyphodium* sp. Lp1, the subject of this current paper, is noteworthy among perennial ryegrass endophytes for its lack of lolitrem production, whereas it produces significant amounts of ergovaline and other ergot alkaloids, as well as peramine (*8*, *11*).

Although ergovaline, a peptide derivative of lysergic acid, is abundantly produced and frequently studied in *Neotyphodium*−grass interactions, additional, simpler ergot alkaloids also have been detected in endophyte−grass associations. These include ergine, the simple amide of lysergic acid, and several precursors to lysergic acid and derivatives of these precursors, collectively referred to as clavines (*8*, *10*, *12*). Structures of these alkaloids and a pathway to the synthesis of lysergic acid and, ultimately, ergopeptines are summarized in **Figure 1A** together with additional branches and compounds described below. The lysergic acid derivatives, and some of the clavines, occur as epimeric pairs differing in configuration at C-8 (8-epimers) that spontaneously equilibrate in hydroxylic solvents (**Figure 1B**). The α-stereoisomers, given the prefix "iso-" or the suffix "-inine", are readily separable from the *β*-epimers by chromatography (*12*−*14*) and typically are pharmacologically inactive (*15*).

Ergovaline is an ergopeptine composed of D-lysergic acid linked via an amide bond to a three-membered peptide derived from L-alanine, L-valine, and L-proline (**Figure 1A**). Numerous ergopeptines, in addition to ergovaline, have been described from the related ergot fungus *Claviceps purpurea* (Fr.:Fr.)Tul. (*12*). The assembly of ergopeptines is catalyzed by a multifunctional peptide synthetase complex named lysergyl peptide synthetase (*16*, *17*). Unlike other eukaryotic peptide synthetases, in which all activities are encoded on a single polypeptide,

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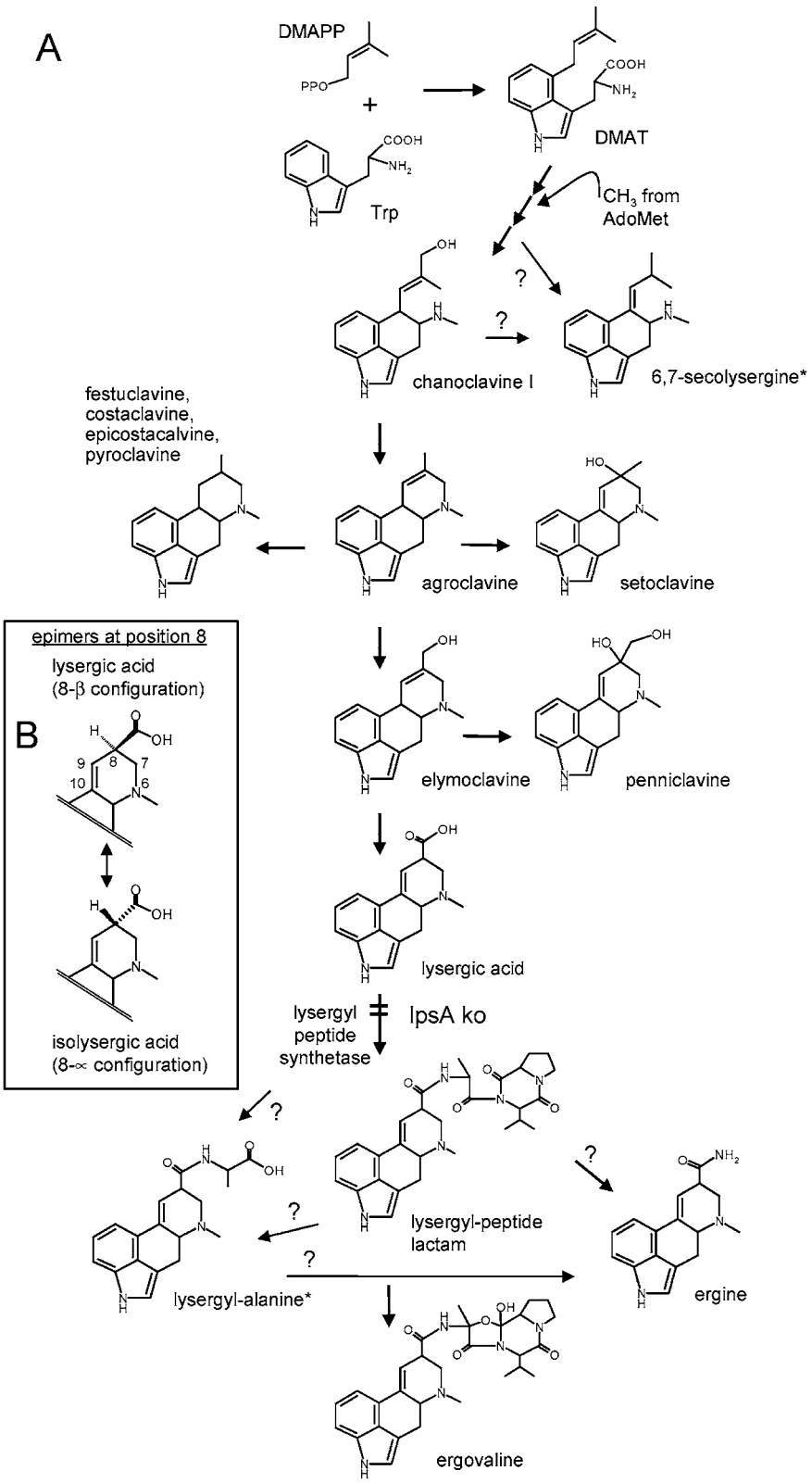


**Figure 1.** Ergot alkaloid pathway. (A) Structures and common names of key intermediates and products of the ergot alkaloid pathway and associated spurs. Provisional structures (described in the text) are marked with an asterisk (*). Abbreviations: DMAPP = dimethylallylpyrophosphate; Trp = tryptophan; DMAT = dimethylallyltryptophan; AdoMet = S-adenosyl-methionine; lpsA ko = step at which the pathway that has been knocked out in that particular mutant. Question marks indicate uncertain relationships between intermediates and/or products discussed in the text. Additional pathway spurs or modifications of listed alkaloids have been omitted in cases where the products were absent or were minor components of the endophyte ergot alkaloid profiles. (B) Carbon numbering and stereoisomerization. Conventional numbering of carbons in the ergoline ring system is shown only for positions mentioned in the text. The alternate configurations of epimers at position 8 is indicated for lysergic acid.

lysergyl peptide synthetase is made up of two separate polypeptides. Lysergyl peptide synthetase 2 (LPS2) activates lysergic acid by adenylation and then binds it covalently as a thioester to enzyme-bound 4′-phosphopantethenate, prior to its transfer

to lysergyl peptide synthetase 1 (LPS1). LPS1 recognizes the three amino acids of the peptide portion of the ergopeptine, binds them as thioesters via adenylate intermediates, and then assembles the lysergyl peptide. The product of this multifunctional enzyme complex is the lysergyl peptide lactam (**Figure 1A**). The lactam is then oxidized at the α-carbon of the first amino acid in the peptide chain (*18*). This hydroxylated intermediate is thought to spontaneously cyclize to the final cyclol ergopeptine product (*18*, *19*).

Genes encoding LPS1 have been cloned from *C. purpurea* (*20*, *21*) and *Neotyphodium* sp. Lp1 (*21*). The genes contain three characteristic peptide synthetase modules, consistent with the role of this enzyme in attaching three amino acids to lysergic acid. With a long-range goal of assessing the contribution of ergopeptines to mammalian toxicity and to the various agronomically beneficial endophyte-associated traits, we have made a targeted gene knockout in the LPS1-encoding gene *lpsA* in the perennial ryegrass endophyte *Neotyphodium* sp. Lp1 (*21*). Analysis of perennial ryegrass infected with this knockout mutant showed that ergovaline was no longer produced. In this study, we have characterized differences in accumulation of other ergot alkaloids, including two previously undescribed ergot alkaloids, that result from blocking the ergot alkaloid pathway at LPS1.

## MATERIALS AND METHODS

**Materials.** Perennial ryegrass cv. "Rosalin" without endophyte or containing endophyte *Neotyphodium* sp. Lp1 of taxonomic group LpTG-2 (*11*) or a derivative of this endophyte in which the *lpsA* gene had been knocked out (*21*) was cultivated in a glasshouse. Tissue for alkaloid analysis consisted of pseudostems, vegetative tissue cut from near the soil line up to and including the ligular zone and consisting primarily of leaf sheaths of mature leaves, small amounts of stem, and some blade tissue of the youngest, unfurled leaf.

All solvents used for HPLC were HPLC grade; solvents used for other procedures were of HPLC or analytical grade. Authentic samples of chanoclavine, agroclavine, elymoclavine, lysergic acid, and ergine were provided by Dr. M. Flieger (Czech Academy of Sciences). Authentic ergovaline was provided by Dr. F. Smith (Auburn University). Ergotamine tartrate and ergonovine maleate were obtained from Sigma-Aldrich Co. (St. Louis, MO).

**Analysis of Ergot Alkaloids by HPLC with Fluorescence Detection.** Ergot alkaloids were extracted and analyzed by a modification of the method developed by Spiering et al. (*14*). Fifty milligrams of freeze-dried and finely ground pseudostems was extracted in 1.0 mL of 2-propanol−water−lactic acid (50:49:1, v/v/v) containing ergotamine tartrate as internal standard at 1.111 μg/mL. Samples in capped 2 mL tubes were agitated in a Fast-Prep 120 (Bio101, La Jolla, CA) at setting 5 for 20 s and then end for end on a rotary mixer for 1 h. Solid material was pelleted by centrifugation at 6000*g* for 10 min. Twenty microliters of extract was analyzed and quantified by HPLC with fluorescence detection as described by Spiering et al. (*14*), except that injected samples were subjected to a gradient (flow rate of 1 mL/min) in which mobile phases A (5% v/v acetonitrile + 95% v/v aqueous 0.1 M ammonium acetate) and B (75% acetonitrile + 25% aqueous 0.1 M ammonium acetate) were mixed in linear progressions as follows: 0 min, 100% A + 0% B; 25 min, 85% A + 15% B; 45 min, 50% A + 50% B; 50 min, 30% A + 70% B; 55 min, 0% A + 100% B; 60 min, 0% A + 100% B; 65 min, 100% A + 0% B. Data presented for each alkaloid are means of eight samples collected in two different harvests of four plants each of perennial ryegrass cv. "Rosalin" infected separately with the indicated endophyte. Data were analyzed by two-factor ANOVA with replication, to account for the two harvests from the same set of plants. Since there were no significant interactions between harvest date and alkaloid content, data from both harvests were pooled in calculating and comparing means.

**Liquid Chromatography−Electrospray Ionization Mass Spectrometry (LC-MS).** Samples were analyzed on a Shimadzu (Kyoto, Japan) HPLC equipped with dual LC-10AD pumps, a SPD-M10Avp photodiode array detector, and a LCMS QP8000α quadrupole mass spectrometric detector operated in positive electrospray ionization mode. Samples of lysergic acid derivatives were prepared as described above. Samples for analysis of clavines were prepared from 1 g of freeze-dried and milled pseudostem tissue which was extracted in 10 mL of 2-propanol−water−lactic acid (50:49:1, v/v/v) in capped tubes, agitated end for end on a rotary mixer for 1 h, and clarified by centrifugation for 15 min at 6000*g*. The clarified extract was made alkaline by the addition of an equal volume of 1% (w/v) aqueous ammonium carbonate and centrifuged again as described above. The supernatant was loaded on a 500 mg non-end-capped C18 silica solid-phase extraction (SPE) column (International Sorbent Technologies (IST), Midglamorgan, U.K.). Columns were washed in succession with 2 mL each of water, 50% (v/v) aqueous 2-propanol, and 80% (v/v) aqueous 2-propanol, before eluting clavines with 2 mL of 80% (v/v) aqueous 2-propanol with 1% (v/v) glacial acetic acid. Eluted clavines were evaporated to dryness in a centrifugal evaporator (Savant Speed Vac, Holbrook, NY) and resuspended in 100 μL of 2-propanol.

For LC-MS analyses, clavines were separated on a 150 mm × 2 mm Phenomenex Synergi 4 μm Polar-RP column with an acetonitrile gradient prepared by mixing mobile phases A (5% v/v acetonitrile + 95% v/v aqueous 0.1 M ammonium acetate) and B (75% acetonitrile + 25% aqueous 0.1 M ammonium acetate) from initial conditions of 86% A + 14% B to 73% A + 27% B at 25 min, 25% A + 75% B at 40 min, and 0% A + 100% B at 42 min. The column was rinsed with 100% B and then 86% A + 14% B for 4 min each, between injecting samples. The flow rate was 0.2 mL/min. Ions with *m*/*z* values of 239 (such as $[\text{agroclavine} + H]^+$), 241 (proposed $[6,7\text{-secolysergine} + H]^+$), 255 ($[\text{elymoclavine} + H]^+$), and 257 ($[\text{chanoclavine I} + H]^+$ and isomers) were detected with a deflector voltage of 45 V for minimal fragmentation and monitored in the selected ion mode. Peaks corresponding to chanoclavine I, agroclavine, and elymoclavine were determined by comparison to authentic standards.

LC-MS data concerning lysergic acid amides were obtained with the same instrument and column described above but with mobile phases A (5% v/v acetonitrile + 1% v/v aqueous formic acid) and B (75% acetonitrile + 1% aqueous formic acid) mixed as follows: 0 min, 98% A + 2% B; 24 min, 85% A + 15% B; 28 min, 50% A + 50% B; 30 min, 0% A + 100% B; 36 min, 0% A + 100% B; 38 min, 98% A + 2% B; 45 min, 98% A + 2% B. The flow rate was 0.2 mL/min.

To gain additional structural information, samples of the unknown lysergic acid amide and the unknown clavine were chromatographed as described above for each class but with the detector on scanning mode. In some analyses, the deflector voltage was raised to 60 V to induce fragmentation.

**Chemical Modification of Ergot Alkaloids.** Lysergic acid amides that were to be subjected to alkaline hydrolysis were collected as individual peaks from chromatographic gradients. The collected molecules were separated from mobile-phase constituents by diluting with an equal volume of water, binding to a non-end-capped 500 mg C18 silica SPE cartridge (IST), washing with water, and then eluting with methanol. Eluates were concentrated to dryness under vacuum. Residues were resuspended in 100 μL of 1.2 M NaOH and incubated at 75 °C for 6 h to hydrolyze peptide bonds, before neutralizing with 50 μL of 2.4 M acetic acid. Controls were neutralized prior to incubation.

Agroclavine and elymoclavine were oxidized to setoclavine/isosetoclavine and penniclavine/isopenniclavine, respectively, as follows. Individual endophyte-free perennial ryegrass plants, with the leaf blade trimmed to 1 cm above the ligular zone and roots trimmed under water to within 3 mm of the stem, were incubated in 2 mL centrifuge tubes containing 0.5 mL of the clavine (50 μg of clavine/mL of 0.8% v/v aqueous 2-propanol + 0.01% v/v lactic acid) at 20 °C. After uptake, the clavine was chased with 50 μL of distilled water, and afterward tubes were replenished with distilled water as needed for a total incubation of 50 h. Plants were then freeze-dried, milled, and extracted as described above for fluorescent ergot alkaloids.

**Analysis of Peramine and Lolitrems.** Peramine was extracted and quantified as described by Spiering et al. (*14*), with homoperamine as

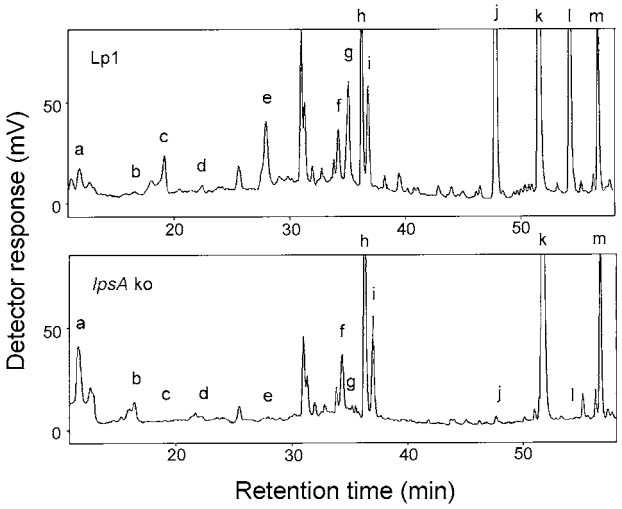


**Figure 2.** Knockout of *lpsA* affects accumulation of several fluorescent ergot alkaloids detected by HPLC. Letters representing peaks or peak positions are shown in both traces, whether or not the peak was present: (a) lysergic acid; (b) isolysergic acid; (c) lysergyl-alanine; (d) isolysergyl-alanine; (e) ergine; (f) setoclavine; (g) erginine; (h) 6,7-secolysergine; (i) isosetoclavine; (j) ergovaline; (k) ergotamine; (l) ergovalinine; (m) ergotaminine. Ergotamine (evident as peak k and its stereoisomer ergotaminine, peak m) was added as an internal standard for quantification of ergot alkaloids. The small peak preceding position j in the *lpsA* knockout eluted before ergovaline and was not present in other traces.

internal standard. Lolitrems were extracted essentially as described above for ergot alkaloids but with a solvent consisting of 90% v/v dichloroethane + 10% v/v methanol. Lolitrems were analyzed by a modification of the procedure of Gallagher et al. (*22*) involving isocratic HPLC with a mobile phase of dichloromethane−acetonitrile−water (86:14:0.1), a 150 mm × 4.6 mm i.d., 5 μm, Alltima silica column (Alltech, Deerfield, IL), and detection by fluorescence (excitation at 265 nm and emission at 440 nm). Perennial ryegrass infected with *N. lolii* isolate Lp19 and endophyte-free perennial ryegrass were included as positive and negative controls, respectively.

## RESULTS AND DISCUSSION

**Increase in the Accumulation of Lysergic Acid with Elimination of Ergine as a Result of Knockout of *lpsA*.** HPLC with the gradient from 5% acetonitrile to 75% acetonitrile separated several polar fluorescent compounds in extracts of endophyte-infected perennial ryegrass. By comparison of chromatograms of extracts of endophyte-free perennial ryegrass with those of perennial ryegrass infected with *Neotyphodium* sp. Lp1 or its *lpsA* knockout derivative, several endophyte-associated peaks with fluorescence properties of ergot alkaloids were noted (**Figure 2**). Comparison with authentic standards revealed peaks corresponding to lysergic acid (peak a, **Figure 2**) and its 8-α epimer isolysergic acid (peak b, **Figure 2**). Accumulation of lysergic acid, measured as both its isomeric forms, was significantly greater ($P < 0.0001$) in associations containing the *lpsA*-knockout mutant (~1 μg/g of dry weight of pseudostem) than in associations containing the parent isolate Lp1 (**Table 1**). However, the amount of lysergic acid that accumulated in the *lpsA* knockout associations corresponded to only 13% of the concentration of lysergic acid derivatives, including ergovaline, that accumulated the Lp1 associations, indicating some type of feedback regulation of the pathway.

Also on the basis of comparisons to authentic standards, peaks corresponding to ergine (peak e, **Figure 2**) and its stereoisomer erginine (peak g, **Figure 2**) were identified and found to be absent from perennial ryegrass associations containing the *lpsA* knockout. The elimination of ergine in the peptide synthetase knockout was not necessarily expected, since the placement of ergine along the ergot alkaloid pathway has not been well established.

**Table 1.** Concentrations in μg/g of Dry Weight (Mean ± Standard Error) of Fluorescent Ergot Alkaloids in Endophyte-Infected Perennial Ryegrass

| alkaloid[a] | *N.* sp. Lp1 | *lpsA* knockout |
|---|---|---|
| 6,7-secolysergine | 0.99 ± 0.11 | 2.02 ± 0.42 |
| setoclavine | 0.85 ± 0.12 | 0.98 ± 0.19 |
| lysergic acid | 0.04 ± 0.02[b] | 1.02 ± 0.16 |
| ergine | 0.29 ± 0.06 | n.d.[c] |
| lysergyl-alanine | 0.39 ± 0.06 | n.d. |
| ergovaline | 7.35 ± 1.25 | n.d. |

[a] Peak areas of listed alkaloids and their 8-epimers, when present, were pooled in calculating concentrations. [b] For this mean only, four samples in which lysergic acid was not detectable were included as 0 in calculating the mean. [c] n.d. = none detected.

**Identification of Lysergyl-Alanine as a Component Eliminated by the *lpsA* Knockout.** Peak c (**Figure 2**) was eliminated from perennial ryegrass−endophyte associations by knockout of *lpsA*. This peak corresponded to a relatively polar compound, intermediate in its retention time between lysergic acid and ergine. This compound did not correspond to any ergot alkaloids for which authentic standards were available to us. Analysis of ergot sclerotia, which had been harvested from infected perennial ryegrass, tall fescue, or rye, revealed that this peak was a minor constituent of ergot, with a relative concentration similar to lysergic acid. Since the unknown accumulated to relatively low concentrations in Lp1-infected perennial ryegrass, ergot sclerotia collected from infected perennial ryegrass were used as a source from which the molecule could be isolated to gain information on its structure. To test whether the molecule was an amide of lysergic acid, a preparation was subjected to alkaline hydrolysis and the products analyzed by HPLC. Lysergic acid resulted from the alkaline hydrolysis (**Figure 3**). Analysis of the unknown by HPLC with a photodiode array detector revealed an absorbance maximum at 309 nm with a preceding minimum at 270 nm, typical of lysergic acid derivatives (*12*). LC-MS in scanning mode with deflector voltage at 45 V revealed an ion at *m*/*z* 340 corresponding to $[\text{lysergyl-alanine} + H]^+$ (not shown). When the deflector voltage was raised to 60 V to induce fragmentation, ions with *m*/*z* 208, 223, and 297 were observed, in addition to the parent ion, *m*/*z* 340 (**Figure 4A**). Fragments with *m*/*z* 208 and 223 are typically abundant fragments for lysergic acid derivatives, with the *m*/*z* 223 fragment hypothesized to be the Δ8,9 ergoline system (corresponding to $[MH - \text{Ala} - CO]^+$) (*23*). The *m*/*z* 297 and 208 fragments, corresponding to $[MH - CH_2NMe]^+$ and $[MH - CH_2NMe - \text{Ala}]^+$, are likely to arise by a retro-Diels−Alder reaction from the parent ion, similar to that observed for fragmentation of lysergic acid diethylamide (*24*). We obtained an analogous fragmentation pattern by ionization of the simple amide ergonovine (not shown). Further evidence for a free acid structure was provided by (i) enhanced LC-MS detection in the positive ionization mode with formic acid rather than ammonium acetate buffer in the mobile phase and (ii) the formation of the predicted mass of *m*/*z* 454 in GC-MS (data not shown) when the molecule was derivatized with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)fluoroacetamide (*25*). These data are consistent with the structure lysergyl-alanine (**Figure 1A**) or 2-[(7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carbonyl)amino]propionic acid.

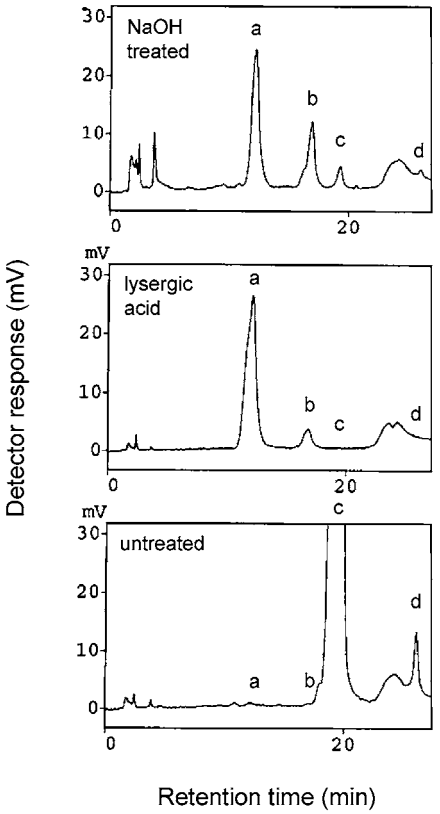


**Figure 3.** Alkaline hydrolysis of lysergyl-alanine (c), yielding lysergic acid. Letters representing peaks (refer to **Figure 2**) or peak positions are shown in every trace, whether or not the peak was present. An untreated sample (bottom panel) was obtained by collection of peak c by elution from HPLC and purification from the mobile phase. Peak d formed spontaneously during this process.

LC-MS with selected ion monitoring at *m*/*z* 340 of preparations from Lp1-infected and *lpsA*-knockout-infected associations provided confirmation that the molecular species observed as differing between the endophyte−perennial ryegrass associations (peak ***c***, **Figure 2**) is likely to be lysergyl-alanine. The Lp1-containing associations had a peak in the *m*/*z* 340 ion chromatogram at the same retention time as lysergyl-alanine purified from *C. purpurea*, and this peak was eliminated from associations containing the *lpsA* knockout (**Figure 5**).

The minor peak d (**Figures 2** and **3**) is proposed to be the 8-α epimer of lysergyl-alanine, isolysergyl-alanine, on the basis of several observations. It co-occurs with lysergyl-alanine, is formed from purified lysergyl-alanine in solution (e.g., **Figure 3**), and is absent from the *lpsA* knockout.

The mean concentration of combined lysergyl-alanine and isolysergyl-alanine in Lp1-containing associations is 0.39 μg/g, which is slightly greater than the amount of ergine and its isomer (0.29 μg/g) that we detected in these same symbiota (**Table 1**). Among nine extracts of Lp1-perennial ryegrass associations, there was no evidence of a correlation between the concentration of ergine and lysergyl-alanine ($r = -0.18$, $P = 0.64$) or ergine and ergovaline ($r = 0.13$, $P = 0.73$), but there was an indication of a correlation between the concentrations of lysergyl-alanine and ergovaline ($r = 0.60$, $P = 0.09$).

Two other previously characterized simple amides of lysergic acid, ergonovine and lysergic acid α-hydroxyethylamide, were not detected in extracts of *Neotyphodium* sp. Lp1 or its *lpsA* knockout mutant.

**Increase in the Levels of the Novel Clavine Alkaloid 6,7-Secolysergine as a Result of the *lpsA* Knockout.** One

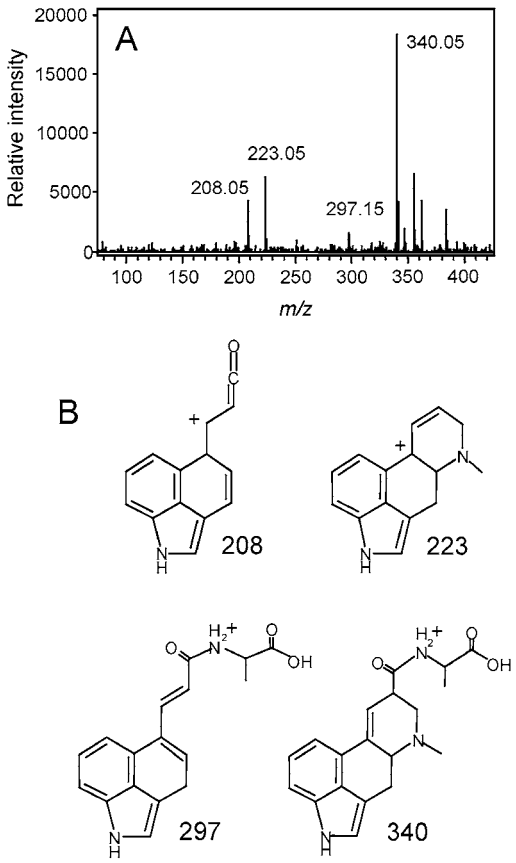


**Figure 4.** Characterization of lysergyl-alanine. (A) LC-MS spectrum showing the parent ion at *m*/*z* 340, $[MH]^+$, which was the sole ion detected at lower ionization energy (45 V) and fragments. (B) Proposed structures of the parent ion, *m*/*z* 340, [lysergyl-alanine + H]$^+$, and fragment ions, *m*/*z* 297 [MH − $CH_2NMe]^+$, *m*/*z* 223 [MH − Ala − CO]$^+$, and *m*/*z* 208 [MH − $CH_2NMe$ − Ala]$^+$.

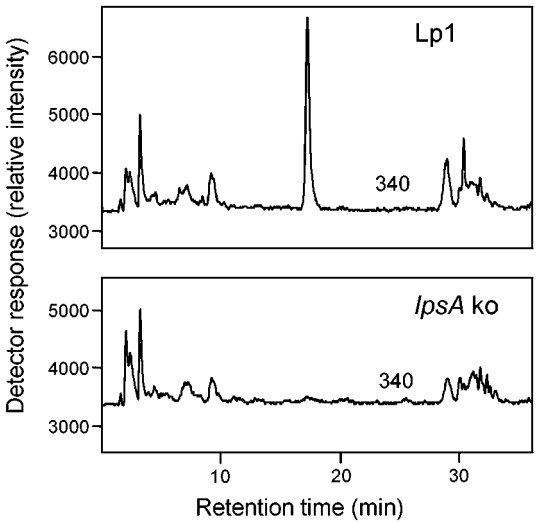


**Figure 5.** Elimination of lysergyl-alanine by the *lpsA* knockout: LC-MS trace with selected ion monitoring at *m*/*z* 340, $[M + H]^+$. The retention time of the peak found only in the Lp1 *m*/*z* 340 ion trace corresponds to the retention time of the fluorescent peak detected in Lp1-infected perennial ryegrass but not in *lpsA* knockout-infected perennial ryegrass.

fluorescent peak showed an increase in concentration as a result of the *lpsA* knockout (peak h, **Figure 2**; 6,7-secolysergine, **Table 1**). The concentration of this alkaloid in Lp1−perennial ryegrass associations was in the range of 1 μg/g of dry weight of

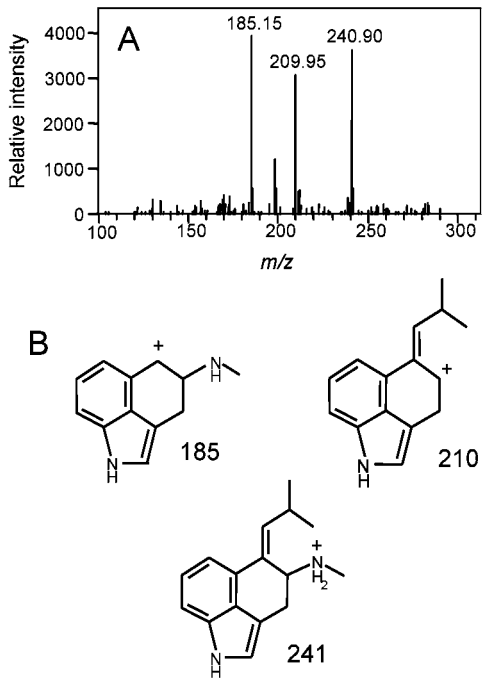


**Figure 6.** Characterization of 6,7-secolysergine. (A) LC-MS spectrum showing the parent ion at *m*/*z* 241 $[M + H]^+$ and fragments. (B) Proposed structures of the parent ion, *m*/*z* 241, [6,7-secolysergine + H]$^+$, and fragment ions *m*/*z* 210 $[MH - NH_2Me]^+$ and *m*/*z* 185 $[MH - CH_2CMe_2]^+$.

pseudostem tissue and increased approximately 2-fold ($P < 0.05$) in the *lpsA* knockout strain (**Table 1**). Interestingly, tall fescue infected with *N. coenophialum* contained a peak with the same retention time, but perennial ryegrass infected with *N. lolii* and the endophyte-free versions of either of these grasses did not (data not shown). The peak did not correspond to any of the available standards and was not present in sclerotia of *C. purpurea* from any source tested. The compound exhibited a basic nature, in that it would not bind to non-end-capped C18 silica under acidic conditions, but when applied under alkaline conditions, it bound very tightly, such that it was not eluted with 80% 2-propanol, a solvent that eluted all of the other fluorescent ergot alkaloids. Elution of the unknown from this absorbent required addition of acid.

Analysis of the unknown by HPLC with photodiode array detection revealed an absorbance maximum at 300 nm and a minimum at 262 nm. This maximum is intermediate to those of the Δ8,9 clavines, which typically are in the range of 280 nm, and the Δ9,10 clavines and lysergic acid derivatives, which have absorbance maxima near 310 nm (*12*). The strong fluorescence properties of the molecule, under conditions of excitation at 310 nm and emission at 410 nm, indicate that it contains a double bond at the 9,10-position, conjugated to the indole ring (*12*). Only ergot alkaloids containing such a chromophore are easily detectable at less than 1 μg/g concentrations by the HPLC procedure described here. LC-MS in the positive ionization mode with deflector voltage of 45 V revealed an unfragmented ion with *m*/*z* 241, corresponding to [6,7-secolysergine + H]$^+$ (not shown). When the unknown was subjected to a deflector voltage of 60 V, major fragments of *m*/*z* 210 and 185 (corresponding to $[MH - NH_2Me]^+$ and $[MH - CH_2CMe_2]^+$) were observed (**Figure 6**). Building upon a base of an indole ring with a conjugated 9,10-double bond and upon the *m*/*z* values of the parent and fragment ions and the absorbance spectrum, we propose that the substance is a 6,7-secoergolene with a double bond in the 9,10-position (**Figures 1A** and **6B**), (5-isobutylidene-1,3,4,5-tetrahydrobenzo-[*cd*]indol-4-yl)methylamine. The stereochemistry of the exocyclic double bond is unknown. A trivial name for this compound, consistent with the nomenclature of Floss (*15*), would be 6,7-secolysergine. The chromatographic behavior of the molecule is consistent with such a structure. The paucity of material available to us has prevented us from confirming and completing the structure determination by additional spectroscopic methods.

**Effect of the *lpsA* Knockout on the Accumulation of Known Clavines.** The fluorescent clavines setoclavine and penniclavine were considered as candidates for the remaining unidentified fluorescent components in the chromatograms (**Figure 2**). We prepared standards for setoclavine (and its 8-α epimer isosetoclavine) and penniclavine (and its 8-α epimer isopenniclavine) by feeding agroclavine (for setoclavine) or elymoclavine (for penniclavine) to endophyte-free perennial ryegrass and allowing the plant to oxidize the introduced clavines (**Figure 1**). The identity of the fluorescent oxidation products as the isomeric pairs of setoclavine/isosetoclavine and penniclavine/isopenniclavine was supported by LC-MS, in which peaks at *m*/*z* 255 or 271 (setoclavine/isosetoclavine and penniclavine/isopenniclavine, respectively) were detected in the oxidized samples from agroclavine and elymoclavine. Scigelova et al. (*26*) had reported that this oxidation could be carried out by peroxidase-rich plant cell cultures such as those of tomato and potato, so it is not surprising that another plant tissue could catalyze this reaction. Nonetheless, this is the first demonstration that some ergot alkaloids produced in *Neotyphodium*−grass associations may be the product of plant as well as fungal enzymatic activities.

HPLC analysis of the prepared standards showed that peaks f and i in **Figure 2** correspond to setoclavine and isosetoclavine. The concentrations of these compounds in perennial ryegrass associations infected with Lp1 and the *lpsA* knockout were not significantly different (**Table 1**). Peaks corresponding to penniclavine/isopenniclavine standards were not reliably detected in either of the endophyte associations examined here.

Other clavines were investigated qualitatively by LC-MS in selected ion monitoring experiments. Chanoclavine I and some of its isomers (similar retention times, same mass, and absence in endophyte-free perennial ryegrass) were present in both Lp1-infected and *lpsA* knockout-infected perennial ryegrass associations, as indicated by the ion at *m*/*z* 257 (**Figure 7**). Within the limitations of the method, levels of accumulation of this intermediate were similar in several extracts of associations containing either endophyte strain. Similarly, agroclavine with an ion at *m*/*z* 239 (**Figure 7**) was detected at similar concentrations in perennial ryegrass associations harboring parental or *lpsA* knockout endophytes. The increased concentration of 6,7-secolysergine in the *lpsA* knockout mutant as compared to the parent isolate, Lp1, as previously demonstrated by HPLC with fluorescence detection, was detected from the ion at *m*/*z* 241 by LC-MS in extracts of several associations of each examined (**Figure 7**). Moreover, additional peaks of *m*/*z* 241 but of lower polarity, as evidenced by greater retention times, were observed (**Figure 7**). These may correspond to the four stereoisomeric forms of the dihydro derivatives of agroclavine named costaclavine, epicostaclavine, festuclavine, and pyroclavine (**Figure 1A**). Elymoclavine was near or below the limits of detection in our clavine preparations, indicating that it does not accumulate in perennial ryegrass infected with Lp1 or its derivatives but presumably is rapidly converted to the next oxidation state,

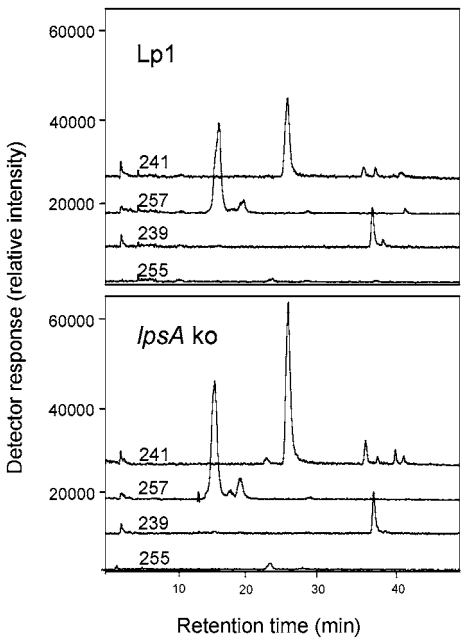


**Figure 7.** Qualitative analyses of selected clavines by LC-MS. Multiple selected ions (indicated above each corresponding trace line) were monitored concurrently in clavine preparations from Lp1-infected perennial ryegrass and *lpsA* knockout-infected perennial ryegrass. Ions monitored correspond to expected $[M + H]^+$ ions of clavines as follows: *m/z* 241, 6,7-secolysergine (also costaclavine, epicostaclavine, festuclavine, and pyroclavine); *m/z* 257, chanoclavine I and isomers; *m/z* 239, agroclavine; *m/z* 255, elymoclavine. The identities of the largest peaks in ion traces of *m/z* 241, 257, and 239 were confirmed as 6,7-secolysergine, chanoclavine I, and agroclavine, respectively, by chromatography of standards.

lysergic acid. An authentic standard for elymoclavine was readily detectable in spiked samples taken through the clavine preparation.

**Effect of *lpsA* Knockout on Accumulation of Other Endophyte Alkaloids Made via Different Pathways but Containing Common Precursors.** The elimination of ergopeptines could conceivably liberate precursors for the formation of peramine (proline) and lolitrems (both tryptophan and isoprene groups). Peramine levels in Lp1−perennial ryegrass associations and *lpsA* knockout associations were not significantly different, with 135 ± 60 $\mu$g/g of dry weight (mean ±1 SE, $n = 7$) and 139 ± 22 $\mu$g/g (mean ±1 SE, $n = 7$) measured, respectively. The parental isolate in our study, *Neotyphodium* sp. Lp1, is not known to produce lolitrems (*11*). Lolitrems were not detectable by HPLC in associations (four each) containing the parental isolate or the *lpsA* knockout mutant.

**Perspectives on the Constitution and Regulation of the Ergot Alkaloid Pathway in *Neotyphodium* sp. Lp1.** The biosynthetic origin of simple amides of lysergic acid (i.e., ergine in *Neotyphodium* spp. and *Claviceps paspali*, ergonovine in *C. purpurea*, and lysergic acid α-hydroxyethylamide in *C. paspali*) has been unknown (*12*, *15*, *19*). We have shown that the production of ergine and a previously uncharacterized simple amide, proposed to be lysergyl-alanine, depends on the activity or products of lysergyl peptide synthetase. The biosynthesis of these simple amides as direct products of the lysergyl peptide synthetase complex would be highly surprising, because intermediates in the synthesis of nonribosomally synthesized peptides are covalently bound to the enzyme and require specific catalysis to be released (*27*, *28*). This possibility, however, cannot be excluded on the basis of the available data. The alternate explanation, that these simple amides are metabolites of a product of the lysergyl peptide synthetase complex, appears more plausible. The lysergyl-peptide lactam intermediate that is formed transiently during ergopeptine biosynthesis (**Figure 1A**) as the direct product of the lysergyl peptide synthetase complex is a reasonable candidate for the source of the simple amides. In the case of the *lpsA* knockout, this lactam intermediate would not be formed and, consequently, its absence could, hypothetically, account for the absence of ergine and lysergyl-alanine.

We emphasize that if the simple amides are breakdown products of more complex ergot alkaloids, then it is highly likely that they are true natural products and not artifacts generated as a result of extraction of ergot alkaloids from fungal or plant material. Several observations support this claim. Ergine is found at only very low levels, and is often not detectable, in extracts of sclerotia of the ergot fungus *C. purpurea* that are extremely rich in other ergot alkaloids, far more so than are any endophyte−grass associations. If ergine was an artifact of extraction of more complex ergot alkaloids, then it would be expected to be an abundant product of extracts of ergot sclerotia. Similarly, a relatively large amount of ergotamine, the equivalent of 10 $\mu$g/g of dry weight of tissue, was routinely added as an internal standard, prior to extraction, to endophyte-free grass tissue or to associations lacking ergopeptines, yet neither ergine nor lysergyl-alanine was ever detected in extracts of these tissues.

The isolation of lysergyl-alanine also is significant because this alkaloid has been proposed as a theoretical intermediate in the biosynthesis of the pharmacologically important simple amide ergonovine (synonym ergometrine) (*12*, *15*, *19*). This molecule is the 2-propanolamide of lysergic acid and could be derived from lysergyl-alanine by complete reduction of the alanine carbonyl group. Our data indicate that ergonovine is not produced by *Neotyphodium* sp. Lp1 in perennial ryegrass. Shelby et al. (*23*) reported that ergonovine is not a constituent of *N. coenophialum* in tall fescue and speculated that previous reports of ergonovine in *N. coenophialum*−tall fescue interactions were due to contamination of plant material with sclerotia or honeydew of *C. purpurea*. Since lysergyl-alanine is produced in *C. purpurea*, where it is proposed to serve as a precursor to ergonovine, it would be interesting to test whether ergonovine would be eliminated from *C. purpurea* by an analogous peptide synthetase gene knockout. Experiments addressing this possibility are complicated by the multiple copies of the LPS1-encoding gene in our strains of *C. purpurea* (*21*).

Analysis of perennial ryegrass associations with the *lpsA* knockout mutant also provided novel information about pathway regulation. The amount of lysergic acid that accumulated in the *lpsA* knockout associations corresponded to only 13% of the concentration of lysergic acid derivatives that accumulated in Lp1 associations (**Figure 1A**, **Table 1**), indicating altered regulation of the pathway. Although only semiquantitative in nature, the LC-MS analyses of chanoclavine and agroclavine indicate similar concentrations of these intermediates in Lp1 and its *lpsA* knockout derivative. Concentrations of setoclavine and isosetoclavine were also similar (**Figure 2**, **Table 1**).

Collectively, these observations indicate that in the *lpsA* knockout mutant the accumulation of lysergic acid feeds back and regulates activity or production of enzymes controlling intermediate steps in the pathway such that the key intermediates between chanoclavine and elymoclavine are maintained at approximately wild-type levels, suggesting that these are under tight genetic control. However, the increased accumulation of the novel clavine provisionally identified as 6,7-secolysergine

in the *lpsA* knockout endophyte indicates that the pathway steps prior to chanoclavine may be regulated in a slightly different manner. The structure of 6,7-secolysergine is consistent with it being derived from chanoclavine or an intermediate between dimethylallyltryptophan and chanoclavine. The structures of these intermediates have not been definitively established (*19*). We hypothesize that some of the flux of early intermediates (prior to chanoclavine) is shunted to metabolites such as 6,7-secolysergine on an early spur from the pathway to ergopeptines (**Figure 1A**). When, as a result of the *lpsA* knockout and resulting lysergic acid accumulation, one or more enzymes in the intermediate or later part of the pathway is down-regulated, more of the early intermediates may be directed into the spur pathway to the metabolite 6,7-secolysergine, which appears to be less tightly controlled.

**Contribution of Ergot Alkaloids to Animal Toxicoses.** There is considerable evidence that ergot alkaloids are major factors in the toxicoses associated with *Neotyphodium*-infected grasses (*3*−*10*), but the contribution of individual ergot alkaloids has been difficult to assess. Much attention has been focused on ergovaline, but simple amides such as ergine have also been proposed to contribute to the toxicoses (*7*, *29*). The potential contribution of clavines to toxicity or other endophyte-associated traits also should be investigated. Effects due to specific ergot alkaloids as opposed to the fungus itself or to other classes of alkaloids have been difficult to separate in in vivo experiments. The genetic approach of altering an endophyte's ergot alkaloid profile provides material that may be useful for future studies designed to determine the contributions of specific alkaloids to the toxicoses or other endophyte-associated traits.

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