Biosynthetic Precursors of Fungal Pyrrolizidines, the Loline Alkaloids

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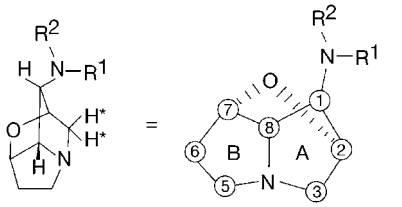
Loline alkaloids are saturated pyrrolizidines with a substituted 1-amino group and an oxygen bridge between C2 and C7, and are insecticidal metabolites of plant-symbiotic fungi (endophytes). Cultures of the endophyte, *Neotyphodium uncinatum*, incorporated labeled L-proline and L-homoserine into the 1-aminopyrrolizidine, *N*-formylloline. The A-ring carbons C1–C3 and the N1 were derived from L-homoserine; the B-ring carbons C5–C8 and the ring nitrogen were derived from L-proline. Incorporation of both deuterium atoms from L-[4,4-$^2H_2$]homoserine and feeding tests with labeled L-methionine indicated that L-homoserine incorporation was not achieved via aspartyl semialdehyde or *S*-adenosylmethionine, but probably involved a highly novel N–C bond-forming γ-substitution reaction.

Introduction

Many temperate grass species maintain systemic, heritable symbioses with *Epichloë* and *Neotyphodium* species (fungal endophytes) that produce loline alkaloids (Scheme 1). The lolines have broad insecticidal activity, can accumulate to levels of up to 2% plant dry mass, and provide host plants with biological protection from insects.[1–2] These alkaloids have an unusual structure, with three heterocyclic rings in a strained arrangement.[3] The biosynthetic pathway of loline alkaloids has not been previously investigated.



	R^1	R^2
N-formylloline (**1**)	HC=O	CH_3
N-acetylnorloline (**2**)	CH_3C=O	H
loline (**3**)	CH_3	H
N-methylloline (**4**)	CH_3	CH_3
N-acetylloline (**5**)	CH_3C=O	CH_3
norloline (**6**)	H	H
N-formylnorloline (**7**)	HC=O	H

Scheme 1. Loline alkaloids produced in *Neotyphodium uncinatum* fermentation cultures. Carbon number and ring assignments are indicated. Compound **1** is generally the primary loline alkaloid produced in the minimal medium cultures.

The loline group of pyrrolizidines comprises saturated 1-aminopyrrolizidines with various substituents on the 1-amino group (Scheme 1) and an oxygen bridge between C2 and C7. In contrast, most plant pyrrolizidines (necines) have an additional carbon atom at C1 and contain neither the ether bridge between C2 and C7 nor the 1-amino group characteristic of lolines.[4] Although the lolines exhibit none of the antimammalian activity associated with necines,[5–7] they show potent activity against a wide array of insect herbivores.[1–2,8–9]

The structural similarity of the loline pyrrolizidine ring system to the necine base of the plant-produced pyrrolizidines has led to the suggestion that lolines and necines might share similar biosynthetic routes.[9] Necines are biosynthetically derived from polyamines, specifically putrescine (Put) and spermidine (Spd). Studies utilizing ^{13}C- and ^{14}C-labeled precursors,[10–13] and characterization of homospermidine synthase,[14–15] indicate that Put and Spd are precursors of homospermidine, which is the first committed intermediate in necine biosynthesis. Bush et al. have proposed Spd as the direct precursor to the loline alkaloid ring system.[9] Prior to this work, this hypothesis had not been tested.

Neotyphodium uncinatum, an endophyte of the grass *Lolium pratense* (= *Festuca pratensis*, meadow fescue), produces lolines in defined-medium fermentation cultures.[16] With the use of these fermentation cultures, sufficient levels of one of the loline alkaloids, *N*-formylloline (**1**), have been obtained to facilitate biosynthetic studies. Here we report the first study in which *N. uncinatum* fermentation cultures were fed labeled amino acids and polyamines to elucidate the origins of all

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  DOI: 10.1002/cbic.200400327

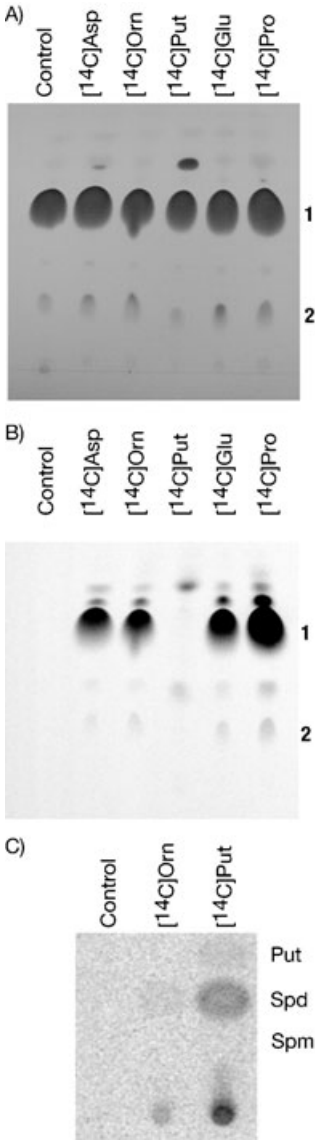


Figure 1. TLC analysis of lolines and polyamines from cultures fed with ^{14}C-labeled compounds. A) Iodine-stained TLC plate. B) Autoradiograph of TLC plate showing incorporation of label from ^{14}C-labeled L-Asp, L-Orn, L-Glu, and L-Pro into lolines. Very little incorporation of label from ^{14}C-Put was observed. C) ^{14}C-Put incorporation into Spd, indicating that Put was taken up by the fungal cells.

carbon and nitrogen atoms of **1**. We demonstrate the incorporation of L-Pro and L-homoserine (L-Hse) into the pyrrolizidine ring structure of the loline alkaloids. L-Met donates the carbon atoms of the *N*-formyl and *N*-methyl substituents of **1**, but not the carbon atoms of the pyrrolizidine ring system. The results indicate that loline alkaloid biosynthesis involves a unique pathway that is distinct from pyrrolizidine-ring formation in necine biosynthesis, and includes carbon–nitrogen bond formation by a novel γ-substitution reaction that involved the N of Pro and C4 of Hse.

Results

Cultures that were fed the radiolabeled amino acids L-[U-^{14}C]Asp, L-[U-^{14}C]Orn, L-[U-^{14}C]Glu, and L-[U-^{14}C]Pro incorporated the label into **1** and **2** (Figure 1A and B). The specific activity of radiolabel in **1** was determined for each treatment. Cultures that were fed with L-[U-^{14}C]Pro gave the highest specific activity in **1** at 5.9 cpm μmol^{-1}. L-[U-^{14}C]Orn and L-[U-^{14}C]Glu, which are both metabolic precursors of L-Pro, were incorporated into **1** at 2.0 cpm μmol^{-1} and 2.8 cpm μmol^{-1}, respectively. L-[U-^{14}C]Asp was incorporated into **1** at 1.3 cpm μmol^{-1}. The polyamine precursor, Put, was also tested, but very little incorporation of [1,4-^{14}C]Put was observed in **1** (0.09 cpm μmol^{-1}). L-[U-^{14}C]Orn and [1,4-^{14}C]Put were incorporated into Spd (Figure 1C). Cultures that were fed with L-[U-^{14}C]Orn incorporated less label in Spd, but greater incorporation was observed in lolines than when [1,4-^{14}C]Put was used.

Stable-isotope-feeding experiments, followed by analysis of **1** by ^{13}C NMR,[17] ^{15}N NMR, and GC-MS,[17] gave positional information for the carbon and nitrogen atoms from loline alkaloid precursors. Selective enrichment of **1** with the stable isotopes was calculated from GC-MS data (see Table 1 and Supporting Information Table S1). Cultures that were fed with L-[^{15}N,U-$^{13}C_5$]Pro gave **1** that was selectively enriched in the B ring carbon atoms C5, C6, C7, and C8 (Figures 2B and 3B). Enrichment (%) for L-[^{15}N,U-$^{13}C_5$]Pro feeding to the cultures indicated complete incorporation of the four ring carbons and nitrogen, for example as a +5 amu shift of the 82 *m/z* ion to 87 *m/z* (Figure 3B; Table 1). The splitting of peaks observed in the ^{13}C NMR spectrum (Figure 2B) was explained by ^{13}C,^{13}C coupling when L-[^{15}N,U-$^{13}C_5$]Pro was incorporated intact. GC-MS data of **2**–**5** also indicated incorporation of the intact L-Pro ring into the B ring (Supporting Information Figure S1). Cultures that were fed with L-[5-^{13}C]Orn gave enrichment in only the C5 position in the B-ring of compound **1** (Table 1 and Figure 2C). Cultures that were fed with L-[1,2-$^{13}C_2$]Orn gave **1** that was selectively enriched in the B-ring position, C8 (Table 1 and Figure 4B). Feeding L-[2,5-$^{15}N_2$]Orn to the cultures labeled the pyrrolizidine-ring nitrogen atom, as shown by enrichment of all +1 peaks relative to unlabeled *m/z* 82, 95, 154, and 182 peaks (Table 1).

The label from L-[4-^{13}C]Asp was incorporated into the C3 of the A-ring of **1** (Table 1 and Figure 2C). Likewise **1** from L-[^{15}N]-Asp-feeding experiments exhibited the label in the 1-amino group; this was evident from the enrichment of the *m/z* = 155 and 183 peaks relative to unlabeled loline (*m/z* = 154) and unlabeled parent ion (*m/z* = 182), respectively (Table 1). Enrichment of the 1-amino group was confirmed by ^{15}N NMR spectroscopy (Figure 5). L-Asp is a precursor of L-threonine, L-isoleucine, and L-Met via an L-Hse intermediate. Examination of **1** from the L-[^{15}N]Hse-feeding experiment also indicated enrichment of the 1-amino group (Table 1). Compound **1** from cultures that were fed L-[4,4-$^{2}H_2$]Hse showed enrichment in the +2 amu shift of 82 *m/z* to 84 *m/z* (Figure 3C and Table 1).

No specific incorporation of label from L-[^{15}N,U-$^{13}C_5$]Met was detected in the pyrrolizidine A-ring of **1** (Table 1, Figure 4C); such incorporation would have been expected if Spd were a loline alkaloid precursor. However, the *N*-formyl and *N*-methyl carbon atoms of **1** were labeled, and this resulted in +1 and +2 amu shifts of the 182 amu parent ion and a +1 amu shift

Table 1. Relative isotopic enrichment in **1** due to incorporation of labeled precursors.

Labeled compound	Conc. [mM]	Unlabeled fragment ions (*m/z*) [amu]			
		82	95	154	182
		% enrichment (amu shift)			
L-[^{15}N,U-$^{13}C_5$]Pro	5	51 (+5)	51 (+4)	52 (+5)	55 (+5)
L-[5-^{13}C]Orn	5	22 (+1)	15 (+1)	19 (+1)	23 (+1)
L-[1,2-$^{13}C_2$]Orn	2	10 (+1)	7 (+1)	10 (+1)	10 (+1)
L-[2,5-$^{15}N_2$]Orn	5	33 (+1)	12 (+1)	34 (+1)	33 (+1)
L-[4-^{13}C]Asp	4	16 (+1)	10 (+1)	14 (+1)	20 (+1)
L-[^{15}N]Asp	5	n/a[a]	n/a	13 (+1)	23 (+1)
L-[^{15}N]Hse	5	n/a	n/a	17 (+1)	16 (+1)
L-[4,4-$^2H_2$]Hse	5	24 (+2)	9 (+1)	26 (+2)	28 (+2)
L-[^{15}N,U-$^{13}C_5$]Met	4	0 (+1)[b]	n/a	24 (+1)	14 (+1)
		n/a	n/a	n/a	28 (+2)
		n/a	2 (+3)[b]	1 (+4)[b]	10 (+4)[b]
L-[methyl-^{13}C]Met	4	n/a	n/a	22 (+1)	36 (+1)
		n/a	n/a	n/a	50 (+2)

[a] n/a = not applicable, because no enrichment of these ions is expected from either pathway shown in Schemes 2 or 3. [b] Enrichment of these ions would be expected according to the alternative pathway shown in Scheme 3 but not as in Scheme 2.

loline alkaloid biosynthetic pathway. The use of radioactive and stable-isotope-labeled precursors has allowed the determination of the origins of the loline-alkaloid pyrrolizidine rings, the 1-amino group nitrogen, and the methyl and formyl substituents on the 1-amino group of **1**. Based on the incorporation of labels from L-[^{14}C]Pro and L-[^{15}N,U-^{13}C]Pro, we conclude that L-Pro contributes all the atoms of the B-ring of the lolines (Scheme 2). Biological conversion of L-Orn to L-Pro retains the position of the α-carbon, so that feeding experiments with posi-

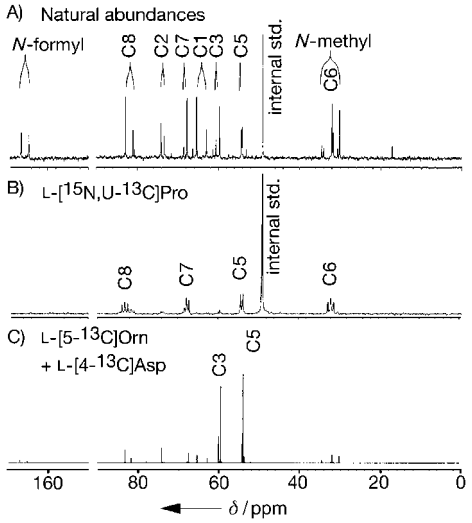


Figure 2. ^{13}C NMR spectra of **1** from selected feeding experiments. The resonances are doubled because of the presence of two rotamers of the tertiary amide.[17] A) ^{13}C natural abundance spectrum of **1**. B) L-[^{15}N,U-^{13}C]Pro (5 mM) feeding to cultures demonstrates enrichment in C5, C6, C7, and C8. The carbon chain is incorporated intact as indicated by ^{13}C,^{13}C coupling of enriched carbons. C) Spectrum of **1** from dual feeding of L-[4-^{13}C]Asp (4 mM) and L-[5-^{13}C]Orn (5 mM) demonstrating enrichment in C3 and C5, respectively.

of the 154 amu loline ion (Table 1). Selective incorporation in the *N*-methyl and *N*-formyl carbon atoms was further demonstrated by ^{13}C NMR (Figure 4C). Analysis of **1** from L-[methyl-^{13}C]Met-fed cultures indicated similar enrichment of the *N*-formyl and *N*-methyl carbon atoms (Table 1; Figure 4D).

Discussion

This study reveals the direct precursors of the loline alkaloids and provides evidence against polyamine involvement in the

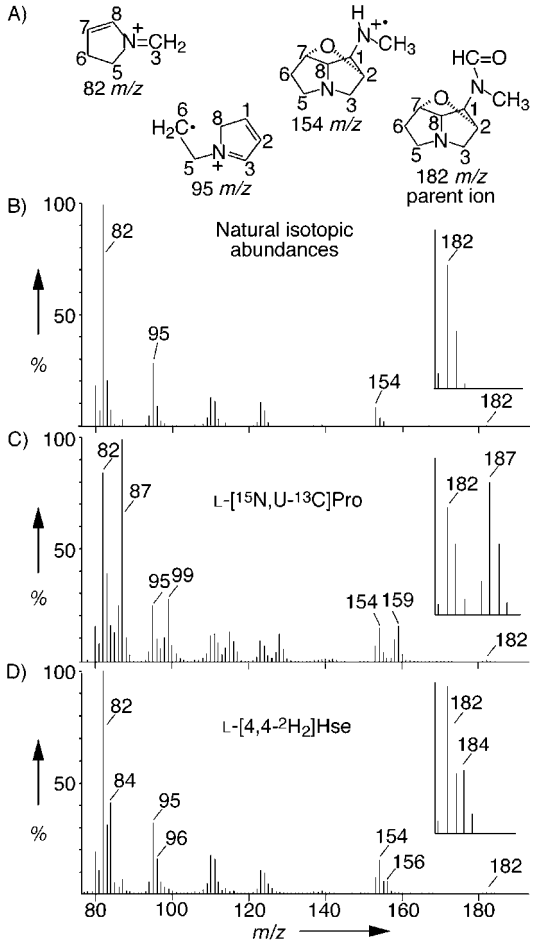


Figure 3. GC-MS of **1** from selected feeding experiments. A) Diagnostic MS ions from **1**. B) Spectrum of **1** isolated from control cultures. C) Spectrum of **1** after feeding of cultures with L-[^{15}N,U-^{13}C]Pro (5 mM), demonstrating selective incorporation of the complete proline ring structure in the ring N and C5 through C8. D) Spectrum of **1** after feeding with L-[4,4-$^2H_2$]Hse (5 mM) demonstrating enrichment at C3 with both deuterium atoms. The numbered peaks are due to the ions in (A) and their isotopically most enriched congeners. Other major peaks represent multiple fragmentation products and are more difficult to interpret.

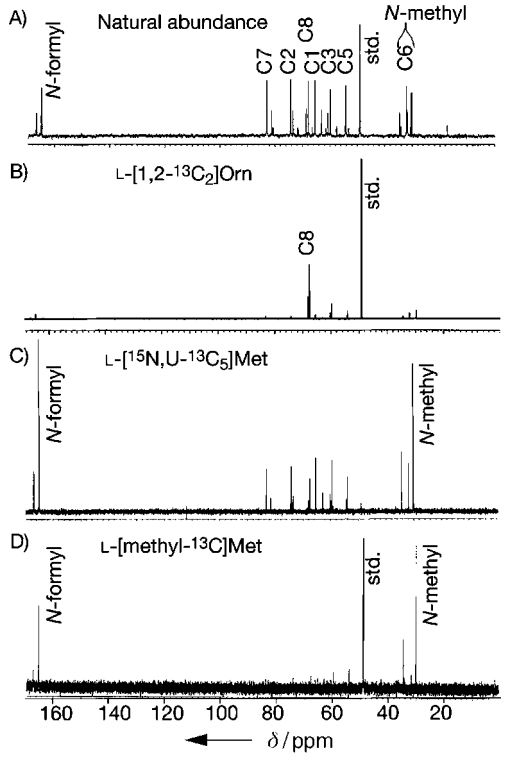


Figure 4. ^{13}C NMR spectra of **1** from selected feeding experiments. A) Chemical shifts of carbon atoms due to naturally occurring ^{13}C in **1**. B) Spectrum of **1** from cultures fed with L-[1,2-$^{13}C_2$]Orn (2 mM), indicating incorporation of label in C8. C) and D) Spectra of **1** from cultures fed with L-[^{15}N,U-$^{13}C_5$]Met (4 mM, C) and L-[methyl-^{13}C]Met (4 mM, D) indicating incorporation of label in the *N*-methyl and N-formyl carbon atoms. std = standard.

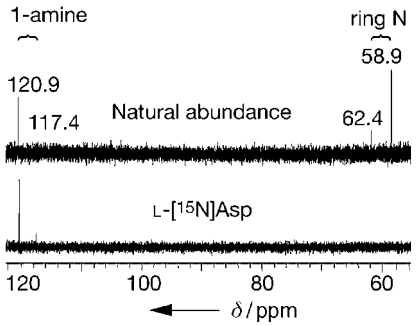
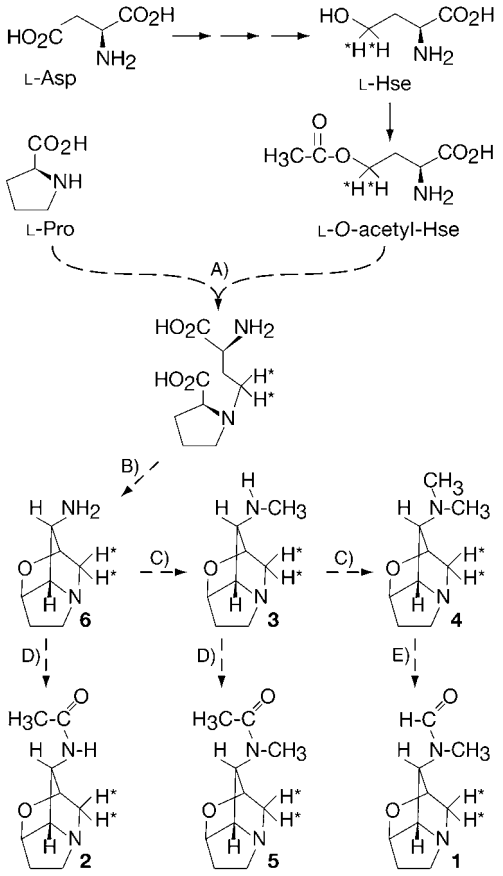


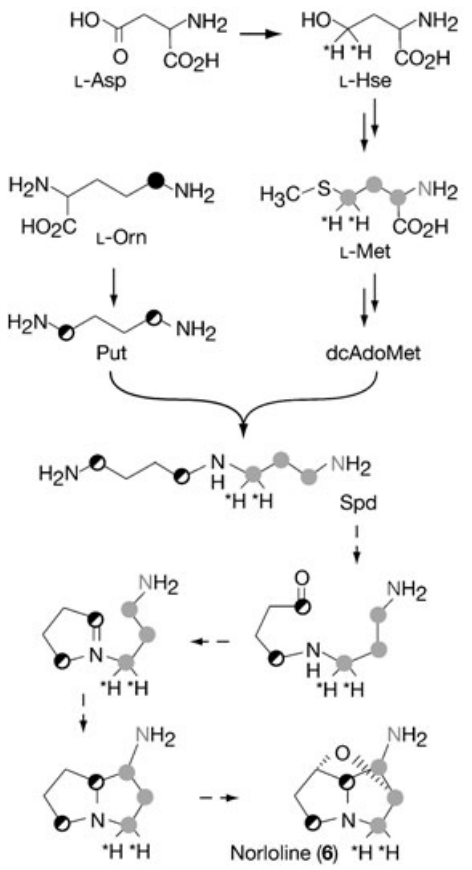
Figure 5. ^{15}N NMR spectra of **1** from selected feeding experiment. A) Chemical shifts due to naturally occurring ^{15}N. B) Spectrum of **1** from cultures fed with L-[^{15}N]Asp (5 mM) demonstrating incorporation of label in the 1-amino nitrogen of **1**.



Scheme 2. Proposed loline alkaloid biosynthetic pathway. A) Condensation of the 2-aminobutyric acid moiety from L-*O*-acetyl-Hse with the N of L-Pro by PLP-mediated γ-substitution; B) A series of steps including oxidative decarboxylation to remove the carboxyl group of L-Pro, cyclization to form the A-ring, and formation of the ether bridge; C) *N*-methylation via AdoMet; D) *N*-acetylation; E) Oxidation of the N-methyl of **4** to form the *N*-formyl moiety of **1**. Similar oxidation of **3** would form **7** (not shown). Solid arrows indicate known steps and dashed arrows indicate proposed steps. The scheme is consistent with the observed retention in **1** of both H atoms (*) at the C4 of L-Hse, and with incorporation of label from the C6 of L-Met into both the formyl and methyl groups of **1**.

tionally labeled L-Orn reveal the likely position of the carbon atoms from L-Pro in **1**: the α-carbon of L-Pro at C8, and the δ-carbon at C5. Furthermore, the L-Orn feeding result is inconsistent with Put as a precursor (Scheme 3). The absence of randomization between C5 and C8 of **1** indicates that polyamines such as Spd and Spm are very unlikely to be loline alkaloid precursors. Taken together, these results indicate that L-Pro is a direct precursor of lolines.

Results of feeding experiments with labeled L-Asp and its metabolic derivative L-Hse, indicate that L-Hse is a loline alkaloid precursor and contributes the 1-amino group nitrogen and C1, C2, and C3 of the A-ring (Scheme 2). The number of carbon atoms in the A-ring and the position of the 1-amino group are consistent with the observed incorporation from L-[^{15}N] and L-[4-^{13}C]Asp, as well as L-[^{15}N] and L-[4,4-$^{2}H_2$]Hse. As evidenced by GC-MS, both deuterium atoms of L-[4,4-$^{2}H_2$]Hse are incorporated into **1**. These incorporation patterns are consistent with either direct incorporation of L-Hse into a loline alkaloid intermediate or with a polyamine precursor. L-Hse is a precursor of L-Met, which (via decarboxylated AdoMet) donates the aminopropyl groups of the polyamines Spd and Spm. According to the ^{13}C NMR data, label from L-[^{15}N,U-^{13}C]Met is not detectably incorporated into the pyrrolizidine ring of **1**, but some enrichment (10%) is evident in the 186 *m/z* ion (**1** parent ion with +4 amu shift) in GC-MS analysis. However, the very low apparent enrichment in 82 *m/z* +1, 95 *m/z* +3, and 154 *m/z* +4 is inconsistent with the incorporation of C2 to C4 of L-Met (Scheme 3). Therefore, the enriched 186 *m/z*



Scheme 3. Previously proposed scheme for loline alkaloid biosynthesis indicating predicted labeling patterns. Asterisks indicate H atoms from the C4 position of L-Hse. Gray circles and lettering indicate the carbon atoms and nitrogen atom contributed by L-Met—via decarboxylated AdoMet—to Spd. The filled circle is the C5 of L-Orn, and the half-filled circles indicate that label from L-[5-^{13}C]Orn contributes equally to C5 and C8 of Spd via the symmetrical intermediate, Put.

ion, together with the 19% enrichment of the 185 *m/z* ion probably reflect the labeling of formyl and methyl carbon atoms, plus additional heavy isotopes of natural abundance, and perhaps metabolized L-[^{15}N,U-^{13}C]Met in the rings. Strong labeling of the *N*-formyl and *N*-methyl groups in cultures that were fed with L-[^{15}N,U-^{13}C]Met or L-[methyl-^{13}C]Met demonstrates that sufficient L-Met enters the cells to expect specific enrichment in C1, C2, and C3, plus the 1-amino group nitrogen, if L-Met were a precursor of the pyrrolizidine ring system of lolines, either directly or via polyamines. Although it is possible that the absence of such incorporation might be due to compartmentalization of different steps in the pathway, it seems unlikely that L-Pro, L-Orn, L-Asp, and L-Hse would enter the appropriate compartments in abundance but L-Met would not. Therefore, L-Hse, but apparently not L-Met, contributes C1, C2, C3, and the 1-amino nitrogen of lolines.

Given the evidence for L-Pro and L-Hse precursors, there are three obvious mechanisms whereby they might enter the pathway: a) The C4 carbon of L-Hse might undertake γ-substitution of the –OH with the ring N of L-Pro, as shown in Scheme 2. b) L-Hse could be oxidized back to L-aspartyl semialdehyde, which could condense with the N of L-Pro. c) L-Aspartyl semialdehyde could be further oxidized to L-Asp, which might then form an amide linkage to L-Pro. Only the first of these possibilities predicts that both deuterium atoms of L-[4,4-$^{2}H_2$]Hse would be retained in lolines as observed (Scheme 2). Although the exact precursor that attaches to the N of L-Pro has not been determined, it is likely to be an activated form of L-Hse, of which L-*O*-acetyl-Hse is common in fungi.[18] It is possible that other intermediates derived from L-Hse and/or L-Pro might be involved. However, the pathway must nevertheless include condensation of an aliphatic group with an amine. In biological systems, *N*-alkylation almost always involves AdoMet (or decarboxylated AdoMet) as the methyl- or aminopropyl-group donor. But our evidence indicates that L-Met is unlikely to be involved in that alkylation (although it is involved in substitutions of the 1-amino group). Thus, loline alkaloid biosynthesis represents a particularly unusual pathway and future identification and mechanistic studies of the enzymes that are involved will be of considerable interest.

Whether L-Pro or a derivative thereof is a proximate precursor has not been determined by this study. The loline alkaloid pathway almost certainly involves loss of the carboxyl group of L-Pro, and this might occur before or after condensation to the A-ring precursor. Oxidative decarboxylation of L-Pro could give 1-pyrroline, 2-pyrroline, or 5-hydroxypyrrolidine, and incorporation of any of these compounds into lolines would be consistent with the results of L-[^{13}C]Orn and L-[^{15}N,^{13}C]Pro-feeding experiments because the appropriate positions of the carbon atoms would be maintained. However, the N of L-Pro seems more likely to undertake the nucleophilic attack on the γ-carbon (C4) of L-*O*-acetyl-Hse in a substitution reaction than the N of either of the other possibilities. Therefore, we suggest that L-Pro is the likely precursor in the first pathway step.

Based on the above considerations and the relationships of likely loline alkaloid-biosynthesis genes identified by Spiering et al.,[19–20] we put forward the pathway summarized in Scheme 2. This scheme involves transfer of the 3-amino-3-carboxypropyl moiety from L-*O*-acetyl-Hse to the N of L-Pro by γ-substitution. The reaction would most likely involve a γ-type pyridoxal phosphate (PLP)-containing enzyme, for which the likely candidate is the product of the *lolC* gene, which is found to be expressed only in loline alkaloid-producing fermentation cultures.[20] This predicted gene product shares highly significant sequence similarity to an *O*-acetylhomoserine-(thiol)lyase. Related enzymes catalyze the transfer of C2 to C4 of L-*O*-acetyl-Hse to the sulfur moiety of L-cysteine to form cystathionine. RNAi constructs of *lolC* introduced into *N. uncinatum* inhibit production of lolines in fermentation cultures. This implicates the *lolC* product in loline alkaloid biosynthesis.[19]

We hypothesize that, once L-Pro and L-Hse or derivatives thereof are linked, a series of reactions by monooxygenases, oxidoreductases, and additional PLP enzymes yield norloline (**6**; Scheme 2). Predicted products of other genes identified in a 25 kb cluster associated with *lolC*, include four with significant similarity to oxygenases, oxidases, and oxidoreductases, and two that are related to known PLP enzymes.[19] These could be sufficient for the synthesis of **6**. Methylation and acet-

ylation reactions are then required to generate the *N*-substituents of **1**–**5** (Scheme 2) and **7**. Candidate genes for these steps remain to be discovered.

In conclusion, the likely origins of all C and N atoms of the core loline alkaloid structure have been identified. L-Hse provides the 1-amino group nitrogen and C1, C2, and C3, and L-Pro provides the ring N and C5, C6, C7, and C8. To help determine the mechanism and timing of ether-bridge closure, future studies are planned with isotopically labeled 1-aminopyrrolizidine without the ether bridge, with or without hydroxyl groups on C2 and C7. Efforts are also underway to detect the proposed first intermediate in fungal mycelium during loline alkaloid production. If identified, further work with this intermediate could elucidate the mechanism of pyrrolizidine-ring formation, a process that also remains undetermined for necines.

Experimental Section

Materials: L-[U-^{14}C]Orn (>250 mCi mmol^{-1}), [1,4-$^{14}C_2$]Put (100–120 mCi mmol^{-1}), L-[U-^{14}C]Pro (200–300 mCi mmol^{-1}), and L-[U-^{14}C]Glu (>200 mCi mmol^{-1}) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). L-[U-^{14}C]Asp (130–250 mCi mmol^{-1}) was obtained from Moravek Biochemicals (Brea, CA, USA). L-[5-^{13}C]Orn (99% ^{13}C) and L-[1,2-$^{13}C_2$]Orn (99% ^{13}C) were purchased from Mass Trace (Woburn, MA, USA) and Icon Stable Isotopes (Mt. Marion, NY, USA), respectively. L-[^{15}N]Asp (>99% ^{15}N) was obtained from Medical Isotopes Inc. (Pelham, NH, USA). Deuterium oxide (99.9%), L-[^{15}N,U-$^{13}C_5$]Met (96–99% ^{15}N, 98%+ ^{13}C), L-[methyl-^{13}C]Met (98% ^{13}C), L-[4-^{13}C]Asp (99% ^{13}C), L-[2,5-$^{15}N_2$]Orn (98% ^{15}N), and $^{15}NH_4NO_3$ (>98% ^{15}N in NH_4^+) were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). *N*-Boc L-Asp 1-*t*-Bu ester was purchased from Bachem Bioscience, Inc. (King of Prussia, PA, USA). All other compounds and L-[^{15}N,U-$^{13}C_5$]Pro (96–99% ^{15}N, 98%+ ^{13}C) were purchased from Sigma–Aldrich. TLC plates for loline alkaloid and polyamine separations were 60 A K6F (Whatman Inc., Florham Park, NJ, USA) with 100 μm thick silica gel.

(*S*)-4,4-Dideuterohomoserine hydrochloride: This compound was previously prepared by Seebach et al.[21] The synthesis of *t*-butyl (*S*)-*N*-(*t*-butoxycarbonyl)-4,4-dideuterohomoserinate was adapted from Rudolph et al.[22] *N*-Methylmorpholine (0.524 g, 5.19 mmol) was added to a stirring solution of *N*-Boc L-Asp 1-*t*Bu ester (1.50 g, 5.19 mmol) in dry THF (40 mL) under N_2 at −10 °C. After 1 min, ethyl chloroformate (0.565 g, 5.19 mmol) was added to the solution slowly, over 5 min. The mixture was stirred for 30 min, and then filtered into a flask that contained cold aqueous sodium borodeuteride (15 mL, 0.450 g, 10.7 mmol, 98% D). The reaction mixture was removed from the ice bath and stirred at RT for 4 h, then cooled to 0 °C and adjusted to pH 2 with 1 N HCl. The solution was extracted with ethyl acetate (3×50 mL), and the combined extracts were washed with brine (100 mL), dried over $MgSO_4$, and evaporated. The colorless gummy mass thus obtained was purified by flash chromatography with silica gel (33% ethyl acetate in petroleum ether as eluant) to give *t*-butyl (*S*)-*N*-(*t*-butoxycarbonyl)-4,4-dideuterohomoserinate (1.15 g, 4.15 mmol, 80% yield) as a colorless gum. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.38 (d, J = 7.3 Hz, 1H), 4.35 (ddd, J = 10.8, 7.8, 3.5 Hz, 1H), 3.01 (br s, 1H), 2.13 (dd, J = 13.8, 3.2 Hz, 1H), 1.47 (dd, 1H, partly obscured), 1.42 (s, 9H), 1.40 (s, 9H); ^{2}H NMR (61.5 MHz, $CHCl_3$, ref. to $CDCl_3$ at 7.26): δ = 3.59 (br s); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 172.2, 156.6, 82.4, 80.1, 57.5 (m), 51.0, 36.5, 28.4, 28.1.

Acetic acid saturated with HCl gas (5 mL) was added to a solution of *t*-butyl (*S*)-*N*-(*t*-butoxycarbonyl)-4,4-dideuterohomoserinate (0.30 g, 1.0 mmol) in CH_2Cl_2 (5 mL), at RT. The mixture was stirred for 4 h. The solid was collected by filtration and dried in vacuo to give a highly hygroscopic solid (0.16 g, 0.94 mmol, 94% yield). $[\alpha]_D$ = −26.3 (c = 0.095 g/100 mL, H_2O); ^{1}H NMR (400 MHz, D_2O): δ = 4.42 (dd, J = 11.6, 9.2 Hz, 1H), 2.81 (dd, J = 12, 9.2 Hz, 1H), 2.41 (t, J = 12 Hz, 1H); ^{2}H NMR (61.5 MHz, H_2O, ref to HOD at 4.80): δ = 4.57 (br s) 4.41 (br s); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 174.3, 58.5 (m), 48.3, 26.3; IR (KBr) 1453, 1745 cm^{-1}; MS (EI): 122 amu.

Fungal cultures and precursor feeding: *Neotyphodium uncinatum* (CBS 102646 at Centraalbureau Voor Schimmelcultures) was maintained in symbiosis with *Lolium pretense*, from which culture the fungus was isolated as described in Blankenship et al.[16] Fungal cultures were maintained on potato dextrose agar (PDA) plates and routinely subcultured by suspension in sterile water and grinding in an Omni Homogenizer Model 17105 (Omni International, Marietta, GA, USA) followed by drop inoculation onto PDA plates. Fermentation cultures of *N. uncinatum* for precursor feeding were set up in minimal medium (MM; 30 mM potassium phosphate/30 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 5.5) with 2 mM $MgSO_4$, 20 mM sucrose, 15 mM urea, 0.6 μM thiamine, and trace elements).[16] Fungal tissue was removed from PDA plates and homogenized in MM. Cultures were prepared in 100× 25 mm polystyrene Petri plates, each with MM (29 mL) and inoculum (1 mL). Plates were wrapped with parafilm and incubated at 21–23 °C on a rotary shaker at 100 rpm. All labeled compounds were diluted with deionized water and filter-sterilized prior to addition to MM fungal cultures at day 5 to 7 (time dependent on onset of loline alkaloid production). ^{14}C-labeled compounds were added at 8 μCi per 30 mL culture. Stable-isotope-labeled compounds were added to a final concentration of 2–5 mM, as indicated. Culture filtrates were harvested 15–20 days later for GC-MS or NMR analysis.

Gas-chromatographic and mass-spectrometric analysis: Conditions for the extraction and GC analysis of loline alkaloids were as described in Blankenship et al.[16] For GC-MS analysis the culture sample (2 mL) was lyophilized and then extracted with NaOH (100 μL, 1 M) and chloroform (1 mL). The aqueous layer was removed, and the organic layer was concentrated. The residue was made up to 100 μL with chloroform, placed in glass GC vials, capped, and 1 μL of each sample for GC-MS was injected into a Hewlett–Packard G1800A GCD that was equipped with a 30 m Restek column (5 Sil MS, 0.25 mm i.d., 0.25 μm film thickness) and an electron ionization detector. Data were collected with GCD ChemStation (G1074A, Version A.00.00) software. Further analysis of GC-MS data utilized WSEARCH32 Version 1.1.2004 (http://minyos.its.rmit.edu.au/~rcmfa/search.htm). Values for enrichment of precursors were calculated from the absolute intensity of the GC-MS data as:

$$\%\ \text{enrichment} = \left[\frac{[M+n]_{\text{labeled}}}{[M]_{\text{labeled}} + [M+n]_{\text{labeled}}} \times \frac{[M+n]_{\text{unlabeled}}}{[M]_{\text{unlabeled}} + [M+n]_{\text{unlabeled}}}\right] \times 100$$

where $[M]$ = intensity of a fragment peak, $[M+n]$ = the intensity of the peak expected to contain the isotopically substituted ion, and "labeled" and "unlabeled" represent the spectra of compounds isolated from cultures that were or were not fed with labeled precursors.

TLC separation of loline alkaloids: Fermentation cultures were centrifuged in 50 mL tubes (700 *g*, 10 min, RT) in order to pellet fungal mycelium. The supernatant was removed and lyophilized. NaOH (2 mL, 1 M) and chloroform (15 mL) were added to the residue obtained from 90–120 mL of culture filtrate. After vigorous shaking, samples were separated in a centrifuge (700 *g*, 5 min, RT), and the chloroform layer was removed. The extraction was repeated, and the combined chloroform fractions were concentrated to approximately 200 μL and spotted onto a silica gel TLC plate. The solvent for the development of TLC plates was 49.5% chloroform, 49.5% methanol, and 1% ammonium hydroxide. After exposure to I_2 vapor to stain the alkaloids, the area corresponding to **1** (R_f = 0.69) was scraped and placed into a 2 mL microcentrifuge tube. Compound **1** was extracted from silica with D_2O (2×400 μL). The pooled extracts were brought up to 750 μL in D_2O for NMR analysis.

NMR analysis: ^{13}C NMR analysis was performed on a Varian Gemini 200 with VNMR 6.1B software (Varian Inc., Palo Alto, CA) at RT with proton decoupling. Methanol (5% by volume) was used as either an internal or external standard, with the methanol peak set to 49.0 ppm. ^{15}N NMR analysis was performed on a Varian 400 with VNMR 6.1B software at RT and proton decoupling. Samples for ^{15}N NMR analysis contained $^{15}NH_4NO_3$ (5 mg) as the internal standard, and the NH_4 peak was set to 20 ppm. Sample tubes were washed thoroughly first with 95% ethanol (2×) and then acetone (2×), and were dried with N_2 gas between sample runs.

Radioactivity-incorporation determination: Samples (1 mL each) from cultures that were fed with ^{14}C-labeled compounds were extracted for TLC separation. Each sample was concentrated to approximately 100 μL and applied to the TLC plate, which was then developed as described above. The plate was autoradiographed with a phosphor cassette and PhosphoImager 445SI Version 4.0 (Molecular Dynamics). The spot associated with **1** was eluted with NaOH (200 μL, 1 M) and then extracted with chloroform (1 mL). Compound **1** was quantified by GC. A sample (100 μL) of the organic phase was placed in a scintillation vial with Bio-Safe II Biodegradable Counting Cocktail (2 mL; Research Products International Corp., Mount Prospect, IL, USA), and the radioactivity was determined with a Packard 1900 TR Liquid Scintillation Analyzer.

Polyamine analysis: Polyamines were extracted from cultures that had been fed with ^{14}C-Orn and ^{14}C-Put by a procedure adapted for tissue or cultured cells.[23] Remaining supernatant was removed from the harvested fungal mycelium by centrifugation, and the mycelium was washed with deionized water. All supernatants were saved for later analysis of labeled lolines. Washed fungal mycelium (100 mg) mixed with 2% perchloric acid (1 mL) in a 1.7 mL microcentrifuge tube was ground with a plastic pestle. This mixture was kept overnight at 4 °C. The homogenate was then centrifuged at 15700 *g* for 10 min. Dansyl chloride in acetone (400 μL, 5 mg mL^{-1}) was added to the supernatant (200 μL) in a 2 mL microcentrifuge tube. Note: dansyl chloride is light sensitive, and the following procedures were carried out with aluminum foil covering. The sample was thoroughly mixed, and saturated sodium-carbonate solution (200 μL) was added to it. The mixture was then thoroughly vortexed and stored overnight at RT. A solution of L-Pro (15 mg) in deionized water (100 μL) was added, and the mixture was vortexed. The L-Pro and excess dansyl chloride were allowed to react at RT for 1 h before extraction with toluene (500 μL). The toluene layer was removed and concentrated to 100 μL, then spotted on a silica gel TLC plate. The plate was developed with cyclohexane/ethyl acetate (40%:60%), then air-dried in the dark. Plates were exposed to UV light and photographed to determine the positions of dansylated compounds. A phosphor cassette (35×43 cm; Molecular Dynamics, Sunnyvale, CA) was then exposed to the TLC plate and the incorporation of the radioactivity was determine with a Molecular Dynamics PhosphoImager 445SI Version 4.0. Spd and Spm spots were examined for radioactive enrichment.

Acknowledgements

This work was supported by the United States National Science Foundation grants IBN/MCB-0213217 and CHE-997841 and the University of Kentucky's Research Challenge Trust Fund. We also thank the United States National Institutes of Health (GM61002) for partial support of this work. We appreciate the advice on NMR analysis provided by Dr. Mark Meier and Dr. Anne-Frances Miller and the technical support provided by Dr. Franklin F. Fannin, W. John Layton, Jennifer L. Wiseman, Mr. Walter Hollin, and Mr. Alfred D. Byrd. Kentucky Agricultural Experiment Station publication number 04-12-166, published with the approval of the director.

Keywords: alkaloids · biosynthesis · natural products · nitrogen heterocycles · NMR spectroscopy

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Received: September 13, 2004
Published online on April 28, 2005

