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Biosynthesis of Slaframine, (1*S*,6*S*,8a*S*)-1-Acetoxy-6-aminooctahydroindolizine, a Parasympathomimetic Alkaloid of Fungal Origin. 3. Origin of the Pyrrolidine Ring†

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ABSTRACT: The phytopathogen *Rhizoctonia leguminicola* has previously been shown to incorporate pipecolic acid into the piperidine alkaloids 1-acetoxy-6-aminooctahydroindolizine (slaframine) and 3,4,5-trihydroxyoctahydro-1-pyrindine. In the experiments described here, resting cultures of *R. leguminicola* were incubated with [1-^{14}C]- and [2-^{14}C]malonic acid and with [1-^{14}C]- and [2-^{2}H]acetic acid. Both acids were incorporated into the ring systems of both alkaloids. Mass spectrometric analysis of ^{2}H-enriched slaframine showed that the label resides in the five-membered ring and that the methyl carbon of acetate is joined to the carboxyl carbon of pipecolate. A pipecolate-dependent decarboxylation of [1-^{14}C]malonate was demonstrated in cell-free extracts of *R. leguminicola*. The results account for previously unattributed carbons in the two alkaloids and suggest the formation of an eight-carbon intermediate common to both alkaloids by acylation of malonate with pipecolic acid.

Slaframine (**6**) is unusual among the piperidine alkaloids derived from lysine in that the carboxyl carbon of the amino acid is preserved in the final product (Guengerich et al., 1973b). Figure 1 illustrates some possible routes from lysine to slaframine within the context of our prior knowledge. Experiments described in the first two papers of this series (Guengerich et al., 1973b; Guengerich & Broquist, 1973) demonstrated that the phytopathogen *Rhizoctonia leguminicola* incorporates lysine into slaframine via pipecolic acid (**1**). It was shown that certain potential bicyclic precursors of slaframine [1-keto- (**4**), 1-hydroxy- (**5**), and 1,6-dihydroxyoctahydroindolizine] could be converted to slaframine by *R. leguminicola*, but no information was gained on compounds intervening between pipecolic acid and the bicyclic compounds.

With the demonstration that acetate as acetyl coenzyme A (acetyl-CoA) is the immediate precursor of the 1-acetoxy substituent of slaframine (Guengerich et al., 1973b), all carbons in the alkaloid were accounted for, except for the two completing the pyrrolidine ring.

Because *R. leguminicola* can convert pipecolic acid to a second piperidine alkaloid with different ring fusion [3,4,5-trihydroxyoctahydro-1-pyrindine, **7** (Guengerich et al., 1973a)], again with retention of the pipecolate carboxyl, it was attractive to postulate (Clevenstine & Broquist, 1976) addition of two carbons to the pipecolate carboxyl carbon to give an intermediate (**2**) capable of cyclization to either product; likely routes from **2** to the alkaloids via known intermediates are shown in Figure 1.

The structures of the two alkaloids would readily accommodate condensations of the C_2 donor with the pipecolyl fragment either at the acid or the aldehyde oxidation states, but Guengerich provided evidence (Guengerich et al., 1973b) favoring condensation at the carboxyl level. These considerations implicated "pipecolylacetate" or a related species (**2**) as the fugitive common intermediate and prompted us to test acetate and malonate as potential C_2 donors. The data acquired enabled us to rule out some of the possible alternatives offered in Figure 1.

Materials and Methods

Isotopic Compounds. Disodium [1-^{14}C]- and [2-^{14}C]-malonate and sodium [1-^{14}C]acetate were purchased from New England Nuclear or Amersham/Searle. Partition chromatography (Ramsey, 1961) showed that the malonate from Amersham/Searle was contaminated with a small quantity of acetate, which was removed by the same technique. DL-(*R*)-[^{3}H]Pipecolic acid (180 mCi/mmol) came from stocks prepared by Guengerich from material synthesized at New England Nuclear by catalytic hydrogenation of picolinic acid with tritium gas (see Guengerich et al., 1973b).

Deuterioacetic acid (Gold Label, 99.5% $C^2H_3COO^2H$) was purchased from Aldrich Chemical Co.

Cultivation of Rhizoctonia leguminicola and Isolation of Alkaloids. The organism (ATCC 26280) was maintained on slants of filtered red clover hay infusion (1000 mL of water per 100 g of dry chopped hay) hardened with 1.5% Bacto-Agar (Difco). Inoculum for experiments was prepared by transferring a tuft of mycelium from a slant to the surface of 240 mL of sterile hay infusion in a 1000-mL Roux bottle. After 2 weeks growth at room temperature, the mycelial mat was blended in 150 mL of sterile distilled water, and approximately

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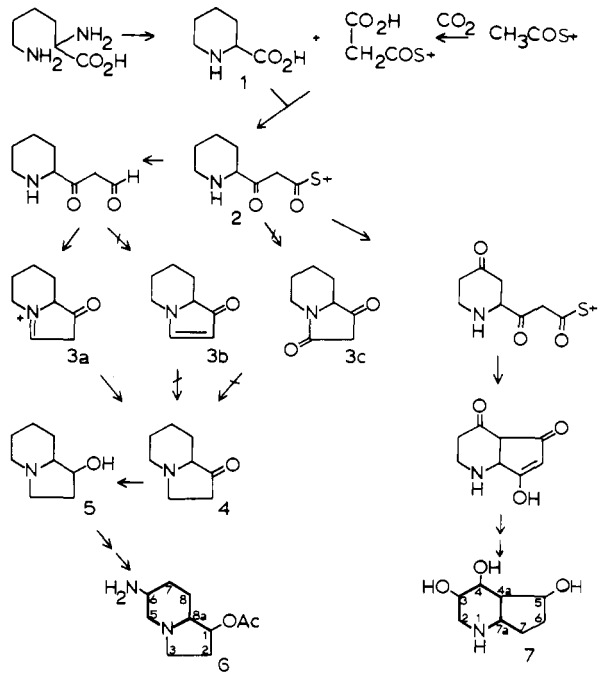

FIGURE 1: Hypothetical scheme of alkaloid biogenesis in *Rhizoctonia leguminicola*.

8-mL aliquots of the resulting suspension were dispensed to Roux bottles containing 240 mL of sterile hay infusion. These experimental cultures were incubated on open shelves at room temperature.

To examine the effect of aeration on growth and alkaloid production, 4-mL aliquots of inoculum were dispensed to 60-mL portions of hay infusion in 250-mL Erlenmeyer flasks. These cultures were incubated at room temperature at 150 rpm in a New Brunswick shaker cabinet.

Radioactive precursors were administered to the stationary cultures by decanting the growth medium after pads had reached confluence (10–12 days) and resuspending the pads on 50 mL of sterile distilled water containing the compounds of interest. After 48-h incubation, cultures were harvested, blended in 95% ethanol, and extracted in Soxhlet extractors for 24 h. Slaframine was recovered from the ethanolic extract essentially by the method of Broquist & Snyder (1971). The resulting slaframine dipicrate could be recrystallized from 20% aqueous ethanol. For comparison of incorporation efficiencies, the compounds to be compared were administered only to cultures of a single batch, in order to minimize the effect of batch-to-batch variations in growth media and alkaloid yield.

Deuterioacetic acid was administered by preparing an 0.8 M solution in water, adjusting the pH of the solution to 5 with concentrated ammonium hydroxide, and injecting aliquots of the filter-sterilized solution under the mycelium of *R. leguminicola* cultures at the following times after inoculation: 168 h (1 mL), 197 h (1 mL), 234 h (2 mL), 266 h (2 mL). After a further 24-h incubation, the hay infusion was decanted from the cultures and they were each resuspended on 50 mL of sterile distilled water containing 0.50 mmol of $C^2H_3COO^2H$ and 0.25 mmol of DL-pipecolic acid, adjusted to pH 5.6 with concentrated ammonium hydroxide. After 48 h, the cultures were harvested and slaframine was extracted as described above.

Radioactive pipecolic acid (1–5 μCi/flask) was administered to the agitated cultures 5 days after inoculation. Cultures were harvested after 48 h and extracted as described above. The aqueous suspension remaining after evaporation of ethanol was treated with 50% aqueous trichloroacetic acid to pH 2 and centrifuged; the clarified supernatant was applied to the ion-exchange column described in the next paragraph.

The second piperidine alkaloid, 3,4,5-trihydroxyoctahydro-1-pyrindine, was monitored and isolated by the method of Guengerich et al. (1973a). This involved elution of the aqueous portion of *Rhizoctonia* extract from a 12 × 500 mm column of Dowex 50-X8 (200–400 mesh) with a pH gradient formed by running 300 mL of 0.25 N NaOH into 300 mL of 0.25 N citrate buffer, pH 3.1. Radioactive fractions eluting at pH 7.5–9.5 were desalted on a second Dowex 50-X8 column (12 × 200 mm; 400 mL of distilled water followed by 400 mL of 1 N HCl) and then purified by thin-layer chromatography (silica gel; chloroform:methanol:concentrated ammonium hydroxide; 10:8:1) or gas–liquid chromatography (Varian 2100; N_2 carrier; FID; 4 mm × 2 m glass column with 10:1 glass splitter; 3% OV-17 on 80–100 mesh Chromosorb W). The identity of the radioactive material on the TLC plate (R_f ca. 0.75) and of the product collected from the gas chromatograph (retention time 2 min at 140 °C and 60 mL of N_2/min) was verified by mass spectrometry (see below). The presence of radioactive 7 could be observed on the ion-exchange chromatogram without further purification, as could the presence of slaframine.

No weighable quantity of the pyrindine alkaloid was isolated. Trace quantities of tritiated 7 were used to track material labeled with stable isotopes.

Slaframine isolated as the dipicrate was deacetylated as follows. Approximately 1 mg of picrate was freed of picric acid by suspension in 0.5 mL of 3 N HCl and extracted four times with equal volumes of diethyl ether. Sufficient 6 N KOH was added to make the solution 1.5 N in OH^-. The solution was heated to boiling, cooled, acidified with 3 N HCl, and applied to a 4 × 70 mm (Pasteur pipet) column of Dowex 50-X8 (200–400 mesh, H^+ form). The column was eluted with 0.25 N citric acid until five 15-drop fractions had been collected. Elution was continued with 0.25 N trisodium citrate (pH 8.3) until 10 fractions had been collected and then with 0.25 N NaOH (pH 12.3) until radioactivity and/or ninhydrin-positive material detectable in the eluate had returned to base-line levels. Elution of ninhydrin-positive material was monitored by adding 0.5 mL of ninhydrin solution (Clark, 1964) to 0.35 mL of each column fraction and developing and reading color according to Clark (1964).

Radioactivity Measurements. Elution profiles of ion-exchange columns were obtained by transferring 0.2-mL (from the large gradient column) or 0.4-mL (from the small stepwise column) aliquots of each fraction to 20-mL polyethylene scintillation vials, adding 10 mL of cocktail (6 g of diphenyloxazole and 200 mL of ethanol per L, made up with toluene), and counting in Nuclear Chicago Mark I or Packard Tri-Carb liquid scintillation spectrometers. Counting efficiencies were determined by adding known quantities (on the order of 0.5 μCi) of the appropriate isotope ([^{14}C]malonate or [^{3}H]pipecolate) to vials representing different eluate compositions and recounting.

The specific activity of slaframine biosynthesized from radioactive precursors was determined by suspending a weighed quantity (1–5 mg) of recrystallized slaframine dipicrate in 0.5 mL of 1 N HCl and removing picric acid as described above. The acidic solution was transferred to a scintillation vial, dissolved in 10 mL of cocktail, and counted. Efficiency was then determined as described above.

Mass Spectrometry. Mass spectra were obtained with an LKB 9000A mass spectrometer, operating in the electron-impact ionization mode at an ionizing voltage of 70 eV and

Table I: [1-^{14}C]- and [2-^{14}C]Malonate Incorporation into Slaframine

disodium malonate fed			slaframine recovered		
label position, C no.	malonate concn (μM)	sp act. (μCi/mmol)	total act. (μCi)	sp act. (μCi/mmol)	sp act. of slaframine/ sp act. of malonate
1	14	9.5×10^3	2.3×10^{-2}	2.19	2.3×10^{-4}
2	14	9.5×10^3	7.1×10^{-2}	4.84	5.1×10^{-4}
1	1000	5.0×10	5.5×10^{-4}	0.22	4.4×10^{-3}
2	1000	5.0×10	2.3×10^{-3}	1.0	2.0×10^{-2}

an accelerating voltage of 3.5 kV.

Slaframine for mass spectrometric analysis was freed of picric acid as described above. The acidic solution was made basic (pH 10) with solid sodium carbonate and extracted with equal volumes of chloroform. The chloroform extract was dried by percolation through anhydrous sodium sulfate in a Pasteur pipet, and an aliquot was transferred to a direct-inlet sample tube for the mass spectrometer and evaporated. Deacetylslaframine prepared as described above was extracted from the hydrolysis mixture with chloroform and transferred to a sample tube as just described.

The octahydropyrindine alkaloid **2** collected in Pasteur pipets from the splitter outlet of the gas chromatograph was rinsed into mass spectrometer tubes with small quantities of methanol. Alkaloid separated by thin-layer chromatography was recovered by scraping silica gel from the TLC plate and transferring it to a Pasteur pipet plugged with paper. Small quantities of methanol were rinsed through the pipet into a small vial, and an aliquot of this extract was transferred to a sample tube and dried.

It was found after most of the experiments described here that **1** and **2** could be analyzed in appropriate desalted ion-exchange fractions by evaporating the material, taking up the solubles in methanol or chloroform, and injecting the material onto a short column (3 × 280 mm; 10% OV-11 on Supelcoport) coupled to the separator of the mass spectrometer. The alkaloids were eluted by raising the column temperature 8 °C/min from an initial temperature of 100 °C. The pyrindine eluted at 150–160 °C and slaframine at 160–170 °C (He carrier gas flow 2.5 mL/min).

Decarboxylase Assay. A 14-day-old mycelial mat of *R. leguminicola* was harvested, washed in tap water, torn into small pieces, and blended in 50 mL of ice-cold 0.1 M sodium phosphate buffer (pH 7.0) to which 10 μL of 2-mercaptoethanol had been added. The suspension was sonicated to homogeneity at 4–10 °C with a Branson sonifier (six to eight 30–45-s bursts at maximum power). Filtration through glass wool yielded 30 mL of a brown suspension. A 20-mL aliquot of this suspension was passed through a refrigerated (7 °C) 24 × 170 mm column of Sephadex G-25. The 20 mL of brown opalescent material eluting after the void volume was collected. Aliquots (2 mL) of this preparation were incubated under nitrogen in stoppered Warburg flasks with [1-^{14}C]malonate and cofactors, as described in Table III, in the presence and absence of DL-pipecolic acid. Reaction was initiated by adding mixed reactants from the sidearm of the flask to extract in the body. Radioactive CO_2 liberated was trapped in 0.2 mL of 6 N KOH in the center well of the flask. The reaction was terminated by addition of 0.5 mL of 20% H_2SO_4 from the other arm of the flask. Aliquots (0.1 mL) of the KOH solution were subjected to liquid scintillation counting as described above. Protein was measured according to Lowry et al. (1951).

Results and Discussion

Figure 2 compares the ion-exchange chromatograms of extracts of *R. leguminicola* incubated with (*R*)-[^{3}H]pipecolic acid (Figure 2a) and with [2-^{14}C]malonic acid (Figure 2b). Peaks corresponding to both piperidine alkaloids are labeled by malonic acid. Chromatograms of extracts of cultures incubated with [1-^{14}C]acetic acid were similar in appearance to the malonate profile, but the alkaloid peaks were less prominent.

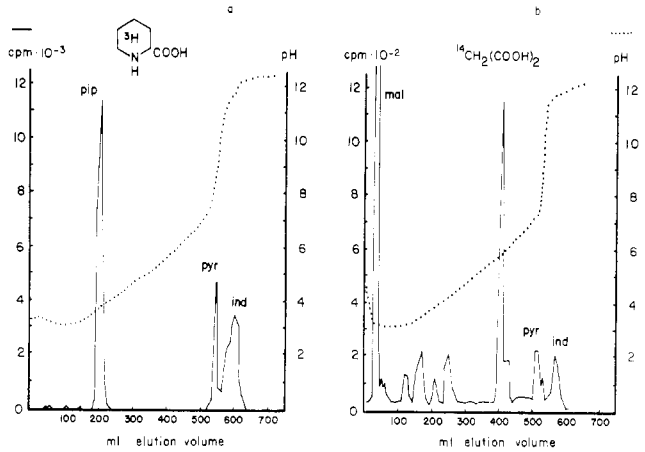

FIGURE 2: Pipecolate and malonate as precursors of piperidine alkaloids in *Rhizoctonia leguminicola*. (a) Incubation with (*R*)-[^{3}H]pipecolic acid. Aliquots (0.5 mL, 70 nmol, 60 μCi/mL) of aqueous (*R*)-[^{3}H]pipecolic acid were injected into the 240 mL of growth medium under each of three mycelial mats of 14 two-week-old cultures 2 days before harvest. An ethanol extract of all the mycelium was prepared and analyzed as described under Materials and Methods. (b) Incubation with [2-^{14}C]malonic acid. Two 11-day-old mycelial mats were incubated on 50 mL of sterile 5 mM pipecolic acid containing 1 μCi (0.11 μmol) of [2-^{14}C]malonic acid for 2 days. Mats were then harvested and an ethanol extract of the mycelium was prepared and analyzed as described under Materials and Methods. Abbreviations: pip, pipecolic acid; pyr, 3,4,5-trihydroxyoctahydro-1-pyrindine; ind, 1-acetoxy-6-aminooctahydroindolizine, slaframine; mal, malonic acid.

Table II: Malonate vs. Acetate Incorporation into Slaframine

precursor fed			slaframine dipicrate recovered		
label position	prec concn (μM)	sp act. (μCi/mmol)	total act. (μCi)	sp act. (μCi/mmol)	sp act. of slaframine/ sp act. of precursor
M-2	3.4	1.2×10^4	3.1×10^{-3}	0.81	6.7×10^{-5}
A-1	40	2.0×10^3	2.2×10^{-3}	0.41	2.0×10^{-4}
M-2	400	4.0×10	5.6×10^{-3}	0.86	2.1×10^{-2}
A-1	400	1.0×10^2	5.0×10^{-3}	0.62	6.2×10^{-3}

Tables I and II show representative values for the efficiency of incorporation of malonate and acetate into slaframine.

The efficiency of isotope incorporation is expressed in the tables as a ratio of specific activities rather than as a percent incorporation because the latter measure depends on recovery of the product; slaframine recoveries are highly variable from run to run (as can be seen by dividing the values in column 4 of Table II or III by corresponding values in column 5). The ratio of total radioactivity recovered in the product to the total

Table III: C^2H_3COOH Incorporation into 3,4,5-Trihydroxyoctahydro-1-pyrindine

m/e	% rel[a] intensity	intensity of m/e + 1 satellite (%)[b] unenriched	enriched
173	21	10	23
156	73	7	26
116	33	7	20
113	100	13	12

[a] Height of peak as percent of height of base peak at *m/e* 113. [b] Height of satellite peak as percent of height of indicated peak.

radioactivity of the precursor fed might not reflect the extent to which the product recovered represents material converted from the radioactive precursor proffered, but comparison of the specific activities of precursor and product does.

Table I shows that label in the carboxyl group of malonate is less efficiently incorporated than is label in the methylene group, as one would expect if decarboxylation is part of the incorporation process. The fact that the ratio of carboxyl to methylene incorporation efficiencies is less than 0.5 implies that there is some equilibration between malonate and acetate before all labeled malonate is either degraded or incorporated into products. More will be said on this point after presentation of mass spectrometric data. In comparing malonate incorporation with the lysine and pipecolate incorporation data of Guengerich et al. (1973b), it can be said in general that malonate is incorporated about as efficiently as lysine but much less efficiently than pipecolate. The latter observation is not surprising in view of the more restrictive metabolic role of pipecolate.

Table II shows that acetate incorporation is of the same order of magnitude as malonate incorporation, but slightly less efficient, as would be expected of a more distal precursor. It was found that, although specific incorporation values are independent of recoveries, they are a function of precursor concentration. With both acetate and malonate, tenfold increase in precursor concentration entails an approximate tenfold increase in specific incorporation.

The premise on which the experiments were undertaken places the label from [2-^{14}C]malonate or [2-^{14}C]acetate in the 2 position of the indolizine nucleus; alternatives include (a) the 1-acetoxy group or (b) the pipecolic acid derived moiety. In previous work, Guengerich has in fact demonstrated (Guengerich et al., 1973b) utilization of acetyl-CoA in the formation of slaframine from deacetylslaframine. Acetate could also find its way into pipecolic acid via the lysine biosynthetic pathway (condensation of acetate with α-ketoglutarate from the tricarboxylic acid cycle to form homocitrate).

Figure 3 shows that hydrolytic cleavage of the acetate group from slaframine which had been obtained from cultures incubated with labeled malonate removes only approximately half the label from the alkaloid. This indicates that the remainder of the label resides in the indolizine nucleus. Such a conclusion is supported by the presence of a radioactive peak corresponding to the pyrindine alkaloid **2** in Figure 2b. Since this compound bears no carbon-containing substituents, label from malonate must be incorporated into the ring system itself.

The site of labeling in the indolizine nucleus could be established by systematic degradation, but methods were not available to carry out the required transformations on the small quantities of material available. As an alternative, incorporation studies were undertaken with acetate containing stable isotopic substituents, so the location of isotope incorporation

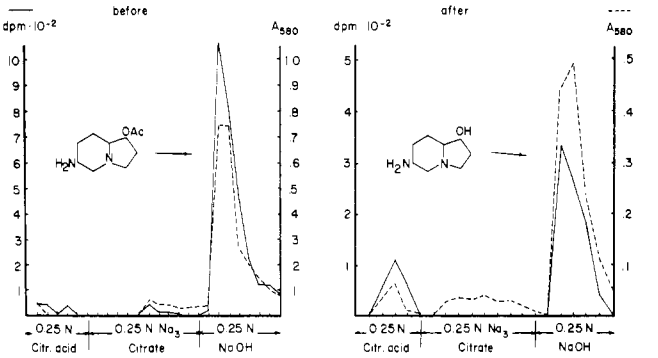

FIGURE 3: [2-^{14}C]Malonate labeling of slaframine and deacetylslaframine. Slaframine dipicrate (0.95 mg, 0.78 μCi/mmol) labeled with [2-^{14}C]malonic acid obtained from an experiment similar to that described in line 2 of Table I was hydrolyzed and chromatographed as described under Materials and Methods. For the unhydrolyzed slaframine control, the acidic solution resulting from extraction of the picric acid from 1.48 mg (0.78 μCi/mmol) of slaframine picrate was chilled in ice and treated with appropriate quantities of KOH and HCl; the resulting slightly acidic solution (0.90 mL) was immediately subjected to chromatography.

into the alkaloid could be established by mass spectrometry.

Incubation of *R. leguminicola* with C^2H_3COOH yielded slaframine (Figure 4) and octahydropyrindine alkaloid (Table III) containing deuterium. Since the methyl carbon of acetate would become the keto carbon of α-ketoglutarate and of α-ketoadipate in the course of lysine biosynthesis, acetate incorporation by this route would not be detected.

Because the proffered acetate is incorporated into slaframine's acetoxy substituent as well as into the octahydroindolizine nucleus, the mass spectrum of the native alkaloid is complicated by enhanced isotope satellites up to five units higher than the nominal molecular weights of the parent and several major fragments (Figure 4a). Removal of the *O*-acetyl group simplifies the mass spectrum.

Comparison of the mass spectra of normal and deuterated deacetylslaframine (Figures 4b and 4c, respectively) reveals that the satellite peaks appearing two mass units above three of the major fragments are greatly enhanced in the deuterated material. The location of the deuterons is indicated by the fact that the fragment ions at *m/e* 100 and *m/e* 113 have substantial P + 2 satellites, while the fragment with *m/e* 70 does not. The *m/e* 100 and 113 ions and their satellites are derived primarily from the pyrrolidine ring (carbons 1, 2, 3, and 8a) plus carbons 5 or 5 and 8 (Gardiner et al., 1968; Gardiner, 1970), while the *m/e* 70 fragment represents predominantly carbons 3, 5, 8, and 8a. Carbon 1 is known from radioisotope studies to arise from the pipecolate carboxyl group rather than from acetate (Guengerich et al., 1973b), and, furthermore, its oxidation state prevents it from carrying two deuterium atoms. The isotopic substituents must, therefore, be on carbon 2. This implies that the methyl carbon of acetate (the methylene carbon of malonate) becomes carbon 2 of slaframine and that the carboxyl becomes carbon 3. This orientation is consistent with the hypothesis offered above.

In the pyrindine alkaloid (Table III), only one of the deuterium atoms is preserved, in contrast to the two observed in the indolizine skeleton of slaframine. It is likely that the deuterium is at the 7 position in **7** reflecting a parallel pathway of formation but this hypothesis cannot be proven until spectroscopic or degradative methods have been developed to establish the location of isotopic label. The difference between the two alkaloids may reflect existence of an intermediate in the formation of **7** for which isotopic exchange with the medium is facilitated. It more likely is an alkene intermediate

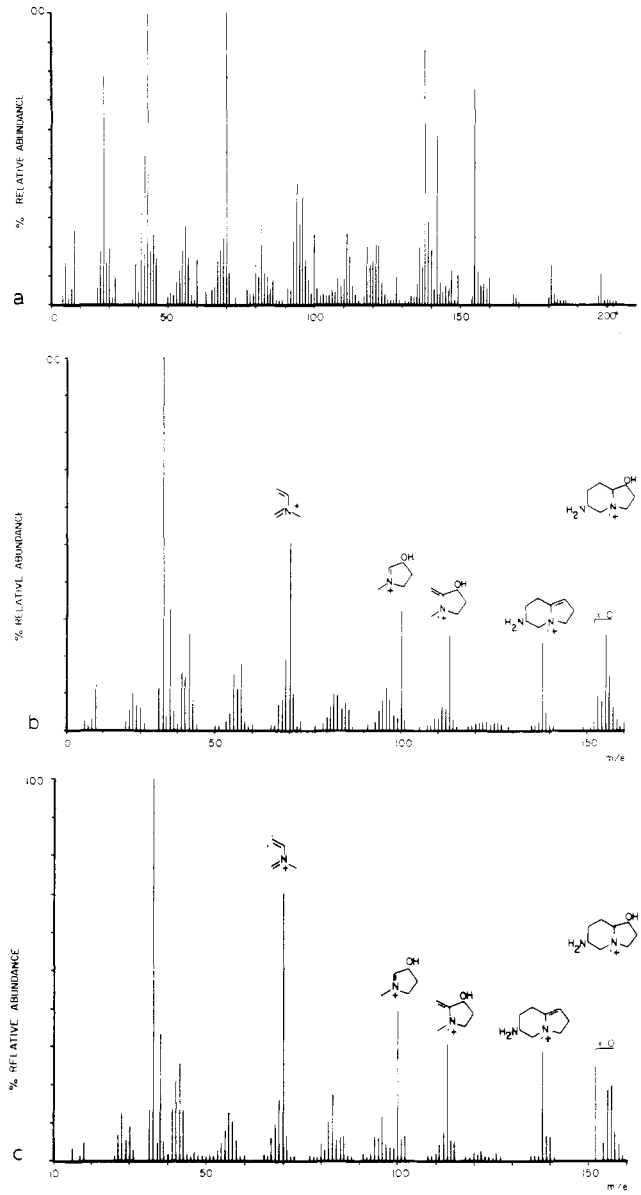

FIGURE 4: (a) Low-resolution mass spectrum of slaframine isolated from *R. leguminicola* incubated with C^2H_3COOH. (b) Spectrum of deacetylslaframine isolated from *R. leguminicola* grown under normal conditions. (c) Spectrum of deacetylslaframine isolated from *R. leguminicola* incubated with C^2H_3COOH. Note enhanced satellites 2 mass units above major fragments.

or other species having only one hydrogen atom at this position.

The results of the deuterium incorporation experiment with slaframine argue against sequential acylation and decarboxylation of malonate. A 2-pipecolylmalonate intermediate would allow the preservation of only one hydrogen. The presence of two isotopic hydrogens on this carbon in the final product implies that the postulated pipecolate–malonate condensation is concerted with decarboxylation, although conceivably the condensing enzyme retains a hydrogen and reinserts it after decarboxylation, as aconitase reinserts a proton after shifting a carboxyl group in the conversion of citrate to isocitrate (Rose & O'Connell, 1967). The preservation of two deuterons also rules out the enamine as the first committed precursor of slaframine.

The likelihood of a concerted reaction is supported by the observations of Arnstadt et al. (1975): in the formation of β-hydroxybutyrate by the condensing enzyme of fatty acid synthetase, exchange of methylene hydrogens from malonate was not detected, either in experiments with labeled malonate

Table IV: Pipecolate-Dependent Decarboxylation of [1-^{14}C]Malonate by Cell-Free Preparations of *Rhizoctonia leguminicola*

system	sp act. (pmol of CO_2 liberated h^{-1} (mg of protein)$^{-1}$)[c]			
	expt 1	expt 2	expt 3	expt 4
complete system[a]	148 ± 12	294 ± 58	730 ± 30	265 ± 8
−pipecolate[b]	90 ± 8	138 ± 43	542	199 ± 30
−pipecolate + proline			570 ± 20	193 ± 4
−coenzyme A				36 ± 4
boiled enzyme			36 ± 4	

[a] Contains 2.0 mL of cell-free *Rhizoctonia leguminicola* extract prepared as described in the text, 1 μCi of disodium [1-^{14}C]malonate, 4.5 μmol of ATP, 0.45 μmol of CoA, 2.6 μmol of malonate, and additions as follows: (expt 1) 5 μmol of DL-pipecolate; (expt 2) 5 μmol of DL-pipecolate; (expt 3) 2.5 μmol of DL-pipecolate, 2.5 μmol of L-proline; (expt 4) 3.7 μmol of DL-pipecolate, 3.7 μmol of L-proline. [b] Omissions replaced with equal volume of phosphate buffer (0.1 M, pH 7.0). [c] Mean ± standard error of duplicates or triplicates.

or with labeled solvent. Although these observations could be explained by retention of hydrogen by the enzyme during formation of a carbanion intermediate, the failure of deuterium at C-2 to exert an isotope effect on the reaction rate argues against any such dissociation.

The postulated condensation of pipecolate and malonate should be detectable as a pipecolate-dependent release of radioactivity from carboxyl-labeled malonic acid. Table III contains data from preliminary experiments which show that such an activity does indeed seem to be present in cell-free preparations of *R. leguminicola*. The various experiments shown represent attempts to maximize the difference between pipecolate-dependent decarboxylation (Table IV) and nonspecific decarboxylation by manipulating buffer, cofactor, and substrate compositions. In all cases, nonspecific activity was responsible for the major decarboxylase activity. Since the nonspecific activity as well as the pipecolate-dependent activity seems to require coenzyme A, it may reflect fatty acid synthesis, although the low efficiency of [1-^{14}C]malonate incorporation into slaframine (Table I) argues that some of the activity is simple decarboxylation without fixation of the remaining carbons in some product. We are continuing to seek an optimum assay system as a starting point for attempts to purify the pipecolate-dependent condensing enzyme.

The results of the experiments described above support the hypothetical biosynthetic pathways to alkaloids **6** and **7** illustrated in Figure 1. We propose that acetate is carboxylated to malonate at the thiol ester level, as in the biogenesis of fatty acids and polyketide secondary metabolites; malonate is acylated by an activated pipecolate, with simultaneous loss of the carboxyl group; the former acetate carboxyl is reduced to the aldehyde level, permitting formation of iminium ion **3a** which is the first committed precursor of slaframine (**1**, Figure 1); alternatively, position 3 of the piperidine ring is activated to promote formation of the first committed precursor of 3,4,5-trihydroxyoctahydro-1-pyrindine (**7**, Figure 1).

The likelihood of the iminium ion being the first committed precursor of slaframine is supported by the retention of two deuteriums at carbon 2 of the final product, as this rules out enamine intermediate **3b**. The lactam of "pipecolylacetate", which would result from cyclization at the acyl level, is not an efficient precursor of slaframine (Clevenstine et al., 1979).

The idea that a common intermediate is directed to slaframine synthesis by a reductive process or to the pyrindine by an oxidative process fits with the observation made by Aust (1965) that growth of *R. leguminicola* in agitated (and thus

more fully oxygenated) medium suppresses formation of slaframine relative to pyrindine. We have repeated this experiment and find that, whereas the combined yield of slaframine and deacetylslaframine is typically 2.2 times that of the pyridine in still cultures, the yields are approximately equal with agitated cultures.

It is interesting from the standpoint of metabolic relationships of host and parasite that *R. leguminicola* should produce characteristic secondary metabolites from two compounds which are characteristic constituents of red clover: pipecolic acid (Greenstein & Winitz, 1961) and malonic acid (Soldatenko & Mazurova, 1957).

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Biosynthesis of Slaframine, (1*S*,6*S*,8a*S*)-1-Acetoxy-6-aminooctahydroindolizine, a Parasympathomimetic Alkaloid of Fungal Origin. 4. Metabolic Fate of Ethyl Pipecolylacetate, 1,3-Dioxooctahydroindolizine, and 1-Hydroxyoctahydroindolizine in *Rhizoctonia leguminicola*†

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ABSTRACT: Known or suspected intermediates in the biosynthesis of slaframine and 3,4,5-trihydroxyoctahydro-1-pyrindine, piperidine alkaloids of the phytopathogenic fungus *Rhizoctonia leguminicola*, were prepared and tested for biological conversions. Ethyl pipecolylacetate, an analogue of the postulated condensation product of pipecolic and malonic acids (two previously identified alkaloid precursors), was insufficiently stable for feeding experiments. The lactam of pipecolylacetate, 1,3-dioxooctahydroindolizine, was degraded by the fungus without direct incorporation into alkaloids. The known slaframine precursor 1-hydroxyoctahydroindolizine was prepared by a novel route which permitted high levels of deuterium enrichment at C-1 and C-3. Mass spectrometric examination of the slaframine biosynthesized from *cis*- and *trans*-[1,3,3-^{2}H]-1-hydroxyoctahydroindolizine strengthened arguments that 1-oxooctahydroindolizine is an intermediate in slaframine biogenesis.

Lysine is converted by the phytopathogenic fungus *Rhizoctonia leguminicola* to two unusual piperidine alkaloids: 1-acetoxy-6-aminooctahydroindolizine (slaframine, **1**) and 3,4,5-trihydroxyoctahydro-1-pyrindine (**2**) (Aust & Broquist, 1965; Guengerich et al., 1973a). As indicated by the heavy lines in the structural drawings in Scheme I, six of the eight carbons in both compounds are derived from the six carbons of lysine via pipecolic acid (Guengerich et al., 1973a,b). In isotope incorporation experiments described in the previous communication in this series (Clevenstine et al., 1979), it has been shown that the remaining two carbons in the five-membered rings of both **1** and **2** are derived from acetate via malonate. These observations are incorporated in the proposed biosynthetic pathway to slaframine shown in Scheme I.

This paper describes two attempts to define further the early stages of slaframine biosynthesis. The first involves feeding ethyl pipecolylacetate (**7**), which is closely related to **3**, the hypothetical common precursor of alkaloids **1** and **2**. The second involves the lactam 1,3-dioxooctahydroindolizine (**4**), which might itself be an intermediate in the pathway or, alternatively, might be able to enter the pathway by conversion to **3**.

During the course of this work a facile synthesis of the 1-hydroxy derivatives (**5a** and **5b**) of octahydroindolizine

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