Sica, V., Nola, E., Parikh, I., Puca, G. A., and Cuatrecasas, P. (1973a), *Nature (London), New Biol. 244*, 36.
Sica, V., Parikh, I., Nola, E., Puca, G. A., and Cuatrecasas, P. (1973b), *J. Biol. Chem.* (in press).
van Heyningen, W., Carpenter, W. B., Pierce, N. F., and Greenough, W. B., III (1971), *J. Infec. Dis. 124*, 415.
Vaughan, T. R. Jr., and Osato, R. L. (1952), *J. Amer. Chem. Soc. 74*, 676.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Biosynthesis of Slaframine, (1*S*,6*S*,8a*S*)-1-Acetoxy-6-aminooctahydroindolizine, a Parasympathomimetic Alkaloid of Fungal Origin. I. Pipecolic Acid and Slaframine Biogenesis†

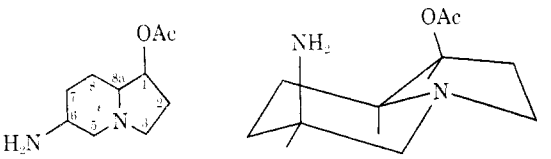
F. Peter Guengerich,‡ J. J. Snyder,§ and Harry P. Broquist*,‡

ABSTRACT: *Rhizoctonia leguminicola*, a fungus that causes black spot disease of red clover, produces an alkaloid that induces excessive salivation in animals. This alkaloid, termed slaframine, has been isolated and characterized as (1*S*,6*S*,-8a*S*)-1-acetoxy-6-aminooctahydroindolizine. Using techniques of radioisotope incorporation and dilution, the biosynthesis of this piperidine alkaloid was studied in growing or resting cultures of *R. leguminicola*. Appropriate radioactive test substances were added to such cultures and, following incubation, slaframine or related compounds were subsequently isolated and examined for radioactivity. DL-[1-^{14}C]Lysine and DL-[6-^{14}C]lysine were equally incorporated into slaframine; such incorporation was effectively blocked by pipecolic acid. Both ring-labeled and carboxyl-labeled pipecolic acid were equally incorporated into slaframine and were approximately 1000 times more effective than lysine as precursors of slaframine. Tritiated 1-ketooctahydroindoindolizine, 1-hydroxyoctahydroindolizine, and 1,6-dihydroxyoctahydroindolizine were readily utilized in slaframine formation, and an NADPH dependent reduction of 1-ketooctahydroindoindolizine to 1-hydroxyoctahydroindolizine was demonstrated in cell-free extracts of *R. leguminicola* cells. Such extracts also catalyzed the acetyl-CoA dependent formation of slaframine from 6-amino-1-hydroxyoctahydroindolizine. A tentative scheme of slaframine biosynthesis stemming from the metabolism of lysine and pipecolic acid is presented.

This laboratory became interested some years ago in the isolation and identification of a mycotoxin elaborated by the fungus, *Rhizoctonia leguminicola*. Consumption by ruminants of legumes such as red clover infested by this fungus results in excessive salivation and can pose problems of some consequence in animal husbandry. General accounts dealing with such aspects together with research on the isolation, chemistry, and physiology of this salivation factor have appeared (Broquist and Snyder, 1971; Aust *et al.*, 1968; Spike and Aust, 1971) and document early work in this field from this and other laboratories.

Chemical studies have now established that this salivation factor, termed slaframine, is (1*S*,6*S*,8a*S*)-1-acetoxy-6-aminooctahydroindolizine (Aust *et al.*, 1966; Gardiner *et al.*, 1968); and its total synthesis has recently been achieved (Cartwright *et al.*, 1970).

In early attempts to increase the yield of slaframine in growing cultures of *R. leguminicola*, and prior to elucidation



of structure, a systematic examination of the effect of amino acids on slaframine synthesis revealed that both DL-[6-^{14}C]-lysine and DL-[1-^{14}C]lysine were incorporated into the alkaloid (Aust, 1965). Brief accounts of progress to date have appeared (Snyder and Broquist, 1968, 1969; Guengerich, 1971) which indicate a role of pipecolic acid in slaframine biosynthesis and will be discussed more fully herein. Using techniques of radioisotope incorporation and dilution, evidence will be presented that slaframine biosynthesis deriving from the lysine metabolism of *R. leguminicola* may include the transformations postulated in Figure 1.

Materials and Methods

Radioactive Lysines. DL-[1-^{14}C]- and [6-^{14}C]lysines were purchased from New England Nuclear. DL-[4,5-^{3}H]Lysine was obtained from Volk Chemical Co.

[^{14}C]Pipecolic Acids, [4,5-^{3}H]Pipecolic Acid. Variously labeled pipecolic acids were synthesized from appropriately labeled lysine in a manner analogous to the synthesis of proline from ornithine (Hamilton, 1952). To DL-lysine·HCl (3.0 mmol) and the desired labeled lysine (*e.g.*, 3 ml of DL-[4,5-^{3}H]lysine·HCl, or 30 μl of DL-[1-^{14}C]lysine·HCl, or 30

† From the Department of Dairy Science, University of Illinois, Urbana, Illinois 61801, and the Department of Biochemistry, Vanderbilt University, Nashville, Tennessee 37232. *Received January 31, 1972.* This work was supported in part by Grants AM-3156 and AM-14338 from the National Institutes of Health, U. S. Public Health Service. F. P. Guengerich is a recipient of a U. S. Public Health Service Predoctoral Fellowship GM-47480.

§ Present address: Abbott Laboratories, North Chicago, Ill. 60064.
‡ Present address: Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tenn. 37232.

μl of DL-[6-^{14}C]lysine·HCl), 75 ml of concentrated HCl was added and the whole mixture was heated to a vigorous reflux; 10 mmoles of nitrous acid in 25 ml of water was added dropwise over a 0.5-hr period, and the mixture was refluxed 2.5 hr. HCl was removed by distillation *in vacuo*, and the residue was left in 25 ml of 1 N NaOH for 12 hr at 25°. The mixture was brought to pH 1 (concentrated HCl), evaporated to dryness *in vacuo* at 80°, and extracted twice with 50 ml of absolute ethanol. The combined extracts were evaporated *in vacuo*. Pipecolic acid was separated from unreacted lysine by applying a solution of the mixture at pH 10 to a Dowex 2 (OH form, 200–400 mesh) column (1 × 25 cm) and eluting with 100 ml of water to remove the lysine. Pipecolic acid was eluted from the column at the 30th 10-ml fraction using 0.25 N HCl. Pipecolic acid fractions were pooled, evaporated to dryness *in vacuo*, dissolved in 2 ml of anhydrous ethanol, and precipitated with two volumes of diethyl ether. Yields ranged from 30 to 40%. The products melted at 256–258° (uncorrected), showed no depression of the melting point of authentic material, and were otherwise shown to be chemically and radiochemically pure by techniques of paper, thin-layer, and ion-exchange chromatography. The position of the label in [1-^{14}C]lysine and [6-^{14}C]lysine was verified by reaction with ninhydrin as in the Moore and Stein assay (1948). [6-^{14}C]-Lysine retained greater than 95%, whereas [1-^{14}C]lysine lost more than 95% of the radioactivity.

[R-^{3}H]Pipecolic Acid. Synthesis of [*R*-^{3}H]pipecolic acid was carried out by New England Nuclear following the procedure of Stevens and Ellman (1950) in which 2-picolinic acid (Aldrich Chemical Co.) was reduced by catalytic hydrogenation with tritium gas. Pipecolic acid, specific activity 250 Ci/mol, was purified by ion-exchange chromatography (Dowex 50, H^+ form, 200–400 mesh, 1.2 × 115 cm column), elution with 1800 ml of 1 N HCl, and subsequent removal of HCl *in vacuo*.

DL-[*R-^{3}H*]*-2-Piperidinemethanol.* [*R*-^{3}H]Ethyl pipecolate was prepared from [*R*-^{3}H]pipecolic acid in the usual manner, using dry HCl as catalyst. The product was extracted into ether. Ethyl pipecolate was reduced with $LiAlH_4$ in ether under nitrogen. The product was purified by ion-exchange (Dowex 50-X8, 1.2 × 25 cm, 200–400 mesh, H^+ form; elution with 1 N HCl) and preparative thin-layer chromatography ($CHCl_3$–MeOH–10% NH_4OH, 40:40:20).

DL-[*1-^{3}H*]*-2-Piperidinemethanol.* Pyridine-2-carboxaldehyde (28 mg, Aldrich) was reduced with 0.2 mmol of [^{3}H]$NaBH_4$ in ethanol. The product was reduced in 40 ml of 0.1 N HCl over 135 mg of PtO_2 for 24 hr at room temperature and 3 atm of pressure. The product was purified by ion-exchange chromatography as before. The product was radiochemically pure as judged by thin-layer chromatography; both labeled 2-piperidine methanols chromatographed with authentic material (Aldrich).

L-[*2-^{3}H*]*Pipecolic Acid.* Δ^1-Piperideine-2-carboxylic acid (188 mg; Meister, 1954) was reduced with [^{3}H]$NaBH_4$ (625 μmol) in ethanol. The resulting DL-[2-^{3}H]pipecolic acid was purified according to Piez *et al.* (1956). The D isomer was removed by treatment with excess hog kidney D-amino acid oxidase (Cooksey and Greenberg, 1965); the L isomer was purified by ion-exchange chromatography (Cooksey and Greenberg, 1965). The product was radiochemically pure as judged by thin-layer chromatography ($CHCl_3$–MeOH–14% NH_4OH, 40:40:20).

1-Ketooctahydroindolizines. Pipecolic acid was esterified by refluxing with hydrogen chloride–saturated ethanol followed by neutralization, extraction, and vacuum distillation.

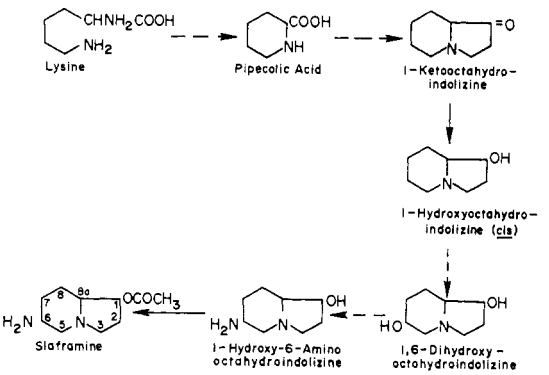


FIGURE 1: A postulated scheme of slaframine biogenesis deriving from the lysine metabolism of *Rhizoctonia leguminicola.*

1-Ketooctahydroindolizine was prepared essentially as described by Clemo and Ramage (1932) by condensation of ethyl pipecolate with ethyl 3-chloropropionate (Aldrich) in the presence of sodium acetate, followed by cyclization using sodium metal. The sodium salt of 1-keto-2-carbethoxyoctahydroindolizine was decarboxylated in 8 N HCl under reflux to yield 1-ketooctahydroindolizine, bp 57° (1.2 mm), which formed a picrate salt from ethanol, mp 171–174° [uncorrected; 175–176° (Leonard *et al.*, 1952)]. The material gave a single peak following gas–liquid chromatography (3% OV-17; 150°), and had a parent ion of *m/e* 139 (mol wt 139) following gas liquid chromatography–mass spectrometry.

The above synthesis was also used to prepare [*piperidine-ring-*^{3}H]-1-ketooctahydroindolizine from [*R*-^{3}H]pipecolic acid in 19% overall yield. Such material following thin-layer chromatography on silica gel G ($CHCl_3$–MeOH, 75:25) appeared as a single spot as visualized either by iodine vapors or Dragendorff's reagent, and virtually all radioactivity on the chromatogram was coincident with this spot. Again the material gave a single peak following gas–liquid chromatography (3% OV-17, 150°).

1-Hydroxyoctahydroindolizines. Reduction of 1-ketooctahydroindolizine with $LiAlH_4$ according to Leonard *et al.* (1952) yielded 1-hydroxyoctahydroindolizine, bp 68–72° (1.2 mm) [93° (18 mm) (Leonard *et al.*, 1952)]. The overall yield based on pipecolic acid was 6–10%. The product gave a molecular ion at *m/e* 141 (mol wt 141) and both the mass fragmentation pattern and nuclear magnetic resonance spectrum were consistent with the presumed structure. Reduction of 1-ketooctahydroindolizine gives rise to *cis*- and *trans*-1-hydroxyoctahydroindolizine with respect to the orientation of the H atoms around carbons 1 and 8a. In certain experiments, as indicated in the tables, such isomers were separated by preparative gas liquid chromatography (Aaron *et al.*, 1966).

[4,5-^{3}H]Pipecolic acid was used as a starting material to obtain [6,7-^{3}H]-1-ketooctahydroindolizine and thence [6,7-^{3}H]-1-hydroxyoctahydroindolizine *via* the above synthesis. [1-^{3}H]-1-Hydroxyoctahydroindolizine was also prepared by the reduction of 1-ketooctahydroindolizine with [^{3}H]$NaBH_4$.

[*^{3}H*]*-1,6-Dihydroxyoctahydroindolizine.* The nitrous acid deamination of slaframine has been found to give rise to 1-acetoxy-6-hydroxyoctahydroindolizine (F. P. Guengerich and S. D. Aust, submitted for publication). [*R*-^{3}H]Pipecolic acid was used to biosynthesize [^{3}H]slaframine, which was treated with nitrous acid and saponified in the usual manner. Gas liquid chromatography–mass spectrometry (% 3OV-17, 150°) showed the presence of essentially one peak, which displayed a parent ion at *m/e* 157 (mol 157) and a fragmen-

TABLE I: Incorporation of [^{14}C]Lysine into Slaframine. Dilution by Pipecolic Acid.[a]

	DL-[1-^{14}C]Lysine		DL-[6-^{14}C]Lysine	
	Sp Act., cpm/mmol			
	1.9×10^{10}		2.8×10^{9}	
	cpm added per culture			
	2.6×10^{6}		1.0×10^{6}	
Group no.	I	II	III	IV
Pipecolic acid, mM	0.0	0.6	0.0	0.6
% ^{14}C incorporated	2.4	1.2	1.4	0.17
Sp act. of slaframine/sp act. of lysine[b]	2.2×10^{-4}	1.1×10^{-4}	3.3×10^{-4}	1.3×10^{-4}
% reduction of sp act.		50		62

[a] 40 Roux bottles containing 250 ml of red clover hay infusion medium were divided into four equal groups and the appropriate amino acid additions, [^{14}C]lysine ± pipecolic acid, made as indicated in the table. The bottles were then autoclaved, cooled, and inoculated with a suspension of *R. leguminicola* cells (28-day old culture) and incubated in stationary culture at 22–24° for 30 days, at which time slaframine was isolated from the mold mycelium, weighed, and counted. [b] No correction has been made in this and other experiments for the lysine or pipecolic acid content of the medium.

tation pattern similar to deacetylslaframine, all peaks being one mass unit higher, as expected. The product, which was positive to Dragendorff's reagent and negative to ninhydrin, was further purified by preparative thin-layer chromatography on layers of silica gel G ($CHCl_3$–CH_3OH, 50:50). The specific activity of the 1,6-dihydroxyoctahydroindolizine was assumed to be essentially the same as that of the slaframine used for the synthesis (0.62 Ci/mol); the yield (from slaframine) was 45%.

Maintenance of Cultures, Slaframine Biosynthesis, and Isolation. Cultures of *Rhizoctonia leguminicola* originally obtained from Dr. G. W. Gerdemann, University of Illinois, were maintained on red clover hay infusion (20%)–agar (2%) medium and subcultured monthly. Liquid cultures were initially prepared by inoculating a suspension of mold mycelium from an agar slant into Roux bottles contained 250 ml of autoclaved, cooled red clover hay infusion medium (Aust *et al.*, 1968) and incubating for 3–4 weeks at 22–24°. Such cultures in turn served routinely as the inoculum to provide mold mycelium *via* Roux bottle fermentation for biosynthetic studies. Slaframine synthesis from radioactive test precursors was studied either in growing cells (stationary cultures in Roux bottles) or under essentially resting cell conditions, the details of which are described in the legends of the tables and in Figure 2. The procedure for the isolation of slaframine as the dipicrate salt is outlined in detail elsewhere (Broquist and Snyder, 1971; Aust, 1965).

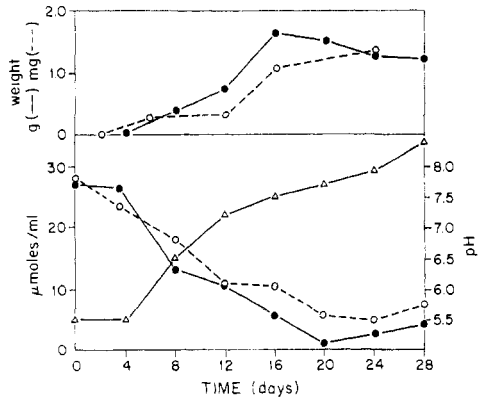


FIGURE 2: Some parameters of growth of *Rhizoctonia leguminicola:* relation to slaframine biosynthesis. A series of Roux bottles containing 250 ml of sterile red clover hay infusion medium were inoculated from a suspension of *R. leguminicola* cells (28-day old culture) and incubated in stationary culture at 22–24°. Cultures were harvested at 4-day intervals for a month; the mycelium was obtained by straining the culture through cheesecloth and washing with water prior to analysis. Top frame: (—) dry weight of mold pad (average of three washed pads dried at 95° for 2 days); (- - - -) weight of slaframine per pad (based on yield of slaframine isolated from 10 Roux bottles). Bottom frame: (△) pH of medium; (—) reducing sugar content of medium equivalent to glucose as determined by Nelson (1944); (- - - - - -) nitrogen content of medium equivalent to ammonia, semi-micro Kjeldahl method of Meneffee and Overmann (1940).

Certain parameters concerning growth of *R. leguminicola* with respect to slaframine yield are depicted in Figure 2. The yield of slaframine approximately parallels the growth of the organism, with slaframine constituting less than one part per thousand of the dry mass. Growth of the organism proceeds at the expense of the nitrogenous material and carbohydrate content of the hay extract. The increase in the pH of the medium may be a factor in limiting the growth of the organism as well. In most experiments *R. leguminicola* cultures were used for biosynthetic studies after 10 or 12 days of growth, as at this time (Figure 2) about half-maximum growth is achieved and slaframine biosynthetis is yet minimal.

Preparation of Cell-Free Extracts. Mycelia of 12-day old *R. leguminicola* cultures were rinsed, blotted dry, and pulverized under liquid nitrogen. The slurry formed by the addition of two volumes of phosphate buffer (0.05 M potassium phosphate and 1 mM 2-mercaptoethanol (pH 7.4)) was sonicated on ice until homogeneous. This crude homogenate was centrifuged at 37,000*g* for 25 min; the supernatant was desalted with a Sephadex G-25 column ("Sephadexed fraction," Table IV). Protein was determined according to Lowry *et al.* (1951).

Expression of Data. Radioactivity was measured by either counting at infinite thinness with a Nuclear-Chicago gas-flow detector or by liquid scintillation counting, using Beckman DPM-100 and Packard 3320 spectrometers. (The cocktail used was 20% ethanol–80% toluene containing 3 g of 2,5-

TABLE II: Incorporation of [*R*-^{14}C]Pipecolate and [*carboxyl*-^{14}C]Pipecolate into Slaframine.[a]

	[*carboxyl*-^{14}C]-Pipecolate[b]	[*R*-^{14}C]-Pipecolate[b]
Yield slaframine, μmol	44.5	16.3
% ^{14}C incorporated in slaframine	2.5	1.0
Sp act. of slaframine/ sp act. of pipecolate	0.33	0.38

[a] 20 Roux bottles containing 250 ml of sterile red clover hay infusion medium were inoculated from a suspension of *R. leguminicola* cells (28-day old culture) and incubated in stationary culture at 22–24° for 10 days. At this point the cultures were divided into two equal groups and supplemented by aseptic addition of the indicated [^{14}C]pipecolic acid. Incubation was continued for 6 days, and the slaframine then isolated from the mold mycelium, weighed, and counted. [b] The medium was supplemented with 0.2 mM [*carboxyl*-^{14}C]-pipecolic acid, sp act. 6.45 μCi/mmol; or 0.2 mM [*R*-^{14}C]-pipecolic acid, sp act. 6.62 μCi/mmol. As indicated in Materials and Methods, establishment of the position of the radioactive carbon atom in [1-^{14}C]lysine was verified by reaction with ninhydrin, but in the case of [6-^{14}C]lysine it could only be concluded by this procedure that the radioactivity was not in carbon 1. Thus pipecolic acid prepared from [6-^{14}C]lysine is simply referred to as [*R*-^{14}C]pipecolate.

TABLE III: Incorporation of Substituted Octahydroindolizines into Slaframine.[a]

	Yield Slaframine (μmol)	% ^{3}H Incorporated into Slaframine	Sp Act. of Slaframine/ Sp Act. of Tracer
[*piperidine-ring*-^{3}H]-1-Ketooctahydroindolizine[b]	51.4	2.29	0.25
[6,7-^{3}H]-1-Hydroxyoctahydroindolizine[c]			
cis	15.5	0.83	0.13
trans	1.4	0.04	0.08
[1-^{3}H]-1-Hydroxyoctahydroindolizine[d]	19.9	0.73	0.20
[^{3}H]-1,6-Dihydroxyoctahydroindolizine[e]	64.2	13.85	0.0147

[a] A series of 10-day old *R. leguminicola* cultures were obtained as described in footnote *a*, Table II as source material for these experiments. Ten such cultures were used in each incorporation experiment shown in which the indicated test precursor, dissolved in a few milliliters of sterile water, was added to each set of cultures at the levels stated in footnotes *b–e*. Incubation was continued for 6 days; slaframine was then isolated from each group of cultures, weighed, and its radioactivity determined. In each case the isolated slaframine was chromatographed to establish the absence of the initial labeled precursor. [b] Sp act. 0.218 mCi/mmol; added at 0.22 mM. [c] Sp act. 6.7 μCi/mmol; added at 0.20 mM. [d] Sp act. 1.11 mCi/mmol; added at 0.22 mM. [e] Sp act. 0.62 mCi/mmol; added at 2.75 μM.

diphenyloxazole/l.) Appropriate corrections were made. Picric acid was extracted from isolated slaframine–dipicrate samples with ether prior to liquid scintillation counting.

The data concerning incorporation of radioactive compounds into slaframine are expressed as the ratio of the specific activity of the isolated slaframine to the specific activity of the radiotracer used, and as the percentage of incorporation of the added radiotracer into slaframine. Conclusions are based primarily on the first procedure, as the latter reflects variables in the isolation procedure.

Results and Discussion

Lysine and Pipecolic Acid as Precursors of Slaframine. Experiments demonstrating the incorporation of lysine into slaframine and the dilution of such incorporation by pipecolic acid are shown in Table I. It is particularly noteworthy that both C-1 and C-6 labeled lysines were utilized in slaframine synthesis, thus, barring any unusual fragmentation of lysine and efficient reutilization of such fragments, it appears that the entire lysine molecule is used in slaframine synthesis. This situation contrasts with the events in the biogenesis of the piperidine alkaloids nicotine and anabasine (Bu'Lock, 1965), in which the pyrrolidine and piperidine rings of these respective alkaloids derive from ornithine and lysine, but decarboxylation of these amino acids precedes eventual ring closure.

The structure of slaframine as a piperidine alkaloid suggested the likely involvement of such cyclic lysine metabolites as Δ^1-piperideine-6-carboxylate, Δ^1-piperideine-2-carboxylate, and pipecolate in the biogenesis of slaframine, and indeed Table I illustrates effective dilution of lysine incorporation into slaframine by pipecolic acid.

The data of Table II show that carboxyl- and ring-labeled pipecolic acids were incorporated equally well into slaframine, implying that pipecolate is incorporated into slaframine *in toto*. These findings, when considered with the data of Table I showing that both [1-^{14}C]lysine and [6-^{14}C]lysine are utilized in the biogenesis of slaframine, are consistent with the sequence: lysine → pipecolate → slaframine, with the entire carbon skeleton of lysine being retained throughout this metabolic pathway.

This metabolic sequence is further strengthened by the finding that lysine was indeed catabolized to pipecolic acid in *R. leguminicola*. Thus, when a mycelial mat of this mold was incubated with either [6-^{14}C]lysine or [1-^{14}C]lysine and the mold pad subsequently examined for radioactive compounds by ion-exchange chromatography (Piez *et al.*, 1956), a radioactive peak coincident with an amino acid was found (region tube 110). Pipecolic acid has been found consistently to elute under these conditions. Concentrates of the peak "pipecolate eluates" could not be distinguished from authentic pipecolate in various chromatographic systems (paper and thin layer). Further studies of the biogenesis of pipecolic acid from lysine together with stereochemical aspects of this conversion are given in an accompanying paper (Guengerich and Broquist, 1973).

1-Hydroxyoctahydroindolizine and Slaframine Biosynthesis. In preliminary isotope dilution experiments, *cis*- and *trans*-1-hydroxyoctahydroindolizine were effective in diluting radioactivity from tritiated pipecolic acid into biosynthesized slaframine. There was no difference between the isomers on the extent of dilution. To establish that 1-hydroxyoctahydro-

TABLE IV: *In Vitro* Reduction of 1-Ketooctahydroindolizine.[a]

System	Activity	
	Cis	Trans
Crude homogenate (9.9 mg of protein)	0.55	0.05
37,000*g* supernatant (4.4 mg of protein)	0.21	0.03
Sephadexed fraction (3.3 mg of protein)	0.29	0.02
Sephadexed fraction, −NADPH system	0.10	0
Sephadexed fraction, −NADPH system, + 7.9 μmol of NADH	0.09	0
Sephadexed fraction, −2-mercaptoethanol	0.03	0.01
Sephadexed fraction, enzyme boiled	0	0

[a] Each incubate consisted of 7.95 μmol of 1-ketooctahydroindolizine, 0.5 ml of (pH 7.8) (0.2 M potassium phosphate and 1 mM 2-mercaptoethanol) buffer, 2 μmol of $NADP^+$, 16 μmol of glucose 6-phosphate, 6.25 units of glucose 6-phosphate dehydrogenase, 1 μmol of $MgSO_4$, and 1.5 ml of the appropriate enzyme preparation (*cf.* Materials and Methods). After 90-min incubation at room temperature, each incubation was stopped by the addition of trichloroacetic acid. After centrifugation and extraction with chloroform, each sample was raised to pH 10 and extracted four times with chloroform. 1-Ketooctahydroindolizine and the cis and trans isomers of 1-hydroxyoctahydroindolizine were separated by gas liquid chromatography (Aaron *et al.*, 1966). Peaks were quantitated by triangulation, and activity is expressed as the ratio of the cis or trans alcohol peak area to the sum of the areas of the ketone and both alcohols.

TABLE V: *In Vitro* Acetylation of Deacetylslaframine.[a]

System	Act.
Complete system, 0.2 ml of homogenate	0.16
Complete system, 0.5 ml of homogenate	0.22
Complete system, 1.0 ml of homogenate	0.23
Complete system, 0.2 ml of boiled homogenate	*0*
Complete system − acetyl-CoA, 0.2 ml of homogenate	0.05

[a] [^{3}H]Deacetylsalframine was prepared by mild alkaline hydrolysis of [^{3}H]slaframine biosynthesized from [*R*-^{3}H]-pipecolic acid; purity was checked by gas liquid chromatography (3% OV-17, 170°). Acetyl-CoA was prepared as previously described (Ochoa, 1955). The complete system contained 4.0 μmol of [^{3}H]deacetylslaframine (1.60 Ci/mol), 0.2 ml of (pH 7.8) (0.2 M potassium phosphate) buffer, 10 μmol of acetyl-CoA, and the indicated amount of crude homogenate. After 1-hr incubation at room temperature, incubations were stopped by the addition of HCl to pH 1. Slaframine was isolated in each case as previously described (Broquist and Snyder, 1971) and chromatographed on layers of silica gel G, using the system: $CHCl_3$–CH_3OH-4% aqueous NH_4OH (40:40:15); 5-mm bands were scraped and counted. The activity is expressed as cpm of slaframine/(cpm of slaframine + cpm of deacetylslaframine).

indolizine was incorporated into slaframine and not simply exerting an inhibitory effect on some portion of the pathway, the compound was prepared with a tritium label. Table III (2nd and 3rd lines) demonstrates that both isomers were incorporated.

The finding that both the cis and trans isomers of 1-hydroxyoctahydroindolizine had biological activity suggested the possible involvement of 1-ketooctahydroindolizine in slaframine biosynthesis (Figure 1). Thus *trans*-1-hydroxyoctahydroindolizine might be dehydrogenated to give 1-ketooctahydroindolizine followed by subsequent reduction to *cis*-1-hydroxyoctahydroindolizine which may lie directly on the pathway of slaframine biosynthesis. This is an attractive postulate since the configuration about carbon 1 and carbon 8a in saframine is cis (Gardiner *et al.*, 1968).

1-Ketooctahydroindolizine interfered with the utilization of pipecolic acid for slaframine biosynthesis in isotope dilution experiments; and the data of Table III demonstrate conversion of tritiated 1-ketooctahydroindolizine to slaframine. [1-^{3}H]-1-Hydroxyoctahydroindolizine (55% cis, 45% trans isomers) was utilized at least as effectively for slaframine synthesis (4th line, Table III) as *cis*-[6,7-^{3}H]-1-hydroxyoctahydroindolizine (compare specific activity ratios last column, Table III). This finding indicates that the tritium label is retained at carbon 1 and is consistent with the view that 1-hydroxyoctahydroindolizine follows 1-ketooctahydroindolizine in slaframine biogenesis as pictured in Figure 1.

The *in vitro* conversion of 1-ketooctahydroindolizine to 1-hydroxyoctahydroindolizine was investigated. Incubation of a crude homogenate of *R. leguminicola* with 1-ketooctahydroindolizine and NADPH under the conditions described in footnote *a*, Table IV, followed by analysis for product by gas liquid chromatography, revealed significant formation of *cis*-1-hydroxyoctahydroindolizine. Such findings further strengthen the evidence for the role of this isomer and the positioning of this intermediate in the slaframine biosynthetic pathway as postulated in Figure 1. Further studies in crude cell-free extracts, Table IV, established that the reductase appeared to be NADPH specific and was stabilized by 2-mercaptoethanol. The activity in the absence of added NADPH is unexplained; perhaps the reductase in its reduced state as prepared, or residual cofactor(s) may be bound to the reductase.

1-Hydroxy-6-aminooctahydroindolizine (Deacetylslaframine). When ethanolic extracts of *R. leguminicola* (from cells incubated with labeled aminoadipic acid, lysine, or pipecolic acid) were chromatographed (Piez *et al.*, 1956), two previously unrecognized basic metabolites were observed (Guengerich, 1971). The more basic of these has been identified as deacetylslaframine by (a) the nature of its chemical properties, and (b) by comparison to authentic deacetylslaframine, using gas liquid and thin-layer chromatographies and mass spectrometry (Guengerich and Broquist, 1971). The levels of incorporation of the above precursors into deacetylslaframine were substantially higher than into slaframine.

The acetylation of 1-hydroxy-6-aminooctahydroindolizine was investigated *in vitro*, as shown in Table V. The conversion of deacetylslaframine to slaframine was confirmed by gas liquid chromatography (3% OV-17, 170°). Thus deacetylslaframine appears to be a direct precursor of slaframine.

1,6-Dihydroxyoctahydroindolizine. A logical precursor of deacetylslaframine might well be the ketone, 1-hydroxy-6-oxoctahydroindolizine, which in turn could derive from 1,6-dihydroxyoctahydroindolizine. The latter diol was synthesized and found to be effectively incorporated into slaframine, Table III, line 5.

The per cent of incorporation of label into slaframine is

quite high, as expected; the low ratio of specific activities is due to the low level of the suspected precursor added to the system.[1] However, from the ratios obtained when other precursors were added at low levels (*vide supra*), it is apparent that this ratio reflects effective incorporation. Provisionally, 1,6-dihydroxyoctahydroindolizine is positioned on the pathway of slaframine biosynthesis as shown in Figure 1. However, it may only be serving as a ready source of 1-hydroxy-6-ketooctahydroindolizine which is then aminated to give deacetylslaframine. Further study is needed to establish the immediate precursor of deacetylslaframine.

Preliminary Studies on the Transformation of Pipecolate to the Protoalkaloid. 2-Piperidinemethanol was found to dilute the incorporation of pipecolic acid into slaframine; it was suspected that perhaps pipecolic acid might be reduced to this alcohol prior to a condensation appropriate for the formation of the octahydroindolizine ring. Tritium from [*R*-^{3}H]-2-piperidinemethanol was incorporated into both pipecolic acid (7%) and into deacetylslaframine (20%), the immediate precursor of slaframine. However, label from [1-^{3}H]-2-piperidinemethanol was incorporated into neither compound. Such results are consistent only with oxidation of the alcohol to pipecolic acid prior to the condensation reaction; thus neither the alcohol nor the aldehyde can participate in the condensation.

L-[2-^{3}H]Pipecolic acid, added at 20 μM to resting cells, was incorporated into slaframine with specific activities ratio of 0.013 (1.5% incorporation), consistent with retention of configuration at carbon 2 of L-pipecolic acid in its conversion to slaframine (compare with a ratio of 0.016 and 0.95% incorporation for DL-[*R*-^{3}H]pipecolic acid added at the same level).

Physiological Role of Slaframine. Slaframine may be regarded as a secondary metabolite of lysine in *R. leguminicola*, but its physiological role, if any, in this fungus is unknown. Its formation in good yield from pipecolic acid (Table II) and certain octahydroindolizines (Table III) indicate ready availability of enzymatic machinery for its synthesis from these sources and may imply that it has utility in *R. leguminicola* metabolism. It is of interest that a brief examination of a series of molds related phylogenetically to *R. leguminicola* revealed the presence of Dragendorf positive materials in certain of such cultures as well (Snyder, 1969). In this regard another piperidine alkaloid, 3,4,5-trihydroxyoctahydro-1-pyrinidine, arising from lysine and pipecolate metabolism in *R. leguminicola* has recently been isolated and characterized (Guengerich *et al.*, 1973).

[1] The low level of addition of the diol is a reflection of the difficulties involved in synthesizing large amounts of the compound from slaframine, as slaframine must be isolated from the fungus. Attempts at the total synthesis of 1,6-dihydroxyoctahydroindolizine were unsuccessful.

References

Aaron, H. S., Rader, C. P., and Wicks, G. E., Jr. (1966), *J. Org. Chem. 31*, 3502.

Aust, S. D. (1965), Doctoral Thesis, University of Illinois.

Aust, S. D., Broquist, H. P., and Rinehart, K. L., Jr. (1966), *J. Amer. Chem. Soc. 88*, 2879.

Aust, S. D., Broquist, H. P., and Rinehart, K. L., Jr. (1968), *Biotechnol. Bioeng. 10*, 403.

Broquist, H. P., and Snyder, J. J. (1971), *in* Microbial Toxins, Vol. VII, Ajl, S. J., Kadis, S., and Montie, T. C., Ed., New York, N. Y., Academic Press, p 319.

Bu'Lock, J. D. (1965), The Biosynthesis of Natural Products, London, McGraw-Hill, p 109.

Cartwright, D., Gardiner, R. A., and Rinehart, K. L., Jr. (1970), *J. Amer. Chem. Soc. 92*, 7615.

Clemo, G. R., and Ramage, G. R. (1932), *J. Chem. Soc.*, 2969.

Cooksey, K. E., and Greenberg, D. M. (1965), *Arch. Biochem. Biophys. 112*, 238.

Gardiner, R. A., Rinehart, K. L., Jr., Snyder, J. J., and Broquist, H. P. (1968), *J. Amer. Chem. Soc. 90*, 5639.

Guengerich, F. P. (1971). *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1067.

Guengerich, F. P., and Broquist, H. P. (1971), Abstract, 23rd Southeastern Regional Meeting of the American Chemical Society, Nashville, Tenn., No. 115.

Guengerich, F. P., and Broquist, H. P. (1973), *Biochemistry 12*, 4270.

Guengerich, F. P., DiMari, S. J., and Broquist, H. P. (1973), *J. Amer. Chem. Soc. 95*, 2055.

Hamilton, P. B. (1952), *J. Biol. Chem. 198*, 587.

Leonard, N. J., Swan, S., Jr., and Figueras, J., Jr. (1952), *J. Amer. Chem. Soc. 74*, 4620.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. S. (1951), *J. Biol. Chem. 193*, 265.

Meister, A. (1954), *J. Biol. Chem. 206*, 577.

Menefee, S. G., and Overmann, O. R. (1940), *J. Dairy Sci. 23*, 1177.

Moore, S., and Stein, W. H. (1948), *J. Biol. Chem. 176*, 367.

Nelson, N. (1944), *J. Biol. Chem. 153*, 375.

Ochoa, S. (1955), *Methods Enzymol. 1*, 685.

Piez, K. A. Irreverre, F., and Wolff, H. L. (1956), *J. Biol. Chem. 223*, 687.

Snyder J. J. (1969), Doctoral Thesis, University of Illinois.

Snyder, J. J., and Broquist, H. P. (1968), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 27*, 764.

Snyder, J. J., and Broquist, H. P. (1969), Abstracts, 158th National Meeting of the American Chemical Society, New York, N. Y., No. 210.

Spike, T. E., and Aust, S. D. (1971), *Biochem. Pharmacol. 20*, 721.

Stevens, C. M., and Ellman, P. B. (1950), *J. Biol. Chem. 182*, 75.