Biosynthesis of the Toxic Indolizidine Alkaloids Slaframine and Swainsonine in *Rhizoctonia leguminicola*: Metabolism of 1-Hydroxyindolizidines

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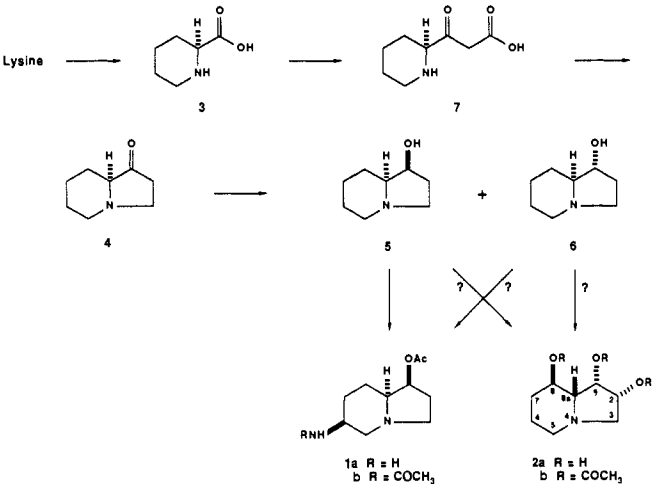
Abstract: Late stages in the biosynthesis of the toxic indolizidine alkaloids slaframine (**1a**) and swainsonine (**2a**) have been investigated with the aid of specifically deuteriated and chiral precursors. Racemic (1,8a)-*cis*- and (1,8a)-*trans*-1-hydroxyindolizidines (**5**, **6**) were prepared with deuterium at the 1-3- and 8a-positions. Racemic **5** was incorporated into **1a** with retention of all deuterium; incorporation into **2a** was accompanied by loss of one deuterium at C-2 and the deuterium at C-8a. Racemic **6** was incorporated to a variable but low extent into **1a** with loss of one deuterium at an undetermined site; incorporation into **2a** occurred with loss of one deuterium at C-2 and the deuterium at C-8a. When chiral [1,3,3′-$^2H_3$,*piperidine ring*-^{3}H]-1-hydroxyindolizidines were fed, **5a** (1*S*,8a*S*) incorporated into **1a**; **5b** (1*R*,8a*R*) and **6a** (1*R*,8a*S*) incorporated equally well into **2a** and also into 1,2-diol **10**; **6b** (1*S*,8a*R*) did not incorporate into either alkaloid. Trapping experiments utilizing DL- and L-[^{3}H]pipecolic acid showed that **6a** is present in the fungus together with traces of **5a**. We propose that the branch point in the biosynthetic pathway to these alkaloids is at 1-oxoindolizidine (**4**). Reduction of ketone **4** gives **5a** and **6a**; **5a** is converted to **1a** through 1,6-dihydroxyindolizidines (**9**) and 1-hydroxy-6-oxoindolizidine. The route to **2a** is postulated to be **6a** → diol **10** → iminium ion **15** → iminium ion **16** → **2a**. Compounds **5b** and **12** (the 8a-epimer of **10**), although efficiently incorporated into **2a**, are considered to be unnatural intermediates.

The fungus *Rhizoctonia leguminicola* produces two toxic indolizidine alkaloids, slaframine (**1a**)[1] which after metabolic activation binds to muscarinic acetylcholine receptor sites,[2] and swainsonine (**2a**),[3] which is an inhibitor of certain α-mannosidases involved in the biosynthesis and catabolism of glycoproteins.[4] Swainsonine is also formed by locoweed (*Astragalus sp.*) in the United States[5] and *Swainsona canescens* in Australia[6] as well as by the fungus *Metarhizium anisopliae*.[7a] The effect of swainsonine on glycoprotein processing is apparently responsible for its role as the causative agent in locoweed poisoning, a serious affliction of livestock in certain areas of this country[4g] and in similar poisoning induced by the Australian plant.[4a,b] Swainsonine has also aroused interest because of its immunostimulatory properties and possible use in cancer chemotherapy.[7]

Scheme I



The biosynthesis of slaframine in *R. leguminicola* was extensively studied in the 1970s by Broquist and co-workers, who showed that the alkaloid is formed from L-lysine via L-pipecolic acid (**3**), 1-oxoindolizidine (**4**), and 1-hydroxyindolizidine (**5**, **6**).[8] Using racemic *cis*- and *trans*-1-hydroxyindolizidines[9] (**5**, **6**) labeled at positions 1 and 3 with deuterium, they observed[8a,8d] that the cis isomer is utilized much better than the trans, which apparently undergoes oxidation back to ketone **4** before conversion to the alkaloid. It is noteworthy that the carboxyl carbon of pipecolic acid is incorporated into slaframine, an unusual occurrence in alkaloid biosynthesis. The two carbons in the pyrrolidine ring are contributed by acetate via malonate.[8c] Presumably, pipecolylacetate (**7**) is an intermediate, although direct proof for its involvement is lacking.

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(9) In this paper cis and trans refer to the orientation of the hydrogens at the 1 and 8a (bridgehead) positions in the indolizidine nucleus.

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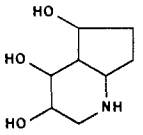
Swainsonine (**2a**) was isolated from the fungus after much of the biosynthetic work on slaframine had been completed and was initially assigned incorrectly as pyrindine **8**.[10] Eventually, the



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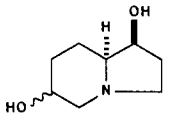
indolizidine structure for the alkaloid was deduced;[3] the absolute configuration, as determined by the Horeau method and subsequently confirmed by several enantiospecific syntheses,[11] was shown to be opposite to that of slaframine at C-1 and C-8a. Swainsonine also arises from pipecolic acid, presumably via ketone **4** and alcohol **5** and/or **6**.[10,12] Feeding experiments with DL-pipecolic-d_9 acid led to the d_7 species of both alkaloids.[12] In slaframine the two deuterium atoms were missing at the 6-position, pointing to a keto precursor of the 6-amino group. In swainsonine one deuterium was missing at the 8-position, excluding involvement of an 8-keto intermediate, and the second one at the 8a-position. The loss of deuterium from the 8a-position of swainsonine was confirmed by feeding [2-^{2}H]pipecolic acid admixed with [^{3}H]-pipecolic acid. Tritium was well incorporated into both alkaloids, but deuterium was incorporated only into slaframine. The biosynthetic pathway as understood at the start of present investigation is shown in Scheme I.

A major unresolved point was the origin of the difference in absolute configurations of slaframine and swainsonine; i.e., at what point in the formation of swainsonine is the 8a-proton lost. A related question was the role of the cis versus trans isomer of 1-hydroxyindolizidine in the swainsonine pathway. The possible involvement of dihydroxylated intermediates in both pathways needed to be explored. Guengerich et al. had shown that a 1,6-diol (**9**) of unknown configuration at C-6 incorporated into slaframine.[8a] In addition, we have found a 1,2-diol (**10**), which can be converted to swainsonine, as a minor metabolite of the fungus.[13] New studies employing chiral and specifically deuteriated intermediates have been undertaken to clarify these points in this interesting and unique biosynthetic pathway.



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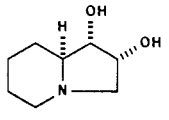

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Results and Discussion

The first intermediates to be examined in further detail were the racemic 1-hydroxyindolizidines **5** and **6**. Guengerich had shown that a cell-free system from *R. leguminicola* could synthesize both the *cis*- and *trans*-1-hydroxyindolizidines from 1-oxoindolizidine (**4**) in the presence of NADPH; in that reaction system cis alcohol **5** predominated over trans alcohol **6**.[8a] A reasonable hypothesis was that slaframine is synthesized from the cis isomer and swainsonine from the trans compound. In our initial experiment, the fungus was incubated with racemic *cis*- and *trans*-[1,3,3′-$^2H_3$]-1-hydroxyindolizidines, which had been used previously in the study of slaframine biosynthesis.[8d] In the new investigation both alkaloids were isolated as their acetates (**1b**, **2b**); Table I shows the mass spectral data that were obtained. These data confirm the previous results for slaframine: i.e., (1) the cis isomer incorporated efficiently with retention of all three deuterium atoms; (2) the trans isomer was utilized poorly with loss of one deuterium atom (presumably at the 1-position via oxidation to ketone **4** and reduction to the cis isomer). Surprising results were obtained for swainsonine: (1) both isomers of [1,3,3′-$^2H_3$]-1-hydroxyindolizidine incorporated efficiently and to approximately the same extent; (2) in *both* cases all three deuterium atoms were retained. We suspected that the explanation for these results lay in the fact that racemic forms of the 1-hydroxyindolizidines had been employed (see Scheme II). In the feeding experiment with cis isomer **5**, enantiomer **5a**, which is 1*S*,8a*S*, has the correct relative and absolute stereochemistry for conversion to slaframine and **5b**, which is 1*R*,8a*R*, is correct for swainsonine. With the trans isomer **6**, neither enantiomer has the correct configuration at both positions 1 and 8a for either alkaloid. However, the incorporation of *trans*-[1,3,3′-$^2H_3$]-1-hydroxyindolizidine into swainsonine with all deuteriums intact indicates that trans isomer **6a** (1*R*,8a*S*) must be the one that is utilized; i.e., the configuration at C-8a in **6a** undergoes inversion, not the configuration at C-1 in **6b** (unless a hydride shift from C-1 to 8a is involved). The observation that pipecolic-d_9 acid showed loss of the hydrogen at C-2 (C-8a in **2a**) en route to swainsonine is in agreement with the hypothesis that trans isomer **6a** is converted to cis isomer **5b**, which then goes on to **2a**.

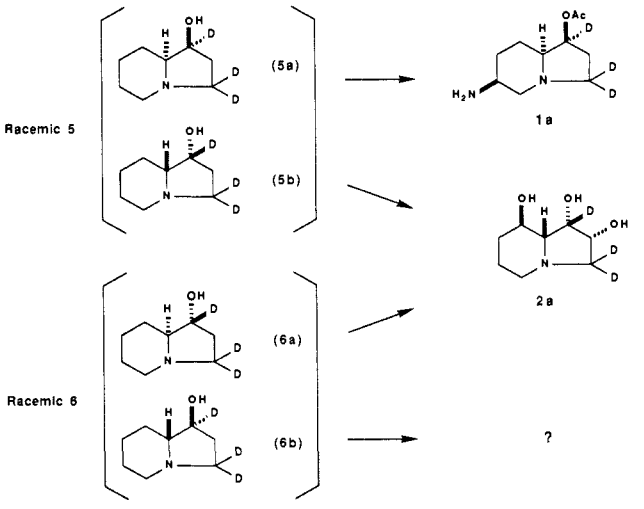
To test this proposal, **5** and **6** were prepared by a route that incorporated a deuterium at the 8a-position as well as at positions 1,2,2′,3, and 3′ (Scheme III). DL-Pipecolic acid was heated with acetic anhydride in the presence of D_2O to incorporate a deuterium specifically at position 2.[14] Ethyl *N*-acetylpipecolate was prepared from the deuteriated amino acid and was shown by NMR and MS to be fully deuteriated at position 2. Cyclization using KH

Table I. Incorporation of DL-[1,3,3′-$^2H_3$]-1-Hydroxyindolizidines (**5**, **6**) into **1a** and **2a**

compd	MS of acetate esters, rel abund[a]			
	d_0	d_1	d_2	d_3
5-d_3 (precursor)[b]				100
1a	100	4	8	243
2a	100	2	6	45
6-d_3 (precursor)[b]				100
1a	100	3	37	
2a	100	3	10	54

[a] Analyzed by NH_3-Cl mass spectroscopy. Relative abundances are derived from peak intensities by correction for natural-abundance ^{13}C contributions. [b] Precursors were fed at 2.2 mM; mats were harvested after 6 days.

Scheme II



(10) The earliest correct structure for swainsonine was assigned by Australian workers to the compound isolated from S. *canescens*;[6] however, the alkaloid was first isolated from *R. leguminicola*: Guengerich, F. P.; DiMari, S. J.; Broquist, H. P. *J. Am. Chem. Soc.* **1973**, *95*, 2055–2056.

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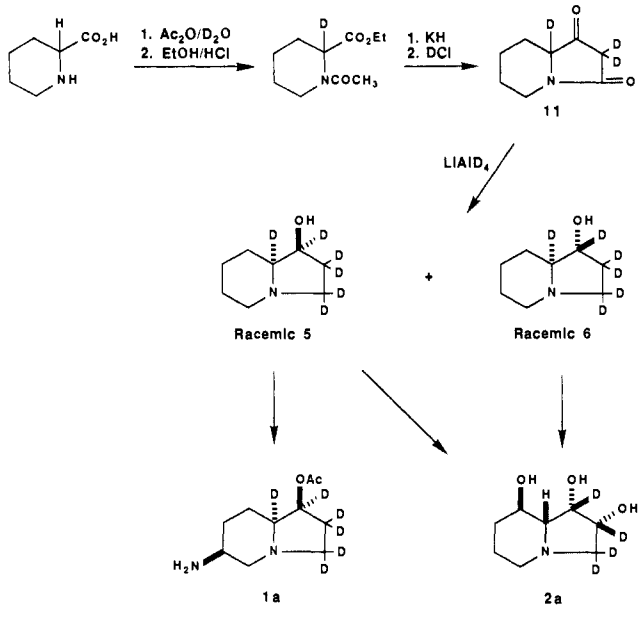
Table II. Incorporation of DL-[1,2,2′,3,3′,8a-$^2H_6$]-1-Hydroxyindolizidines (**5**, **6**) into **1a** and **2a**

compd	MS of acetate esters, rel abund[a] d_0	d_1	d_2	d_3	d_4	d_5	d_6
5-d_6 (precursor)[b]		4	7	23	35	85	100
1a	100	2	3	6	18	71	98
2a	100	2		4	15		

compd	MS of acetate esters, rel abund[a] d_0	d_1	d_2	d_3	d_4	d_5	d_6
6-d_6 (precursor)[b]		16	13	22	29	78	100
1a	100	1		3	5	1	
2a	100	2		9	18	1	
6 (recovered)	40	4	6	16	39	136	100

[a] Analyzed by NH_3-Cl mass spectroscopy. [b] Precursors were fed at 1.86 mM; mats were harvested after 2 days.

Scheme III



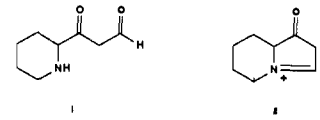
gave 1,3-dioxoindolizidine (**11**);[8d] the reaction was quenched with D_2O to avoid loss of the deuterium from C-8a. Reduction of **11** with $LiAl^2H_4$ gave the *cis*- and *trans*-[1,2,2′,3,3′,8a-$^2H_6$]-1-hydroxyindolizidines (**5**-d_6, **6**-d_6), which were separated by chromatography. In spite of precautions, some loss of deuterium from position 8a occurred by an unknown mechanism[15] and products **5** and **6** were only about 60% deuteriated at that position; however, the extent of deuteriation was high at positions 1,2, and 3. The data obtained from feeding of the d_6 alcohols are shown in Table II. Again, both isomers incorporated into swainsonine to the same extent and *both* showed loss of two deuterium atoms, the obligatory deuteron at position 2 plus the one at 8a. The site(s) of deuteriation in each case were established from the ^{13}C spectra on the basis of the deuterium-induced upfield shift of carbon atoms near the site of deuteriation or from ^2H spectra. The finding that the cis isomer also lost the 8a-hydrogen in transformation to swainsonine was quite unexpected and raised further questions about the later stages in the biosynthesis. The pathway apparently is not a simple epimerization of **6a** to **5b** followed by transformation to swainsonine. It is noteworthy that some starting material was recovered from the feeding of **6**-d_6; mass spectroscopy revealed that a substantial fraction of the material was undeuteriated, which is the first direct evidence for **6** being synthesized by the fungus.

While these experiments were in progress, additional intermediates in the biosynthesis of slaframine and swainsonine were being sought. From feeding experiments using high concentrations (12 mM) of a mixture of *cis*- and *trans*-[1-^{3}H]-1-hydroxyindolizidine, a new metabolite was found, which was shown by spectroscopic studies and by synthesis to be 1,2-dihydroxyindolizidine (**10**) in which the 1- and 2-protons are cis and the 1- and 8a-protons are trans.[13] The absolute configuration of **10** was inferred to be 1*S*,2*R*,8a*S*. Diol **10** could also be isolated from cultures to which only tracer levels of the labeled **5** and **6** had been added, indicating that it is a normally occurring metabolite, albeit present in very small quantities. When [1-^{3}H]-diol **10** was reincubated with the fungus it was incorporated into swainsonine with exceptional efficiency (45%). Thus, it appeared that **10** is the intermediate between 1-hydroxyindolizidine and swainsonine. A reasonable scenario would involve epimerization of the *trans*-(1*S*,2*R*,8a*S*)-diol **10** to *cis*-(1*S*,2*R*,8a*R*)-diol **12**, but a careful search failed to yield any **12**; this search was made both by ion-exchange chromatography and by GC–MS, aided by an authentic sample of **12**. However, involvement of **12** in the pathway cannot be ruled out, since our failure to detect it may only be a reflection of too small a metabolic pool. In a further attempt to define the involvement of 1,8a-*cis*-diol **12**, the compound was prepared with a deuterium label at C-8a from the acetonide **13** of 1,8a-*trans*-diol **10** (Scheme IVa). Acetonide **13** was oxidized with mercuric acetate to the 4,8a-iminium ion, which underwent stereospecific reduction with $NaCNB^2H_3$ to 1,8a-*cis* isomer **14**; **14** gave 1,8a-*cis*-[8a-^{2}H]diol **12** upon hydrolysis. For feeding, the [8a-^{2}H]**12** was mixed with [1-^{3}H]**12**, prepared by deoxygenation of swainsonine (Scheme IVb), which had been biosynthesized from [1-^{3}H]-1-hydroxyindolizidine.[13] Diol **12** incorporated into swainsonine almost as well (31%) as **10**, but the deuterium label from the 8a-position was lost. Thus, the fungus is able to metabolize both 8a-epimers of (1*S*,2*R*)-1,2-dihydroxyindolizidine as well as the 8a-epimers of (1*R*)-1-hydroxyindolizidine. Furthermore, while only with **12** has the fate of deuterium at position 8a been established, almost certainly similar loss would occur from position 8a in **10**. This evidence suggests involvement of iminium ions **15** and **16** (see Scheme VII) in the final stages of the biosynthesis of swainsonine.

To probe the involvement of such iminium ions, a feeding experiment was carried out with [1,1-$^2H_2$]ethanol, prepared by $LiAl^2H_4$ reduction of acetyl chloride. The rationale was that $CH_3C^2H_2OH$, as a substrate for dehydrogenase enzymes, could serve as a source of deuteriated nicotinamide coenzymes, [4-^{2}H]NAD(P)H, which might well be involved in reduction of iminium ions formed during swainsonine biosynthesis. Deuteriated ethanol (50 mM) was fed together with pipecolic acid (0.2 mM). The alkaloids were isolated as their acetates and analyzed by mass spectrometry and ^2H NMR. Both alkaloids showed deuterium enrichment at C-1, C-3, C-5, and C-8a; total enrichment in both alkaloids ranged from 3 to 6%. Deuterium would have been introduced at C-1 and C-3 during conversion of keto acid **7** to hydroxyindolizidines **5** and **6**.[16] Deuterium at C-5 would have been introduced during the formation of pipecolic acid.[17] For

(15) A reviewer has suggested that partial loss of deuterium at position 8a may occur by a pathway involving peroxides in the ethereal solvents converting the indolizidine to its *N*-oxide, which could lose the 8a-proton by conversion to the iminium ion.

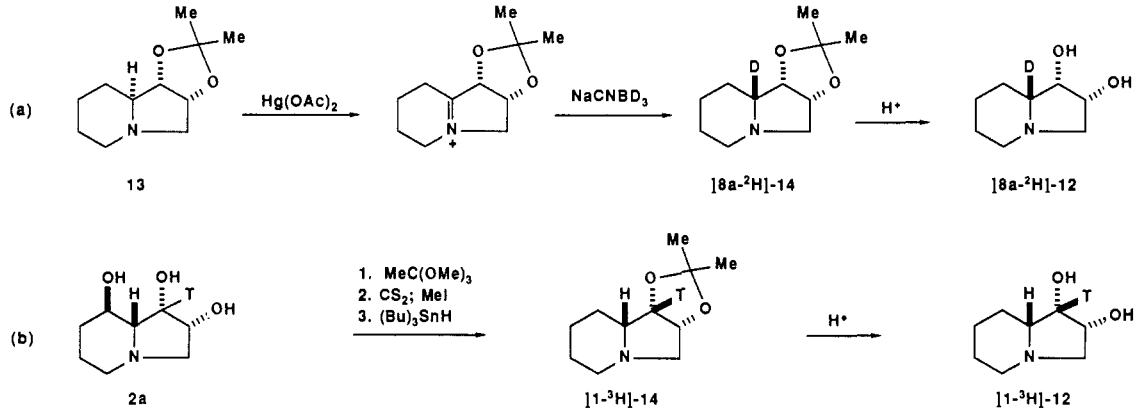
(16) We feel that the most probable intermediate between pipecolylacetate **7** and ketone **4** is keto aldehyde *i*, which would give **4** after cyclization to iminium ion *ii* and reduction.



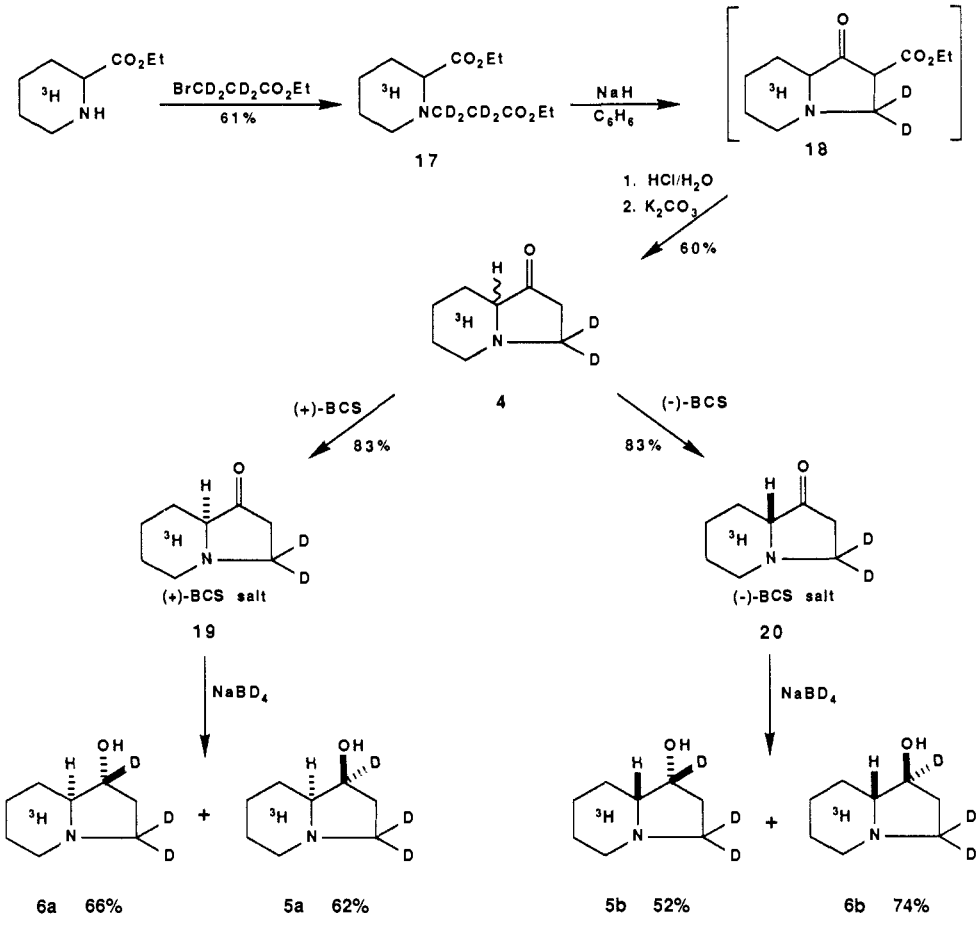
The other plausible intermediate, keto lactam **11**, has been fed; it is degraded to pipecolic acid before incorporation into the alkaloids.[8d]

(17) Initial evidence pointed to Δ^1-piperideine-2-carboxylic acid (from lysine) as the precursor to L-pipecolic acid in *R. leguminicola*;[8b] however, more recent results suggest that the route through Δ^1-piperideine-6-carboxylic acid is the major one: Wickwire, B. W.; Broquist, H. P., unpublished results. The former would lead to label at C-8a and the latter to label at C-5 upon reduction.

Scheme IV



Scheme V



slaframine, deuterium at position 8a could also arise during formation of pipecolic acid. However, in the case of swainsonine, we have shown that none of the potential precursors thus far identified (**3**, **5a**,**b**, **6a**,**b**, **10**, **12**) yield alkaloid retaining label at C-8a, and hence the deuterium from the ethan-d_2-ol must be introduced at a very late stage in the synthesis, which strongly supports the intermediacy of iminium ions **15** and **16**.

At this point we undertook feeding experiments with the individual enantiomers of the [1,3,3′-$^2H_3$]-1-hydroxyindolizidines (**5a**, **5b**, **6a**, **6b**) to confirm the conclusions drawn from feeding the racemates. Optically active 1-hydroxy compounds were prepared by reducing separated enantiomers of 1-oxoindolizidine (**4**).[18,19] Syntheses of the optically active [1,3,3′-$^2H_3$,*piperidine ring*-^{3}H]-1-hydroxyindolizidines are shown in Scheme V. The ethyl ester of randomly tritiated pipecolic acid was alkylated with ethyl [2,2,3,3-$^2H_4$]-3-bromopropionate to give diester **17**, which was cyclized with sodium hydride to β-keto ester **18**.[20] Decarboxylation of **18** in situ gave amino ketone [3,3-$^2H_2$]**4**, which was resolved with (+)-3-bromo-8-camphorsulfonic acid [(+)-BCS]. We had envisioned feeding the individual enantiomers of ketone **4** itself, but their facile racemization ($t_{1/2}$ = 7.4 min in D_2O, pD 7.7, 21 °C) precluded this experiment. Chiral alcohols **5a**, **5b**, **6a**, and **6b** were obtained in high optical purity by reduction of (+)- and (−)-BCS salts **19** and **20** with NaB^2H_4. Optical purity was evaluated by ^{1}H NMR and HPLC of the dibenzoyl-D-tartrate

(18) (a) Kunieda, T.; Koga, K.; Yamada, S. *Chem. Pharm. Bull.* **1967**, *15*, 337–344. (b) Yamada, S.; Kunieda, T. *Chem. Pharm. Bull.* **1967**, *15*, 490–498.

(19) Harris, C. M.; Harris, T. M. *Tetrahedron Lett.* **1987**, *28*, 2559–2562.

(20) Clemo, G. R.; Ramage, G. R. *J. Chem. Soc.* **1932**, 2969–2973. These authors used Na in the original synthesis.

(21) In the original experiment only one isomer was detected; the second one may well have been overlooked. Unfortunately, the sample had decomposed and could not be rerun after the authentic **23a** and **23b** were available.

Table III. Incorporation of Optically Active [1,3,3′-$^2H_3$,*piperidine ring*-^3H]-1-Hydroxyindolizidines (**5a**, **5b**, **6a**, **6b**) into **1a**, **2a**, and **10**

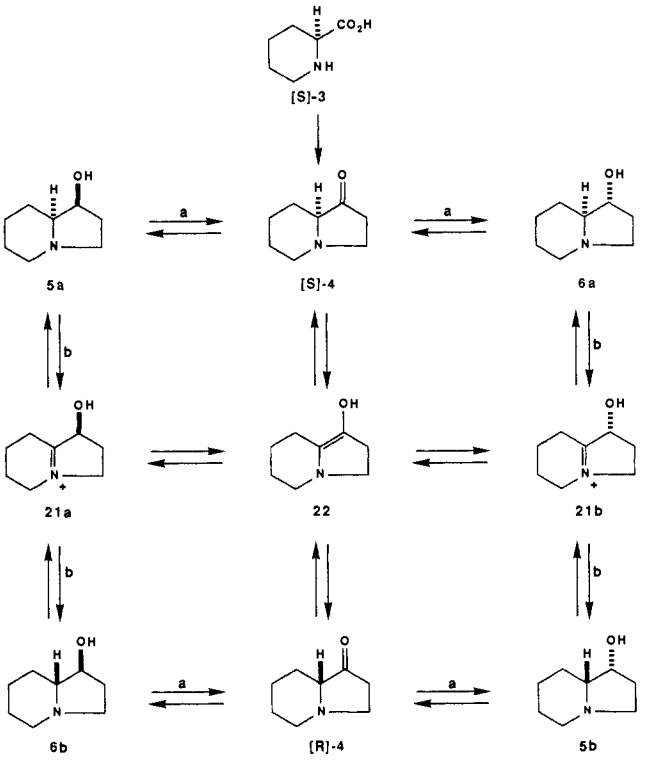
compd	MS of acetate esters, rel abund[a] d_0	d_1	d_2	d_3	sp act., product/ precursor	incorp, %
5a-d_3 (fed)[b]			22	100		
1a	37		21	100	0.48	11
2a	100				0.02	0.5
diol (unknown)[c]	27		21	100	*d*	*d*
5[c] recovered			64	100	*d*	*d*
6[c]	20		66	100	*d*	*d*
5b-d_3 (fed)[b]			22	100		
1a	100	2			*h*	
2a[e]	100		15	35	0.26	8
10	27		39	100	0.84	5.6
5[c] recovered			60	100	*d*	*d*
6[c]			55	100	*d*	*d*
6a-d_3 (fed)[b]			22	100		
1a	100	5			*h*	
2a[f]	100		22	41	0.26	13
10	19		43	100	0.73	9
5[c]	100				*d*	*d*
6[c] recovered	13		66	100	*d*	*d*
6b-d_3 (fed)[b]			22	100		
1a	100	4			*h*	
2a	100				*d*	*d*
5[g]					*d*	*d*
6[c] recovered			66	100		

[a] Analyzed by GC-MS (CH_4-CI). [b] Precursors were fed at 1.4–1.5 mM; mats were harvested after 5 days. [c] GC–MS identification only. [d] None determined. [e] Measurement of isotope ratios by ^{13}C NMR gave d_0:d_2:d_3 = 100:6:28. [f] Measurement of isotope ratios by ^{13}C NMR gave d_0:d_2:d_3 = 100:9:32. [g] Not detected. [h] Highly radioactive impurity made determination unreliable.

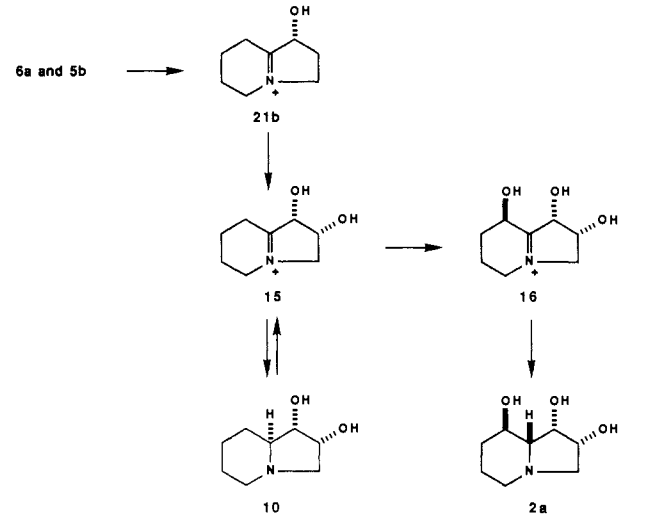
esters of the alcohols.[19] Each alcohol was incubated with three mats; after 5 days the cultures were harvested, and the metabolites were isolated as their acetates. Both the crude mixture of acetylated alkaloids and the purified metabolites were examined by GC–MS (Table III). The sites of deuteriation were confirmed by ^{13}C and ^2H NMR. The results fully substantiate the hypotheses developed from the feeding of racemic intermediates that (1) cis diastereomer **5a** (1*S*,8a*S*) incorporates efficiently into slaframine, while the other three diastereomers are only sparingly and indirectly utilized, and (2) both cis diastereomer **5b** (1*R*,8a*R*) and trans diastereomer **6a** (1*R*,8a*S*) incorporate efficiently into swainsonine. The fate of the remaining diastereomer **6b** (1*S*,8a*R*) is uncertain in that it failed to incorporate into any isolable metabolites. It is of interest that the yield of slaframine was markedly improved by addition of racemic **5** or enantiomer **5a** to the culture.

Certain important ancillary results were obtained from this experiment. *trans*-1-Hydroxyindolizidine-d_3 was detected among the metabolites isolated from cultures fed cis isomers **5a** and **5b**. Cultures fed trans isomer **6a** contained d_3 and d_2 trans species and d_0 cis species but no d_2 or d_3 cis species, indicating that interconversion between the 1-hydroxy isomers is primarily in the direction of cis → trans. Scheme VI shows the various pathways by which the 1-hydroxy isomers could hypothetically interconvert. The cis–trans interconversion can occur either by reoxidation to the ketone followed by reduction from the other face (path a) or through an iminium ion (path b). Interconversion by path a would lead to a d_2 species (unless, of course, the deuterium that was removed is readded). The increase in d_2 relative to d_3 in the recovered 1-hydroxy compounds seen here and in recovered **6a,b** (Table II) argues that the path a process does occur to some extent; path b, through iminium ion **21a** or **21b**, would give rise to a d_3 species, although tautomerization of the iminium ion to enamine **22** could also lead to a d_2 species. The observation that the d_3 cis diastereomers led to d_3 trans diastereomers supports the hypothesis that path b is operative when H-1 and H-8a are in the cis orientation, but not when they are trans (no d_3 cis is formed from d_3 trans). It is doubtful whether interconversion among the 1-hydroxy diastereomers is of any great significance in the bio-

Scheme VI



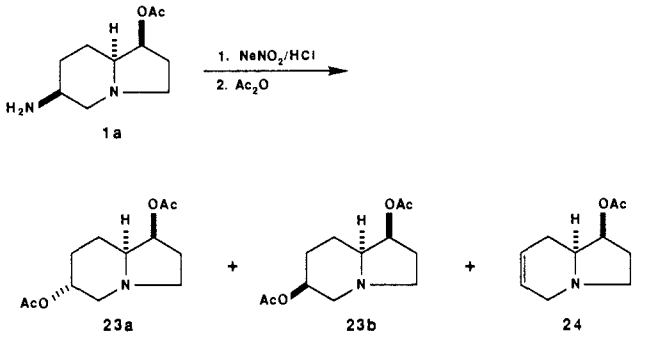
Scheme VII



synthesis of the alkaloids, however, except perhaps for **5b**→**6a** conversion (see below). For example, **5a** is incorporated exclusively into **1a**, hence path a to **6a** cannot be significant; path b to **6b** does apparently occur to some extent; but the fact that **6b** is not incorporated into **1a** or **2a** means that neither the conversion of **6b** to **5b** via path a nor the conversion of **6b** to **5a** via path b is significant. Early feeding experiments[8d] (and Table I) did show measurable incorporation of racemic **6** into slaframine, but in subsequent experiments (Tables II and III) little incorporation was seen. The reason for this is not clear, although it is possible that enzyme levels in the fungus have changed over time to the point that there is now little conversion of **6** to **5**.

A second noteworthy finding is that *trans*-diol **10** resulted from incubations of both **5b** and **6a**; no *cis*-diol **12** was detected by GC–MS; i.e., the cis 1-hydroxy compound was converted to the same diol as the trans. Sufficient diol was isolated from both feeding experiments to confirm the structure and sites of deuteriation by NMR spectroscopy. The failure to detect diol **12** is in agreement with the results obtained during the initial isolation and identification of diol **10**. These results, taken in conjunction with those on the conversion of **5b** to **6a** discussed above, suggested

Scheme VIII



that the pathway to **2a** is **5b** → **6a** → **10** → **2a**. However, the alternative had to be considered that both hydroxylation steps occur through iminium ions and that diol **10** is only a side product that can readily reenter the pathway (Scheme VII).

To test the latter possibility, *trans*-1-hydroxy diastereomer **6a** was prepared with deuterium at positions 1,2,2′, and 8a. Ketone **4** was equilibrated with D_2O to introduce deuterium at positions 8a,2, and 2′, and the deuteriated ketone was resolved as before and reduced with NaB^2H_4 to give **5a**-d_4 and **6a**-d_4. Compound **6a**-d_4 was fed, and the metabolites were isolated as before. If the biosynthetic pathway involved hydroxylation of iminium ion **21b**, then diol **10** would be devoid of deuterium at position 8a. NMR spectra of the diacetate of **10** showed, however, that **10** had retained 89% of the deuterium at position 8a and, therefore, iminium ion **21b** must not be an obligatory intermediate in the hydroxylation of **6a** to give **10**. Swainsonine showed no incorporation of deuterium at position 8a in this experiment; this observation is further evidence for the involvement of iminium ions **15** and **16** in the conversion of **10** to **2a**.

A third useful observation coming out of feeding the optically active alcohols was the detection of minute quantities of a new diol from the feeding of cis diastereomer **5a** (Table III). The retention time and fragmentation pattern of its diacetate were clearly different from the acetates of **10** and **12**. Guengerich et al. found that labeled 1,6-diol **9**, of unknown stereochemistry at the 6-position, was incorporated into slaframine, but they detected no 1,6-diol in fermentations.[8a] To identify the unknown diol, the C-6 epimers of 1,6-dihydroxyindolizidine were prepared from slaframine by the procedure of Guengerich in which slaframine is deaminated with nitrous acid (Scheme VIII).[8a] The crude product mixture from the deamination was acetylated and separated by column chromatography to give the acetate esters of (1*S*,6*R*,8a*S*)- and (1*S*,6*S*,8a*S*)-1-acetoxy-6-hydroxyindolizidines (**23a**, **23b**) in a combined yield of 15% and olefin **24** as the major product (45%). The stereochemistry at C-6 in the major diastereomer (**23a**) was assigned by NMR on the basis of coupling constants of the 6-proton [δ 4.87 ($J_{6,5eq}$ = 4.7 Hz, $J_{6,5_{ax}}$ = 11.6 Hz, $J_{6,7_{eq}}$ = 4.7 Hz, $J_{6,7_{ax}}$ = 11.6 Hz, H-6_{ax}], which indicated that the acetoxy group was equatorial in this isomer. In the spectrum of diastereomer **23b**, the 6-proton appeared at δ 5.03, in agreement with the predicted downfield location of an equatorial proton relative to an axial one and the coupling pattern appeared almost identical with that of the 6-proton of slaframine itself. The chemical ionization (CH_4) mass spectra of **23a** and **23b** showed *m/z* 182 as the base peak (loss of HOAc), whereas the isomeric 1,2-diacetates of **10** and **12** exhibit *m/z* 122 as their base peaks, probably attributable to a protonated pyrrole structure. Structure **24** was assigned for the olefin by NMR on the basis of the COSY spectrum, which clearly showed that the double bond was 6,7 not 5,6.

Identification of the 1,6-diol was carried out with metabolites obtained from feeding [1-^{2}H]**5a**, which had been synthesized by reduction of the (+)-BCS salt of **4** with NaB^2H_4 to give 5a-d_1 and **6a**-d_1. The acetates of unfractionated metabolites resulting from feeding [1-^{2}H]**5a**, as well as an enriched fraction from column chromatography of the acetylated material, were analyzed by GC–MS. These data as well as mass spectral data of synthetic

Table IV. Identification of (1*S*,6*R*,8a*S*)- and (1*S*,6*S*,8a*S*)-1,6-Dihydroxyindolizidine (**23a**, **23b**) in Cultures of *R. leguminicola* from Feeding (1*S*,8a*S*)-[1-^{2}H]-1-Hydroxyindolizidine (**5a**)

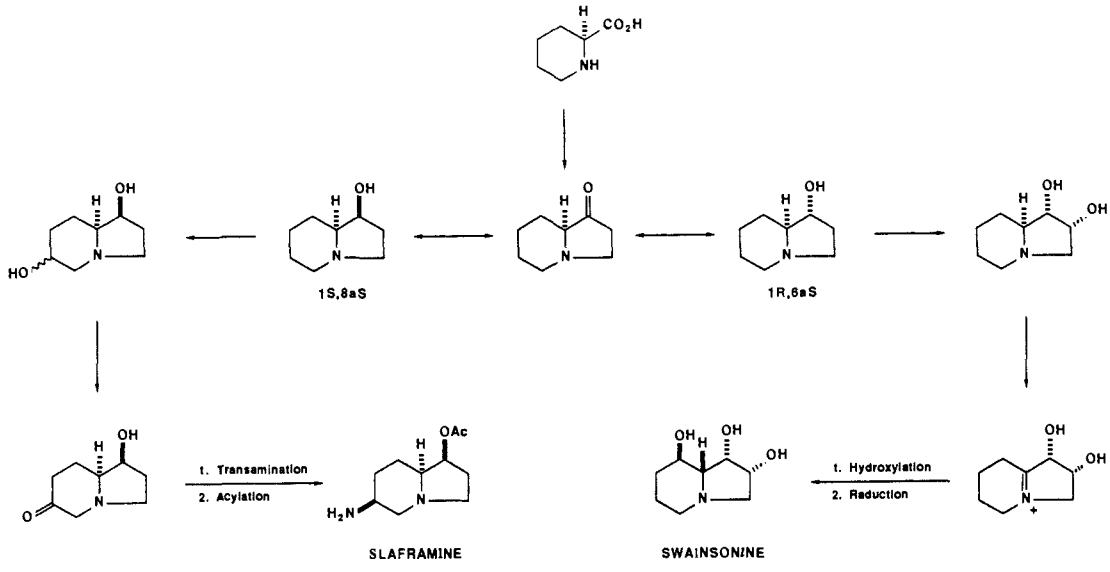
compd	ret time, min	GC–MS,[a] *m/z*	rel intens
5a-*d* (precursor,[c] as acetate ester)		185 (d_1)	44[b]
		184 (d_0)	19
		123	100
		122	52
23a	8.03	243 (d_1)	30
		242 (d_0)	23
		183	98
		182	100
		123	20
		122	39
23b	7.20	243 (d_1)	61
		242 (d_0)	55
		183	81
		182	100
		123	40
		122	59
10 (diacetate)	6.39	242	38
		182	49
		122	100
1b	11.05	242 (d_1)	100
		241 (d_0)	66
synthetic **23a**	8.17	242	29
		182	100
		122	16
		121	26
synthetic **23b**	7.29	242	84
		182	100
		122	27
		121	40

[a] Analyzed by Cl-MS (CH_4); the listed *m/z* values represent M + H^+ ions. [b] By ^{1}H NMR the ratio of d_1 to d_0 was 100:27. [c] Precursor was fed at 0.84 mM concentration; mats were harvested after 5 days.

23a,**b** are shown in Table IV. From the retention times and fragmentation patterns it is apparent that in this experiment both diastereomers of **9** were formed from deuteriated **5a**.[20] The mass spectra of **23a** and **23b** both exhibit d_0 species, indicating that the corresponding diols are natural metabolites of the fungus. Diol **10** was also formed in this fermentation but was undeuteriated. Unfortunately, these results do not answer the question of which epimer of the 1,6-diol is the natural precursor of slaframine. It is conceivable but unlikely that both are natural precursors; more probably one arises by direct oxidation of **5a** at the 6-position and the other is the product of reduction of the 6-oxo derivative.

Although the experiments with optically active 1-hydroxyindolizidines yielded valuable information, certain ambiguities remained in the swainsonine pathway, namely whether under normal circumstances the fungus produces and utilizes both 8a-epimers of the (1*R*)-1-hydroxy- and (1*S*,2*R*)-1,2-dihydroxyindolizidines. Mass spectral results indicated that both *cis*- and *trans*-1-hydroxyindolizidines are present (in very small amounts), but no 1-hydroxyindolizidine has been isolated from the fungus. A trapping experiment was undertaken to probe which 1-hydroxy diastereomers are actually formed. In separate experiments the fungus was incubated with DL-[^{3}H]- and L-[^{3}H]pipecolic acid together with cis and trans racemic 1-hydroxyindolizidines; the mats were harvested after 90 min and extracted after introduction of additional nonradioactive *cis*- and *trans*-1-hydroxyindolizidine as carrier. Although only L-pipecolic acid has been isolated from the fungus,[8b] we felt it necessary to examine the possibility that the D isomer might be the precursor to **5b** and **6b** even if not previously detected. After a series of purification steps the recovered 1-hydroxy compounds were derivatized with dibenzoyl-D-tartaric anhydride to give dibenzoyl-D-tartrate esters, which were separated by HPLC. The UV absorbance at 254 nm and ^{3}H content of the four esters were measured; the results are shown in Figure 1. The following can be seen: (1) DL-Pipecolic and L-pipecolic acid gave similar patterns of products, reinforcing the conclusion[8b] by Guengerich and Broquist that the L isomer is the

Scheme IX



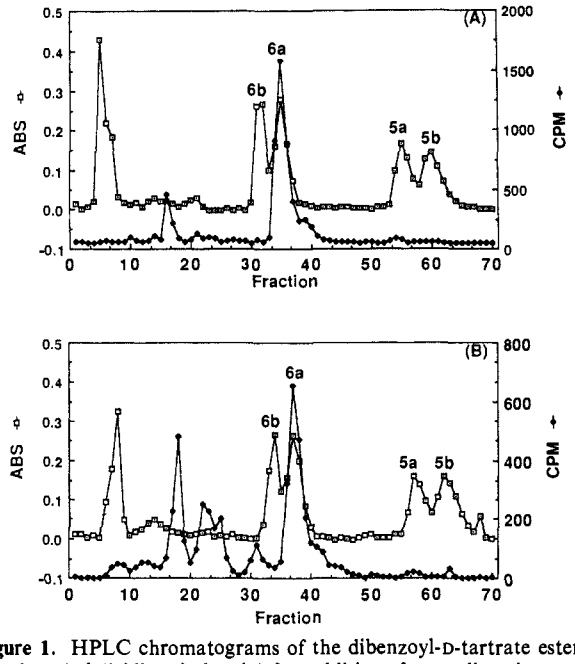


Figure 1. HPLC chromatograms of the dibenzoyl-D-tartrate esters of 1-hydroxyindolizidines isolated (after addition of nonradioactive carrier) from feeding DL-[^{3}H]pipecolic acid (A) and L-[^{3}H]pipecolic acid (B).

true precursor of both alkaloids. (2) The predominant diastereomer accumulated by the culture under these conditions is **6a**, the (1*R*,8a*S*) trans compound; isomer **5a** could be detected in trace amounts. These results are at variance with the report by Guengerich et al. that formation of the cis isomer predominated in a cell-free system.[8a] It is noteworthy that, in their study, the isolate of the fungus produced primarily slaframine while the culture used in the present study produced substantially more swainsonine than slaframine.

Our current view of alkaloid biosynthesis in *R. leguminicola* is summarized in Scheme IX. We feel that compounds **5b** and **12** are not natural intermediates in the biosynthetic pathway, although the organism is able to utilize them efficiently in the synthesis of swainsonine. The incorporation of unnatural precursors into naturally occurring alkaloids has been observed previously, for example, in the biosynthesis of nicotine.[22] The proposed epimerization of **5b** and **12** via iminium ions **21b** and **15** is similar to that suggested by Battersby et al.[23] for the interconversion of (+)- and (−)-reticuline; (−)-reticuline is the direct precursor of the opium alkaloids, but (+)-reticuline is also well incorporated but with loss of label at the 2-position. In that case the investigators were able to prepare the intermediate iminium compound, 1,2-didehydroreticuline, which incorporated very well. Our attempts at isolation and synthesis of iminium ions **15**, **16**, and **21b** have not yet been successful. These species can readily lose a proton from the 1-position to give the enol of **4**. Further clarification of the biosynthetic pathway to these indolizidine alkaloids in *R. leguminicola* may well require isolation and study of individual enzymes. A very interesting question to be answered about the biosynthesis of swainsonine is whether a similar pathway is operative in the plants that produce it; it is quite conceivable that in plants the five-membered ring (as proline) could be the starter unit followed by annelation of the piperidine ring. Studies to answer this question are in progress.

Experimental Section

General Procedures. NMR spectra were recorded on JEOL FX-90Q, IBM NR-300, and Bruker AM-400 spectrometers (^{1}H at 90, 300, and 400 MHz, respectively). ^{2}H spectra of the compounds isolated from the feeding of $CH_3C^2H_2OH$ were obtained at 92 MHz on the Carnegie-Mellon spectrometer operated in the rapid-scan correlation mode with homonuclear lock on the solvent. Mass spectra were recorded on LKB-9000, VG-Micromass 7070, Ribermag R-1010B, and Ribermag R-1010C spectrometers. GC–MS was carried out on an SPB-1 (10-m) column attached to a Ribermag 1010-C mass spectrometer with detection by chemical ionization, either with CH_4 or with NH_3 as the ionizing gas. The latter gave less fragmentation. Radioactivity was measured on a Beckman LS-100 liquid scintillation counter with [^{3}H]toluene as an internal standard; samples were routinely counted in duplicate to <3% error. Distillations were carried out using a Kugelrohr apparatus. HPLC was performed using an IBM 9533 instrument. During the workup of reactions, extracts were routinely dried over $MgSO_4$ prior to evaporation on a rotary evaporator at aspirator pressure. Chromatographic solvents are expressed as percent by volume of the first component in the solvent mixture.

Growth Conditions for the Fungus. *R. leguminicola* Gough et E.S. Elliot (ATCC No. 26280) was obtained on agar slants from American Type Culture Collections, Rockville, MD, and stored on slants of filtered red clover hay infusion (10% w/v) hardened with 1.5% Bacto-Agar (Difco). Inocula were maintained on sterile, filtered red clover hay infusion (240 mL, 10% w/v) in 1-L Roux bottles at 20–25 °C. At 3–4-week intervals subcultures were prepared by blending a mycelial mat with 100 mL of sterile water and transferring 10-mL aliquots of the resulting suspension to fresh hay infusion in Roux bottles. A fresh culture line was begun from a slant every 3–4 months.

Metabolite Production. The growth conditions described by Clevenstine et al.[8c] were employed with the following modification made for

(22) Leete, E. *In Alkaloids, Chemical and Biological Perspectives*; Pelletier, S. W., Ed.; Wiley: New York, 1983; Vol. 1, Chapter 3.

(23) Battersby, A. R.; Foulkes, D. M.; Binks, R. *J. Chem. Soc.* **1965**, 3323–3332.

certain experiments: the red clover hay infusion medium was replaced by Czapek Dox medium containing 0.3% Difco Bacto yeast extract.[24]

Cultures for feeding studies were young mats (5–7 days after inoculation from another mycelial mat), which were grown in 1-L Roux bottles to the point of confluence. The medium was decanted, and sterile water containing the substrate (50 mL passed through 0.45-μm sterile filter or autoclaved) was added so that the mycelial mat returned to the surface. After growth at room temperature for the desired time, the mat was removed, homogenized in 95% EtOH, and extracted with EtOH in a Soxhlet apparatus for 24 h. After evaporation of the EtOH the residue was dissolved in a large excess of Ac_2O and stirred at room temperature for 2 days. The solvent was evaporated, and 1 M HCl was added to the residue. The solution was washed with CH_2Cl_2, K_2CO_3 was added until pH 10, and the mixture was extracted with CH_2Cl_2. The CH_2Cl_2 extract was reextracted with 1 M HCl and the extraction cycle repeated in order to remove all nonalkaloidal material. The final CH_2Cl_2 solution was concentrated. The residue was chromatographed on a short column of TLC silica gel (Merck 60F-254) packed and eluted with $CHCl_3$–MeOH (98:2). **2b** eluted first, followed by diol diacetates, swainsonine diacetates, and finally **1b**. **2b**: ^1H NMR ($CDCl_3$) δ 5.52 (H-1), 5.21 (H-2), 4.96 (H-8), 3.17 (H-3), 3.05 ($H\text{-}5_{eq}$), 2.57 (H-3′), 2.18 (H-8a), 2.07, 2.04, 1.98 (3 $COCH_3$), 2.00 ($H\text{-}7_{eq}$), 1.85 ($H\text{-}5_{ax}$), 1.73 ($H\text{-}6_{eq}$), 1.71 ($H\text{-}6_{ax}$), 1.25 ($H\text{-}7_{ax}$); ^{13}C NMR ($CDCl_3$) δ 170.02, 169.80, 169.80 (3 $COCH_3$), 70.17 (C-2), 69.79 (C-1), 69.20 (C-8a), 68.01 (C-8), 59.12 (C-3), 51.70 (C-5), 29.76 (C-7), 23.26 (C-6), 20.88, 20.50, 20.39 (3 $COCH_3$). **1b**: ^1H NMR ($CDCl_3$) δ 6.40 (N*H*), 5.25 (H-1) 4.20 ($H\text{-}6_{eq}$), 3.07 (H-3), 3.03 ($H\text{-}5_{eq}$), 2.27 (H-2), 2.17 ($H\text{-}5_{ax}$), 2.08 (CH_3CO), 2.03 (H-3′), 1.99 (CH_3CO), 1.95 ($H\text{-}7_{eq}$), 1.93 (H-8a), 1.77 (H-2′), 1.60 ($H\text{-}8_{eq}$), 1.57 ($H\text{-}8_{ax}$), 1.46 ($H\text{-}7_{ax}$); ^{13}C NMR ($CDCl_3$) δ 170.60 ($OCOCH_3$), 169.06 ($NHCOCH_3$), 74.79 (C-1), 67.41 (C-8a), 57.49 (C-5), 53.02 (C-3), 43.85 (C-6), 30.51 (C-2), 28.16 (C-7), 23.47 ($NHCOCH_3$), 21.05 ($OCOCH_3$), 20.55 (C-8).

Preparation and Feeding of DL-[1,3,3′-$^2H_3$]-1-Hydroxyindolizidines (5-d_3, 6-d_3). 1-Hydroxyindolizidines-d_3 **5** and **6** were prepared and separated by the method of Clevenstine et al.[8d] They were fed at a concentration of 2.2 mM to fungal mats, which were harvested after an additional 6 days of growth.

Preparation of DL-[2-^2H]-Pipecolic Acid.[14] A solution of pipecolic acid (1.3 g, 0.010 mol) in D_2O (3.7 mL) was evaporated to dryness, freshly distilled Ac_2O (21.7 mL) and D_2O (2.5 mL) were added, and the mixture was refluxed under N_2 in a 170 °C oil bath for 2 min. The solvent was removed, treatment with Ac_2O/D_2O repeated, and the solution evaporated. The residue was refluxed in 1 M HCl for 12 h. The solution was evaporated and the residue recrystallized from $EtOH/Et_2O$ (1:1.5) to give 1.1 g (66%) of DL-[2-^2H]-3-HCl: mp 255 °C dec [lit.[25] mp 258–261 °C]; No ^1H at C-2 could be detected by ^1H or ^{13}C NMR; MS (EI) *m/e* (rel intens) 130 (M^+, 7), 129 (3), 86 (31), 85 (100), 84 (26), 57 (40), 56 (27), 55 (16).

Preparation of Ethyl DL-[2-^2H]-*N*-Acetylpipecolate. [2-^2H]3-HCl (0.5 g, 3.0 mmol) was refluxed for 12 h in EtOH saturated with HCl gas. The mixture was basified with K_2CO_3 to pH 10 and extracted with CH_2Cl_2. The extract was evaporated, the residue was dissolved in Ac_2O, and the mixture was stirred for 12 h at room temperature. After removal of the solvent, the residue was distilled [95 °C (0.1 mm)] to yield 0.49 g (82%) of ethyl [2-^2H]-*N*-acetylpipecolate as a colorless oil: MS (EI) *m/e* (rel intens) 200 (M^+, 4), 157 (2), 155 (2), 127 (43), 85 (100).

Preparation of DL-[1,2,2′,3,3′,8a-$^2H_6$]-1-Hydroxyindolizidines (5-d_6, 6-d_6). Ethyl [2-^2H]-*N*-acetylpipecolate (0.6 g, 3.0 mmol) in dry THF was added dropwise to a stirred suspension of KH (1.3 g, 23.4% oil dispersion, washed twice with pentane) in dry THF under N_2.[8d] After 2 h, D_2O (5 mL) was added dropwise and the THF was evaporated. The solution was acidified to pH 5 with 12 M DCl and rapidly extracted with CH_2Cl_2. The solvent was evaporated, and the residue, dissolved in dry THF, was added to $LiAl^2H_4$ (0.4 g) in THF and heated (under N_2) with stirring at 39 °C for 2 h. The solution was cooled to 0 °C, H_2O (0.4 mL), 10% NaOH (0.4 mL), and H_2O (1.2 mL) were added in succession; a large excess of Et_2O was added, and the mixture was stirred for 12 h. The suspension was filtered and the filtrate evaporated. The residue, dissolved in a minimum of H_2O, was chromatographed on Bio-Rad AG 50W-X8 cation exchange resin at 50 °C with a 0.1 M $NaHCO_3$/0.1 M NaOH gradient. The fractions containing **5**-d_6 and **6**-d_6 were identified by development with Dragendorff's reagent of fractions spotted on a silica gel TLC plate. The indolizidines were obtained from the aqueous solution by continuous extraction with CH_2Cl_2. **6**-d_6 eluted first, followed by **5**-d_6. **6**-d_6: 58.6 mg, 13%; ^{13}C NMR ($CDCl_3$) δ 74.98 (t, C-1), 70.68 (s, C-8a without ^2H), 70.05 (t, C-8a with ^2H), 53.20 (C-5), 28.47 (C-8 without ^2H at C-8a), 28.35 (C-8 with ^2H at C-8a), 24.82 (C-7), 23.96 (C-6), C-2 and C-3 were not observed; MS (Cl, NH_3) *m/e* (rel intens) 148 ($M + H^+$, 12), 147 (23), 146 (13), 145(7), 144 (2), 101 (11), 100 (100), 99 (50), 98 (10). **5**-d_6: 56.7 mg, 13%; ^{13}C NMR ($CDCl_3$) δ 72.07 (t, C-1), 68.87 (C-8a without ^2H), 68.33 (t, C-8a with ^2H), 53.45 (C-5), 25.02 (C-7), 24.94 (C-8), 24.83 (C-8 with ^2H at C-8a), 23.79 (C-6); MS (CI, NH_3) *m/e* 148 ($M + H^+$, 28), 147 (31), 146 (17), 145 (9), 144 (3), 118 (13), 117 (14), 116 (9), 115 (3), 100 (100), 99 (61), 98 (11).

Feeding Studies with DL-[1,2,2′,3,3′,8a-$^2H_6$]-1-Hydroxyindolizidines. Groups of three mats were fed **5**-d_6 and **6**-d_6 (1.86 mM) and harvested after 2 days. From feeding **5**-d_6, **2b** (12.3 mg) and **1b** (10.9 mg) were isolated as described above (see mass spectral results in Table II). **1b** was significantly enriched in ^2H, and evidence could be seen in the ^{13}C spectrum: ^{13}C NMR ($CDCl_3$) δ 170.60 ($OCOCH_3$), 169.06 ($NCOCH_3$), 74.77 (C-1, with ^1H at C-1 and C-8a, very weak), 67.37 (C-8a with ^1H at C-8a and C-1, very weak), 67.30 (C-8a with ^1H at C-8a and ^2H at C-1, very weak), 57.48 (C-5), 53.01 (C-3, very weak), 43.87 (C-6), 30.56 (C-2, very weak), 28.16 (C-7), 23.48 ($NCOCH_3$), 21.01 ($OCOCH_3$), 20.58 (C-8, weak), 20.48 (C-8 with ^2H at C-8a, weak). Sites of deuteriation in **2b** were determined to be C-1, C-2, C-3, and C-3′ by ^2H NMR. No starting material was recovered from this feeding.

From the feeding of **6**-d_6, **2b** (12.8 mg) and **1b** (3.1 mg) were isolated. The mass spectrometry results are shown in Table II; ^{13}C and ^2H NMR spectra were used to confirm the sites of deuteriation in **2b**; the level of incorporation into **1b** was too low to be detected by NMR. Some **6** was recovered from this feeding, the mass spectrum of which showed a d_0 signal in addition to highly deuteriated species.

Preparation of DL-[8a-^2H]-1,2-Dihydroxyindolizidine (12). A mixture of acetonide **13**[13] (0.20 g, 1.0 mmol) and $Hg(OAc)_2$ (2.4 g, 7.5 mmol) in deoxygenated 5% aqueous HOAc (10 mL) was refluxed for 2 h under N_2.[26] Hg salts were removed by filtration, treatment of the filtrate with H_2S, and a second filtration through Celite. The filtrate was treated with $NaCNB^2H_3$ (0.10 g, 1.5 mmol) for 1 h. The aqueous mixture was washed with Et_2O, made alkaline to pH 10, and extracted with CH_2Cl_2. The extract was concentrated and chromatographed on neutral alumina (50 g, 50% EtOAc/hexane) to give 15% of [8a-^2H]**14**. The ^1H NMR spectrum of the material was similar to that of undeuteriated **14** except that the H-1 signal at δ 4.57 appeared as a doublet ($J_{1,2}$ = 6.3 Hz) rather than as a quartet. The H-8a signal for **14** appears in a poorly resolved area of the spectrum near δ 2.0; small differences could be detected in that portion of the spectrum of the deuteriated material but were not readily interpreted. The ^{13}C NMR spectrum was identical to that of undeuteriated **14** except that only a trace of the signal at δ 68.0 was present, which indicated that position 8a was 66% deuteriated. Chromatography also yielded some unaltered acetonide **13**; NMR spectra showed that it was undeuteriated. Acetonide **14** was converted to the free diol by treatment with 2 M HCl for 6 h at 80 °C, followed by lyophilization.

Preparation of (1*S*,2*R*,8a*R*)-[1-^3H]-1,2-Dihydroxyindolizidine (12). [1-^3H]Swainsonine (1.00 mg, 14.8 μCi), obtained from feeding experiments with [1-^3H]**5** and -**6**, was diluted with 0.100 g (0.587 mmol) of unlabeled material and converted to diol **12** by the published procedure[13] in an overall yield of 23% (3.41 μCi).

Feeding Study with (1*S*,2*R*,8a*R*)-[1-^3H]-1,2-Dihydroxyindolizidine (12). Radioactive **12** (0.24 mM, 0.831 μCi, 23.1 μCi/mmol) was divided among three mats; after 2 days the mats were harvested. The resulting **2a** (19 mg) contained 2.42 μCi/mmol, representing a 32% incorporation of label with 10-fold dilution by endogenous **2a**.

Feeding Study with (1*S*,2*R*,8a*R*)-[8a-^2H,1-^3H]-1,2-Dihydroxyindolizidine (12). The labeled **12** (21 mg, 66% *d*, 0.150 μCi, 1.10 μCi/mmol) in sterile H_2O (150 mL) was added to three mats. After 2 days the mats were harvested and **2b** (50 mg, 0.27 μCi/mmol) was isolated. ^2H NMR and mass spectral (Cl) analysis indicated no incorporation of ^2H at position 8a or elsewhere in **2b**. On the basis of ^3H incorporation, position 8a of swainsonine would have been 17% deuteriated if the deuterium had remained intact.

Preparation of [1,1-$^2H_2$]Ethanol. Freshly distilled acetyl chloride (3.4 mL, 47.6 mmol) was added dropwise to $LiAl^2H_4$ (Aldrich; 1.0 g, 23.8 mmol) in dry Et_2O (35 mL) with ice bath cooling. The mixture was refluxed for 3 h, the Et_2O was evaporated, H_2O (50 mL) was added, and the [1,1-$^2H_2$]EtOH/H_2O was distilled, bp 78–100 °C. ^1H NMR was used to determine the concentration of the [1,1-$^2H_2$]EtOH in the distillate.

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Feeding Studies with [1,1-$^2H_2$]Ethanol. Several different feeding experiments were carried out. Fungal mats (6–8 days old) were incubated with [1,1-$^2H_2$]ethanol (50 mM) and unlabeled pipecolic acid hydrochloride (0.2 mM). The mats were harvested after 6–7 days and the alkaloids isolated by the usual procedure. Deuterium enrichment in **1b** and **2b** ranged from 3 to 6%.

Preparation and Resolution of [3,3′-$^2H_2$,*piperidine ring*-^3H]-1-Oxoindolizidine.[18,20] Ethyl [^3H]pipecolate (3.77 g, 0.024 mol, 2.98 μCi/mmol), ethyl [2,2,3,3-$^2H_4$]-3-bromopropionate (MSD Isotopes; 5 g, 0.027 mol), NaOAc (1.97 g, 0.024 mol), and KI (10 mg) were heated together on a steam bath for 1 h. The mixture was cooled and partitioned between H_2O and CH_2Cl_2. The aqueous layer was basified with K_2CO_3 and extracted with CH_2Cl_2. The residue from evaporation of the extract was distilled (bp 100–110 °C, 0.25 mm) to yield 3.72 g (61%) of ethyl [2,2′,3,3′-$^2H_4$,*ring*-^3H]-2-(ethoxycarbonyl)-1-piperidinepropionate (**17**, 2.67 μCi/mmol): ^1H NMR ($CDCl_3$) δ 4.20 (2 H, q), 4.13 (2 H, q), 3.15 (1 H, m), 3.0 (1 H, m), 2.87 (1 H, m, not detected), 2.70 (1 H, m, not detected), 2.50 (2 H, m, not detected), 1.80 (2 H, m), 1.60 (3 H, m), 1.38 (1 H, m), 1.28 (3 H, t), 1.25 (3 H, t).

Diester **17** (3.70 g, 0.014 mol, dissolved in 5 mL of dry C_6H_6) was added dropwise to a suspension of NaH (0.043 mol, prepared from 0.172 g of a 60% suspension of NaH in mineral oil that was washed three times with dry pentane before use) in dry C_6H_6 (35 mL) in a 250-mL flask. The mixture was refluxed for 1.25 h. (*Caution*! Foaming commences 30–45 min after application of heat.) Heat was stopped, and the reaction was stirred an additional 4 h. The C_6H_6 was removed under aspirator pressure and 6 M HCl (20 mL) was added cautiously to the pasty residue while the reaction flask was cooled in an ice bath. After addition was complete, the reaction was heated under reflux overnight, cooled, neutralized with saturated NaOH, and extracted with Et_2O. The extract was evaporated and the residue distilled [bp 50–60 °C (0.25 mm)] to give 1.18 g (60%) of [3,3′-$^2H_2$]-1-oxoindolizidine, which was immediately stored in the freezer: ^1H NMR ($CDCl_3$) δ 3.15 (1 H, m, H-3, not detected), 3.00 (1 H, m, H-5_{eq}), 2.35 (1 H, m, H-3′, not detected), 2.22 (2 H, m, H-2, H-2′), 2.08 (1 H, m, H-5_{ax}), 1.95 (1 H, m, H-8a), 1.85 (1 H, m, H-8_{eq}), 1.75 (1 H, m, H-7_{eq}), 1.55 (1 H, m, H-6_{eq}), 1.45 (1 H, m, H-6_{ax}), 1.16 (1 H, m, H-8_{ax}), 1.10 (1 H, m, H-7_{ax}); ^{13}C NMR δ 213.7 (C=O), 69.35 (C-8a), 53.62 (C-5), 50.00 (C-3, not detected), 35.81 (C-2 with ^1H at C-3, not detected), 35.60 (C-2), 25.09, 24.86, 23.60 (C-6, C-7, C-8).

Resolution of **4** was carried out by the method of Kunieda et al.;[18] **4**-d_2 (0.550 g, 3.90 mmol) was mixed with (+)-3-bromo-8-camphorsulfonic acid [(+)-BCS] in acetone (2.20 mL), the yellow solution was stored at −20 °C overnight, the resulting white solid was filtered, suspended in acetone, and filtered again to give 0.74 g (83%) of (+)-BCS salt **19** of (*S*)-[3,3′-$^2H_2$,*ring*-^3H]**4**: mp 160–161 °C; $[\alpha]^{26}_D$ +32° (*c* 1.22, H_2O) [lit.[18a] mp 160–161.5 °C; $[\alpha]^{15}_D$ +38.7° (*c* 1.25, H_2O)]. The same procedure employing (−)-BCS yielded salt **20** (0.74 g, 83%) of enantiomeric (*R*)-[3,3′-$^2H_2$,*ring*-^3H]**4**: mp 158–159 °C; $[\alpha]^{26.5}_D$ −36° (*c* 1.06, H_2O).

Preparation of [1,3,3′-$^2H_3$,*piperidine ring*-^3H]5a, -5b, -6a, and -6b.[19] (+)-BCS salt **19** (0.74 g, 1.72 mmol) was added as a solid in small portions to a cooled (ice bath) solution of NaB^2H_4 (0.207 g, 4.93 mmol) in absolute EtOH. The reaction mixture was stirred as it was allowed to warm up to room temperature for a total reaction time of 5 h. The EtOH was evaporated, H_2O (2 mL) was added, and the solution was extracted with CH_2Cl_2. The extract was evaporated to give a mixture of (1*R*,8a*S*)-[1,3,3′-$^2H_3$,*piperidine ring*-^3H]- and (1*S*, 8a*S*)-[1,3,3′-$^2H_3$,*piperidine ring*-^3H]-1-hydroxyindolizidines as a colorless oil (0.248 g, 100%). The diastereomeric mixture, dissolved in 0.1 M $NaHCO_3$ (1 mL) was applied to a column (1.2 × 50 cm) of Dowex 50-X2 (200–400 mesh, 49 °C, equilibriated with 0.1 M $NaHCO_3$); the column was eluted with a gradient of 0.1 M $NaHCO_3$ (150 mL) and 0.1 M NaOH (150 mL), and 2.5-mL fractions were collected. The compounds were deteeted by measuring radioactivity (50-μL aliquots) and by TLC (alumina, 5% MeOH/$CHCl_3$; **5**, R_f 0.58; **6**, R_f 0.47) examination of Et_2O (1-mL) extracts of every fifth tube. Isomer **6a** eluted in fractions 150–190, isomer **5a** in fractions 205–245. The aqueous fractions were extracted continuously with CH_2Cl_2; the extract was concentrated and distilled to give **6a** [81 mg, 66%); $[\alpha]^{27}_D$ −51° (*c* 1.12, EtOH) [lit.[19] $[\alpha]^{28}_D$ −49° (*c* 0.90, EtOH)]] and **5a** [76 mg, 62%; $[\alpha]^{27}_D$ + 23° (*c* 0.8, EtOH) [lit.[19] $[\alpha]^{27}_D$ +27° (*c* 0.95, EtOH]]; the specific activity of **6a** and **5a** was 2.45 μCi/mmol. **6a**: ^1H NMR ($CDCl_3$) δ 3.85 (0.15 H, m, H-1), 2.95 (m, 1 H, H-5_{eq}), 2.90 (1 H, m, H-3, not visible), 2.30 (1 H, m, H-3′, not visible), 2.15 (1 H, m, H-2), 2.00 (1 H, m, H-5_{ax}), 1.90 (1 H, m, H-8_{eq}), 1.75 (1 H, m, H-7_{eq}), 1.70 (1 H, m, H-8a), 1.55 (1 H, m, H-6_{eq}), 1.45 (2 H, m, H-2′, H-6_{ax}), 1.20 (2 H, m, H-7_{ax}, H-8_{ax}). **5a**: ^1H NMR ($CDCl_3$) δ 4.05 (0.15 H, m, H-1), 3.13 (1 H, m, H-5_{eq} and H-3 not visible), 2.17 (1 H, m, H-2), 2.00 (1 H, m, H-3′ not visible), 1.95 (1 H, m, H-5_{ax}), 1.85 (1 H, m, H-8_{eq}), 1.73 (1 H, m, H-8a), 1.70 (1 H, m, H-2′), 1.68 (1 H, m, H-7_{eq}), 1.65 (1 H, m, H-6_{eq}), 1.54 (1 H, m, H-6_{ax}), 1.25 (1 H, m, H-8_{ax}), 1.20 (1 H, m, H-7_{ax}).

The same procedure was followed for (−)-BCS salt **20** to give **6b** [74%; $[\alpha]^{28}_D$ +45° (*c* 1.16, EtOH) [lit.[19] $[\alpha]^{28}_D$ +51° (*c* 0.54, EtOH)]] and **5b** [52%; $[\alpha]^{27}_D$ −23° (*c* 1.12, EtOH) [lit.[19] $[\alpha]^{28}_D$ −33° (*c* 1.0, EtOH)]]; the specific activity of **6b** and **5b** was 2.45 μCi/mmol. The NMR spectra of **5b** and **6b** were identical with those of **5a** and **6a**.

The optical rotations determined on the purified alcohols were of low accuracy because of the small amount of material and the difficulty of removing all solvent from the relatively volatile (particularly the cis isomer) amino alcohols. Therefore, the enantiomeric purity of the resolved amino alcohols was checked by preparing the dibenzoyl-D-tartrate esters of the purified alcohols by treatment with 1 equiv of dibenzoyl-D-tartaric acid anhydride[27] in CH_2Cl_2, overnight at room temperature. Analysis of the esters by ^1H NMR and HPLC indicated optical purity of greater than 98%.[19]

Feeding of Labeled 5a, 5b, 6a, and 6b. Each precursor (1.40–1.50 mM) was fed to three mats; the mats were harvested after 5 days. The crude acetates were analyzed by GC–MS; the results are shown in Table III. Purified metabolites were also analyzed by ^1H NMR, ^2H NMR, and ^{13}C NMR to verify sites and extent of deuteriation. Yields of **2b** include material obtained by reacetylation of partially acetylated **2a** (both 1,2- and 1,8-diacetoxy compounds were found). The control (no added hydroxyindolizidine) gave 2.7 mg of **1b** and 35.3 mg of **2b**; **5a** gave 13.1 mg of **1b** and 23.3 mg of **2b**; **5b** gave 4.6 mg of **1b**, 20.8 mg of **2b**, and 3.7 mg of the diacetate of *trans*-diol **10** [^1H NMR ($CDCl_3$) δ 5.24 (1 H, m, H-2), 4.77 (0.26 H, m, H-1), 3.60 (0.22 H, m, H-3, cis to H-2), 3.03 (1 H, m, H-5_{eq}), 2.4–1.0 (all remaining protons)]; **6a** gave 9.9 mg of **1b**, 33.2 mg of **2b**, and 7.0 mg of the diacetate of *trans*-diol **10** [^1H NMR ($CDCl_3$) δ 5.24 (1 H, m, H-2), 4.77 (0.21 H, m, H-1), 3.60 (0.14 H, m, H-3 (cis to H-2)), 3.03 (1 H, m, H-5_{eq}), 2.4–1.0 (all remaining protons)]; **6b** gave 3.6 mg of **1b** and 30.9 mg of **2b**.

Preparation of (1*R*,8a*S*)-[1,2,2′,8a-$^2H_4$,1-^3H]-1-Hydroxyindolizidine 6a-d_4). 1-Oxoindolizidine (0.5 g, 3.59 mmol) was stirred in D_2O (2 mL) for 1 h, extracted, distilled, and reequilibrated (1 mL D_2O) for an additional 1 h. Distillation gave [2,2′,8a-$^2H_3$]-1-oxoindolizidine (0.36 g, 70%), which was 100% deuteriated at position 8a and 95% deuteriated at each of the two C-2 sites, as determined by ^1H and ^2H NMR.

The ketone was resolved as described above utilizing (+)-BCS, which had been lyophilized from D_2O. The (+)-BCS salt was obtained in 21% yield [0.30 g; mp 159–161.5 °C, $[\alpha]^{27}_D$ + 38.9° (*c* 1.30, H_2O)] and reduced with a mixture of NaB^2H_4 (42 mg, 1.0 mmol) and [^3H]$NaBH_4$. The 1-hydroxyindolizidines were isolated as above. [1,2,2′,8a-$^2H_4$,1-^3H]**6a** was obtained in 37% yield (122 μCi/mmol); **5a** was obtained in 12% yield. NMR analysis showed the following distribution of deuterium: C-1 0.84, C-2 0.78, C-8a 0.71. The deuterium loss at C-2 and C-8a relative to the parent ketone probably resulted from exchange with H_2O in the bromocamphorsulfonic acid, which is difficult to dry thoroughly.

Feeding of [1,2,2′,8a-$^2H_4$,1-^3H]6a. Two mats were incubated with **6a**-d_4 (0.97 mM). After 5 days the mats were harvested and the metabolites were isolated as before to yield **1b** (2.2 mg), **2b** (18.7 mg, 23.1 μCi/mmol, 11% incorporation), and the diacetate of **10** (<2 mg after additional purification by TLC, 1.1% incorporation). The specific activity of the diacetate of **10** could not be established due to (nonradioactive) contamination of the sample; insufficient material was available for further purification. The deuterium distribution in the products was determined by integration of the ^1H NMR spectrum and the relative distribution confirmed by ^2H NMR: (**2b**) C-1 0.24, C-2 0.21, C-8a 0.0; (diacetate of **10**) C-1 0.56, C-2 0.48, C-8a 0.42. No deuterium was detected in **1b** by NMR or GC–MS.

Synthesis of (1*S*,8a*S*)-[1-^2H,1-^3H]-1-Hydroxyindolizidine (5a-d_1). To a cooled solution of a mixture of NaB^2H_4 (42 mg, 1.0 mmol) and [^3H]$NaBH_4$ (1 mg, 10 mCi in 0.1 mL of 0.01 M NaOH) in absolute EtOH (5 mL) was added the (+)-BCS salt of 1-oxoindolizidine (0.398 g, 0.88 mmol) as a solid over 30 min. The reaction was stirred 2 h as it warmed to room temperature. Chromatography and distillation gave **5a**-d_1 (14.1 mg, 22%) and **6a**-d_1 (25 mg, 40%). ^1H NMR showed d_1:d_0 = 100:22; specific activity 1.96 mCi/mmol.

Feeding of [1-^2H,1-^3H]5a. Compound **5a**-d_1 (0.84 mM) was fed as described above to two mats, which were harvested after 5 days and the metabolites isolated to give **2b** (8.5 mg, 0.03 mCi/mmol, 0.45% incorporation) and **1b** (6.9 mg, 1.03 mCi/mmol, 15.9% incorporation). The silica gel column fraction eluting directly after **2b** showed small amounts of three constituents with R_f values corresponding to **23b**, **23a**, and the diacetate of **10**. This fraction was analyzed by GC–MS for the presence of 1,6-dihydroxyindolizidines; see Table IV.

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Synthesis of (1*S*,6*R*,8a*S*)- and (1*S*,6*S*,8a*S*)-1,6-Diacetoxyindolizidines (23a, 23b). Compounds **23a** and **23b** were prepared from **1a** essentially as described by Guengerich et al.[8a] A solution of $NaNO_2$ (3.6 M, 1.0 mL) was added dropwise over the course of 1 h to a cooled (ice bath) solution of **1a** (52 mg, 0.26 mmol) in 0.5 M HCl (5 mL). The ice bath was removed, and the reaction was stirred at room temperature for 1 h. The mixture was adjusted to pH 10 with K_2CO_3 and then extracted with CH_2Cl_2. Evaporation of the extract gave a dark oil (30 mg), which was treated with Ac_2O (1 mL) and 4-(dimethylamino)pyridine (5 mg) for 12 h. The Ac_2O was evaporated and the residue was chromatographed on silica gel (4 g, Merck 60F-254) with elution by $CHCl_3$/MeOH (98:2). The products eluted in the order: **23a**, **23b**, **24**. Compound **23a**: (6.6 mg, 13%; ^1H NMR ($CDCl_3$) δ 5.22 (1 H, m, H-1), 4.87 (1 H, m, $J_{6,5_{eq}}$ = 4.7 Hz, $J_{6,5_{ax}}$ = 9.6 Hz, $J_{6,7_{eq}}$ = 4.7 Hz, $J_{6,7_{ax}}$ = 9.6 Hz, H-6_{ax}), 3.36 (1 H, m, $J_{5_{eq},5_{ax}}$ = 10.0 Hz, H-5_{eq}), 3.14 (1 H, m, H-3), 2.29 (1 H, m, H-2), 2.13 (1 H, m, H-7_{eq}), 2.06 (1 H, m, H-3′), 2.07 (3 H, s, CH_3CO), 2.03 (3 H, s, CH_3CO), 1.92 (2 H, m, H-8a, H-5_{ax}), 1.83 (1 H, m, H-2′), 1.73 (1 H, m, H-8_{eq}), 1.54 (1 H, m, H-8_{ax}), 1.31 (1 H, m, H-7_{ax}); MS (see Table IV). Compound **23b** (1.5 mg, 2%) was obtained not completely free of **23a**: ^1H NMR ($CDCl_3$) δ 5.22 (1 H, m, H-1), 5.03 (1 H, m, H-6_{eq}), 3.34 (1 H, m, H-5_{eq}), 3.14 (1 H, m, H-3), 2.29 (1 H, m, H-2), 2.13 (3 H, s, CH_3CO), 2.09 (3 H, s, CH_3CO), 2.15–1.95 (2 H, m, H-7_{eq}, H-3′), 1.95 (2 H, m, H-8a, H-5_{ax}), 1.75 (1 H, m, H-2′), 1.55 (1 H, m, H-8_{ax}) 1.35–1.25 (1 H, m, H-7_{ax}); MS (see Table IV). Compound **24**: 21 mg, 45%; ^1H NMR ($CDCl_3$) δ 5.85 (1 H, m, H-7), 5.67 (1 H, m, H-6), 4.16 (1 H, m, H-1), 3.53 (1 H, m, H-5), 3.25 (1 H, m, H-3), 2.78 (1 H, m, H-5′), 2.48 (1 H, m, H-8a), 2.25 (1 H, m, H-2), 2.07 (2 H, m, H-3′ + H-8), 2.04 (CH_3CO), 1.79 (2 H, m, H-2′ + H-8′); MS (Cl, NH_3) *m/e* ($M + H^+$) 182 (100), 122 (14), 121 (29), 120 (58), 119 (14), 118 (29).

Identification of 1-Hydroxyindolizidines in Cultures of *R. leguminicola*. L-Pipecolic acid was prepared by treatment of DL-pipecolic acid with D-amino acid oxidase.[28] Individual mats were fed L-[^3H]- or DL-[^3H]-pipecolic acid (0.31 mmol, 42.5 μCi) and DL-1-hydroxyindolizidines (**5-6**; 5.25 mg) in sterile H_2O (55 mL). The mats were incubated for 2 h, removed from the Roux bottles, air dried for 2 days, ground with sand, and continuously extracted with 95% EtOH. Additional nonradioactive **5-6** (18.7 mg) was added to the pulverized mats before extraction. The extracts were acidified with dilute HCl and evaporated. The residue was taken up in H_2O (10 mL) and filtered; the filtrate was continuously extracted with CH_2Cl_2. The aqueous layer was made basic with K_2CO_3 and extracted continuously with CH_2Cl_2. The latter extract was evaporated, and the residue was chromatographed on a short alumina column (1g) in $CHCl_3$. Compounds **5** and **6** eluted shortly after the solvent front. The recovered 1-hydroxyindolizidines (9.3 mg from DL-pipecolic acid and 11.5 mg from L-pipecolic acid) were derivatized by stirring with 1 equiv of dibenzoyl-D-tartaric acid anhydride (0.025 M in CH_2Cl_2) for 2 days at room temperature. The esters were analyzed by HPLC (C-18 column, 10 × 250 mm, 0.01 M Et_3N–PO_4, pH 7.5/MeOH, (45:55), 3 mL/min). Fractions (1.5 mL) were collected and analyzed by UV and liquid scintillation counting.

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