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Lentiginosine, a Dihydroxyindolizidine Alkaloid That Inhibits Amyloglucosidase†

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ABSTRACT: Lentiginosine, a dihydroxyindolizidine alkaloid, was extracted from the leaves of *Astragalus lentiginosus* with hot methanol and was purified to homogeneity by ion-exchange, thin-layer, and radial chromatography. A second dihydroxyindolizidine, the 2-epimer of lentiginosine, was also purified to apparent homogeneity from these extracts. Gas chromatography of the two isomers (as the TMS derivatives) showed that they were better than 95% pure; lentiginosine eluted at 8.65 min and the 2-epimer at 9.00 min. Both compounds had a molecular ion in their mass spectra of 157, and the NMR spectra demonstrated that both were dihydroxyindolizidines differing in the configuration of the hydroxyl group at carbon 2. Lentiginosine was found to be a reasonably good inhibitor of the fungal α-glucosidase, amyloglucosidase ($K_i = 1 \times 10^{-5}$ M), but it did not inhibit other α-glucosidases (i.e., sucrase, maltase, yeast α-glucosidase, glucosidase I) nor any other glycosidases. The 2-epimer had no activity against any of the glycosidases tested.

Plants produce many different types of alkaloids, but the biochemical mode of action of most of these has not been elucidated. On the other hand, a number of plant indolizidine and pyrrolizidine alkaloids have been shown to be potent inhibitors of various glycosidases (Elbein & Molyneux, 1987; Elbein, 1987). For example, swainsonine (1,7,8-trihydroxyindolizidine) is a toxic alkaloid that is found in the leaves and stems of *Swainsona* species that grow in western Australia (Colegate et al., 1979), and in *Astragalus* species that grow in the western U.S. (Molyneux & James, 1982; Davis et al., 1984; Molyneux et al., 1985). Members of both of these genera are considered to be poisonous to animals (Hartley, 1978). Swainsonine was shown to be a potent inhibitor of lysosomal α-mannosidase (Dorling et al., 1980), as well as other arylmannosidases (Kang & Elbein, 1983a). This alkaloid also inhibited the glycoprotein processing mannosidase II (Tulsiani et al., 1982) and in cultured cells caused the formation of N-linked glycoproteins having hybrid types of oligosaccharides (Elbein et al., 1981; Tulsiani et al., 1984; Arumughan & Tanzer, 1983; Kang & Elbein, 1983b).

Another indolizidine alkaloid (1,6,7,8-tetrahydroxyindolizidine), castanospermine, was crystallized from the seeds of the Australian tree *Castanospermum australe* (Hohenschutz et al., 1980) and found to be a potent inhibitor of α- and β-glucosidases (Saul et al., 1983). This alkaloid also inhibited the processing of N-linked glycoproteins. In this case, the inhibition was at the glucosidase I stage (Pan et al., 1983), resulting in the accumulation of glycoproteins with $Glc_3Man_9(GlcNAc)_2$ structures in cultured cells incubated in the presence of castanospermine (Repp et al., 1985; Arakaki et al., 1987; Pan et al., 1983).

After removal of the castanospermine from the seed extract by crystallization, several other new alkaloids were identified in these extracts. One of these was purified to homogeneity and identified as 6-epicastanospermine. Although this tetrahydroxyindolizidine alkaloid still had good activity against the amyloglucosidase, it had lost the inhibitory activity toward β-glucosidase (Molyneux et al., 1986). A second alkaloid was purified from these extracts and shown to be a tetrahydroxypyrrolizidine alkaloid. This compound, australine, had a unique substitution pattern and was also found to inhibit α-glucosidases (Molyneux et al., 1988). In addition, it inhibited glycoprotein processing at the glucosidase I stage, although not as effectively as castanospermine, and caused the accumulation of $Glc_3Man_{7-9}(GlcNAc)_2$ structures in cultured cells (Tropea et al., 1989). The fact that a pyrrolizidine alkaloid can also inhibit these glycosidases indicates that a six-membered ring is not essential for inhibitory activity. Previously, a polyhydroxypyrrolidine called DMDP had also been shown to inhibit α- and β-glucosidases, as well as the

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glycoprotein processing glucosidase I (Elbein et al., 1984).

In this paper, we demonstrate that a dihydroxyindolizidine alkaloid, named lentiginosine and isolated from *Astragalus lentiginosus*, also inhibits the α-glucosidase amyloglucosidase. Lentiginosine and its 2-epimer were purified from these extracts and characterized by mass spectrometry and high-resolution NMR. While lentiginosine did inhibit the α-glucosidase, neither it nor its 2-epimer showed any effect on other α-glucosidases such as sucrase or maltase or on the glycoprotein processing enzymes glucosidase I or II. Although other dihydroxyindolizidines have been isolated from natural sources, none of these has been shown to possess biological activity. Thus, these studies demonstrate that compounds with only two hydroxyl groups may show some glycosidase inhibitory activity.

Experimental Procedures

Materials. The enzymes amyloglucosidase (from *Aspergillus niger*), β-glucosidase (from almonds), α-galactosidase (from *A. niger*), β-galactosidase (from bovine liver), and α-mannosidase (from jack beans) and all the *p*-nitrophenyl glycoside substrates were obtained from Sigma Chemical Co. β-Mannosidase was purified from *A. niger* as previously described (Elbein et al., 1977). Intestinal sucrase was purified to homogeneity from the small intestine of white rats or rabbits (Y. T. Pan, J. Ghidoni, and A. D. Elbein, unpublished results). Crude homogenates of rat intestine were used as a source of maltase. Glucosidase I and glucosidase II were purified from mung bean seedlings (Szumilo et al., 1986; K. Hatnaka, G. P. Kaushal, I. Pastuszak, and A. D. Elbein, unpublished results). [^{3}H]Glucose-labeled $Glc_3Man_9GlcNAc$ and $Glc_2Man_9GlcNAc$ for use as substrates for the glycoprotein processing enzymes were prepared in influenza virus infected MDCK cells as previously reported (Szumilo & Elbein, 1985).

Enzyme Assay. (*a*) *Arylglucosidases.* The enzymatic activities of amyloglucosidase, β-glucosidase, α- and β-galactosidase, and α- and β-mannosidase were determined colorimetrically by monitoring the release of *p*-nitrophenol from the appropriate *p*-nitrophenyl glycoside substrate (Rudick & Elbein, 1974). All reaction mixtures contained 20 μmol of sodium acetate buffer, pH 5.0, 2 μmol of *p*-nitrophenyl glucoside, and enzyme in a final volume of 0.4 mL. Incubations were for 15 min at 37 °C, and the reactions were stopped by the addition of 2.5 mL of 0.4 M glycine buffer, pH 10.5. The *p*-nitrophenol liberated in the reaction was measured at 410 nM. Assays were done under conditions where the amount of *p*-nitrophenol released was linear with both time and protein concentration.

(*b*) *Sucrase and Maltase.* Intestinal sucrase and maltase were determined by measuring the formation of reducing sugar from sucrose and maltitol, respectively. The reaction mixture contained 7.5 μmol of sodium citrate buffer, pH 6.0, 9 μmol of substrate (sucrose or maltitol), and enzyme in a final volume of 0.3 mL. The mixtures were incubated at 37 °C for 10 min, and the formation of reducing sugar was determined by the Nelson method (Nelson, 1944). For both enzymes, activity was proportional to time and amount of protein.

(*c*) *Glucosidase I and Glucosidase II.* Glucosidase I activity was determined by measuring the release of [^{3}H]glucose from [^{3}H]glucose-labeled $Glc_3Man_9GlcNAc$ as described previously (Szumilo & Elbein, 1985). Glucosidase II activity was monitored by determining the release of *p*-nitrophenol from *p*-nitrophenyl α-glucoside. The incubation mixture for both enzymes contained 50 mM MES buffer, pH 6.5, 0.1% Triton X-100, enzyme, and substrate (25 000 cpm of $Glc_3Man_9GlcNAc$ for glucosidase I or 1.25 μmol of *p*-nitrophenyl glucoside for glucosidase II) in a final volume of 0.25 mL. A typical incubation was for 1 h at 37 °C and was linear with both time and protein concentration for the duration of the assay.

Isolation of Lentiginosine. Spotted locoweed, *Astragalus lentiginosus* var diphysus, was collected near Holbrook, AZ, air-dried, and ground to pass through a 1-mm screen. One kilogram of material was placed in a stainless steel pot with 5 L of 80% methanol and heated on a hot plate for several hours. The residue was removed and heated with another portion of fresh methanol. The two methanol extracts were pooled and concentrated to dryness, and the residue was taken up in water. The alkaloid fraction was initially isolated by ion-exchange chromatography on large columns of Dowex 50–NH_4^+. The above extract was divided into three or four portions that were applied individually to a 7 × 80 cm column of resin. After thorough washing of the column, the alkaloids were eluted with 1 M NH_4OH. The eluate was concentrated to dryness and reapplied to a column of Dowex 50–NH_4^+ which was eluted with a gradient of NH_4OH (0–0.5 M). Fractions were assayed for inhibitory activity against a variety of glycosidases (α- and β-glucosidase, α- and β-mannosidase, α- and β-galactosidase) by using the assay described above. Active fractions were pooled and further purified by thin-layer and radial chromatography. The solvent system for these separations was usually $CHCl_3$:CH_3OH:NH_4OH:H_2O (70:26:2:2), although some variations of this solvent were used. The alkaloids were monitored for purity by TLC and by gas chromatography as described below.

Structural Elucidation of Indolizidine Alkaloids. Thin-layer chromatography (TLC) was performed on Merck 0.25 mm silica gel 60, precoated, glass-backed plates developed with $CHCl_3$:CH_3OH:NH_4OH:H_2O (70:26:2:2). Detection was achieved by spraying the plates with 10% acetic anhydride in benzene, followed by Ehrlich's reagent (Molyneux et al., 1985). The alkaloids were monitored for homogeneity by gas chromatography (GC) of the trimethylsilyl ether (TMS) derivatives, prepared by treatment with *N*-methyl-*N*-(trimethylsilyl)fluoroacetamide (MSTFA) in pyridine, on a Hewlett-Packard 5830 instrument, equipped with a flame ionization detector, on-column injector, and a 30-m × 0.32-mm i.d. SE-30 fused silica column. Low-resolution electron-impact mass spectra (MS) were obtained on a VG Micromass 7070 mass spectrometer; high-resolution mass measurements were determined on the same instrument. Nuclear magnetic resonance (NMR) spectra were determined on a Nicolet NTC 200FT spectrometer, with software package, at 200 MHz (^{1}H) and at 50.3 MHz (^{13}C), using solutions in deuterium oxide with 3-(trimethylsilyl)-1-propanesulfonic acid (TSP) as an internal standard or in deuteriochloroform with tetramethylsilane (TMS) as an internal standard. Multiplicities for ^{13}C signals were determined by application of the carbon-attached proton test (CAPT) sequence. Two-dimensional spectra were obtained by homonuclear ^{1}H–^{1}H shift spectroscopy (CSCM). Optical rotations were measured in methanol or chloroform solution in a 1-dm cell on a Perkin-Elmer 241 automatic polarimeter.

2-Epilentiginosine [*(1S,2R,8aS)-1,2-Dihydroxyindolizidine*] (**4a**). The alkaloid (*cis*-diol) was obtained as a colorless oil, with a retention time on GC of the TMS ether of 9.00 min, identical with that of the synthetic diol and its enantiomer (Heitz & Overman, 1989). EIMS *m/z* 157 (M, 29%), 140 (11), 122 (9), 97 (100), 84 (19); high-resolution MS mass measurement $[M]^+$ *m/z* 157.1141 (calcd for $C_8H_{15}NO_2$, 157.1103); ^{1}H NMR ($CDCl_3$) δ 4.24 (ddd, $J_{2,1}$ = 7, $J_{2,3\alpha}$ = 5.3, $J_{2,3\beta}$ = 7, H2), 3.70 (dd, $J_{1,2}$ = 7, $J_{1.8a}$ = 7.7, H1), 3.54

(dd, $J_{3\beta,2}$ = 7, $J_{3\beta,3\alpha}$ = 10, H3β), 3.04 (br d, $J_{5\beta,5\alpha}$ = 11, H5β), 2.22 (dd, $J_{3\alpha,3\beta}$ = 10, $J_{3\alpha,2}$ = 5.3, H3α), 2.10 (ddd, $J_{5\alpha,5\beta}$ = 11, $J_{5\alpha,6\beta}$ = 11, $J_{5\alpha,6\alpha}$ = 3.5, H5α), 1.98 (br dd, $J_{8a,1}$ = 7.7, H8a), 1.94–1.20 (m, H6ax, H6eq, H7ax, H7eq, H8ax, H8eq); ^{13}C NMR ($CDCl_3$) δ 75.0 (C2), 67.9 (C1), 67.3 (C8a), 61.9 (C3), 52.8 (C5), 28.7 (C6), 25.0 (C8), 23.8 (C7); $[\alpha]^{24}_D$ −32.5°, $[\alpha]_{578}$ −33.3°, $[\alpha]_{546}$ −38.6°, $[\alpha]_{436}$ −65.2° (c 0.13, $CHCl_3$).

2-Epilentiginosine Diacetate [*(1S,2R,8aS)-1,2-Diacetoxyindolizidine*] (**4b**). The *cis*-diol (**4a**) (8 mg) was dissolved in pyridine (0.3 mL) and treated with acetic anhydride (0.5 mL), and the solution was kept at room temperature overnight. The reaction mixture was poured into water, the solution extracted with $CHCl_3$, and the extract washed with water. The $CHCl_3$ solution was dried over anhydrous Mg_2SO_4 and evaporated to give a colorless oil. ^{1}H NMR ($CDCl_3$) δ 5.23 (ddd, $J_{2,1}$ = 7.3, $J_{2,3\alpha}$ = 5.5, $J_{2,3\beta}$ = 7, H2), 4.75 (dd, $J_{1,2}$ = 7.3, $J_{1,8a}$ = 8.7, H1), 3.59 (dd, $J_{3\beta,3\alpha}$ = 10, H3β), 3.03 (br dd, $J_{5\beta,5\alpha}$ = 10.5, H5β), 2.25 (dd, $J_{3\alpha,2}$ = 5.5, $J_{3\alpha,3\beta}$ = 10, H3α), 2.15 (br d, $J_{5\alpha,5\beta}$ = 10.5, H5α), 2.07 (s, $OCOCH_3$), 2.05 (s, $OCOCH_3$), 2.04 (dd, $J_{8a,1}$ = 8.7, H8a), 1.90–1.25 (m, H6ax, H6eq, H7ax, H7eq, H8ax, H8eq); ^{13}C NMR ($CDCl_3$) δ 170.0 (2 × O*C*OCH$_3$), 74.9 (C2), 68.3 (C1), 65.0 (C8a), 59.3 (C3), 52.7 (C5), 28.5 (C6), 24.9 (C8), 23.5 (C7), 20.7 (OCO*C*H$_3$), 20.5 (OCO*C*H$_3$).

Lentiginosine [*(1S,2S,8aS)-1,2-Dihydroxyindolizidine*] (**7a**). The alkaloid (*trans*-diol) was obtained as a colorless oil, with a GC retention time as the TMS ether of 8.48 min. EIMS *m/z* 157 (M, 29%), 140 (17), 97 (100), 84 (22), 69 (27); high-resolution MS mass measurement [M^+] *m/z* 157.1098 (calcd for $C_8H_{15}NO_2$, 157.1103); ^{1}H NMR (D_2O) 4.08 (ddd, $J_{2,1}$ = 4, $J_{2,3\alpha}$ = 7.7, $J_{2,3\beta}$ = 2, H2), 3.66 (dd, $J_{1,8a}$ = 8.5, $J_{1,2}$ = 4, H1), 2.95 (br d, $J_{5\beta,5\alpha}$ = 11, H5β), 2.85 (dd, $J_{3\beta,2}$ = 2, $J_{3\beta,3\alpha}$ = 11, H3β), 2.63 (dd, $J_{3\alpha,3\beta}$ = 11, $J_{3\alpha,2}$ = 7.7, H3α), 2.06 (ddd, $J_{5\alpha,5\beta}$ = 11, $J_{5\alpha,6\beta}$ = 3, $J_{5\alpha,6\beta}$ = 11, H5α), 1.95 (br ddd, $J_{8a,1}$ = 8.5, H8a), 1.85–1.2 (m, H6ax, H6eq, H7ax, H7eq, H8ax, H8eq); ^{13}C NMR (D_2O) δ 85.5 (C2), 78.2 (C1), 71.2 (C8a), 62.8 (C3), 55.3 (C5), 30.2 (C6) 26.6 (C8), 25.7 (C7); the ^{1}H–^{1}H COSY NMR spectrum is illustrated in Figure 4; $[\alpha]^{24}_D$ −3.3°, $[\alpha]_{546}$ −3.9°, $[\alpha]_{436}$ −7.5° (*c* 0.33, MeOH).

Lentiginosine Diacetate [*(1S,2S,8aS)-1,2-Diacetoxyindolizidine*] (**7b**). The diacetate derivative was prepared in the same manner as that of the *cis*-diol (**4a**) and was obtained as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 5.00 (ddd, $J_{2,3\alpha}$ = 1, $J_{2,3\beta}$ = 6.5, $J_{2,1}$ = 2.7, H2), 4.95 (dd, $J_{1,2}$ = 2.7, $J_{1,8a}$ = 8, H1), 3.95 (br ddd, $J_{5\beta,5\alpha}$ = 11, $J_{5\beta,6\alpha}$ = 2, $J_{5\beta,6\beta}$ = 7, H5β), 3.69 (br dd, $J_{3\beta,2}$ = 1, $J_{3\beta,3\alpha}$ = 11, H3β), 3.02 (br ddd, $J_{5\alpha,5\beta}$ = 11, $J_{5\alpha,6\alpha}$ = 5.5, H5α), 2.62 (dd, $J_{3\alpha,2}$ = 6.5, $J_{3\alpha,3\beta}$ = 11, H3α), 2.39 (m, H8a), 2.08 (s, 2 × $OCOCH_3$), 2.1–1.4 (m, H6ax, H6eq, H7ax H7eq, H8ax, H8eq); ^{13}C NMR ($CDCl_3$) δ 170.9 (O*C*OCH$_3$), 170.3 (O*C*OCH$_3$), 82.1 (C2), 76.8 (C1), 67.7 (C8a), 59.8 (C3), 53.0 (C5), 28.7 (C6), 24.6 (C8), 23.7 (C7), 21.1 (OCO*C*H$_3$), 20.9 (OCO*C*H$_3$).

RESULTS

Isolation and Purification of Lentiginosine. Crude methanolic extracts of *A. lentiginosus* were found to have activity that inhibited the α-glucosidase, amyloglucosidase. As indicated under Experimental Procedures, the inhibitor was initially purified by chromatography on columns of Dowex 50–NH_4^+ and then on TLC plates. The active band from the TLC plates was eluted and further purified by radial chromatography as shown in Figure 1. It can be seen that the amyloglucosidase-inhibitory activity emerged in fractions 9–12 and was clearly separated from the major alkaloid, swainsonine,

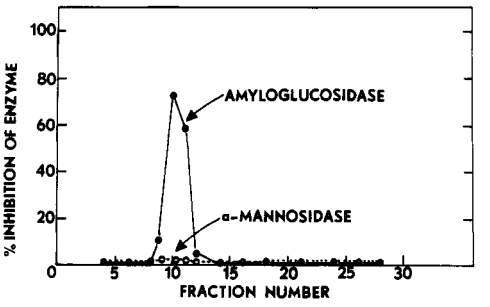

FIGURE 1: Separation of amyloglucosidase-inhibitory activity from swainsonine by radial chromatography. Extracts of *A. lentiginosus* were prepared and purified by ion-exchange chromatography and TLC as described under Experimental Procedures. The active material (i.e., inhibitory material against amyloglucosidase) was eluted from the plates and applied to the Chromatotron and run in $CHCl_3$:CH_3OH:NH_4OH:H_2O (165:31:2:2). Fractions were collected and assayed for their ability to inhibit amyloglucosidase and α-mannosidase.

which inhibits α-mannosidase. The peak of α-glucosidase-inhibitory material was pooled and further purified on 0.25-mm TLC plates in $CHCl_3$:CH_3OH:NH_4OH:H_2O (165:31:2:2). This procedure resulted in separation of the two isomers in essentially homogeneous form. Although it is difficult to be certain of the actual yield of alkaloid obtained during the isolation, we did obtain about 10 mg of lentiginosine from 1 kg of dried plant material.

The lentiginosine and its 2-epimer were also examined by gas chromatography of their trimethylsilyl ethers as demonstrated in Figure 2. The upper profile (A) shows that the lentiginosine gave only one major peak on the GC which eluted at 8.48 min. This elution position was clearly distinct from that of the 2-epimer which, as shown in the middle profile, emerged from the column at 9.00 min. The lower profile shows the elution of swainsonine which emerges at 13.95 min. These data indicate that the lentiginosine and its 2-epimer had been separated from each other by the purification procedure, and each appeared to be homogeneous.

Structural Characterization of Lentiginosine and Its 2-Epimer. The high-resolution mass spectra established that the two alkaloids were isomeric, with the molecular formula $C_8H_{15}NO_2$. Acetylation gave diacetate derivatives, indicating that both must be dihydroxyindolizidine alkaloids. The electron-impact mass spectra were virtually identical, showing a parent ion at *m/z* 97, indicative of a lack of hydroxyl substitution in the six-membered ring. The metabolites must therefore be stereoisomeric 1,2-dihydroxyindolizine alkaloids.

The alkaloid that was eluted from the GC at 9.0 min (Figure 2) and which lacked glucosidase inhibitory activity was shown by ^{1}H and ^{13}C NMR spectroscopy of the parent compound and its diacetate derivative to be (1*S*,2*R*,8a*S*)-1,2-dihydroxyindolizidine (Figure 6, **4a**) or its enantiomer, having a cis configuration of the diol moiety.

Since lentiginosine (8.48-min peak on the GC) is stereoisomeric with the *cis*-diol (**4a**), only three structures are possible, namely, a *cis*-diol with inverted stereochemistry at the bridgehead position C8a or the two structures with a trans relationship between the diol groups.

Examination of the ^{13}C NMR spectrum showed that major shift differences from the spectrum of *cis*-diol (**4a**) were observable only for the methine carbon atoms C1, C2, and C8a, consistent with any of these three possibilities. However, the ^{1}H NMR spectrum exhibited a coupling constant of 8.5 Hz for H1–H8a, very similar in magnitude (8.7 Hz) to that of the *cis*-diol (**4a**), indicative of a trans configuration between these two protons. This therefore requires a trans relationship

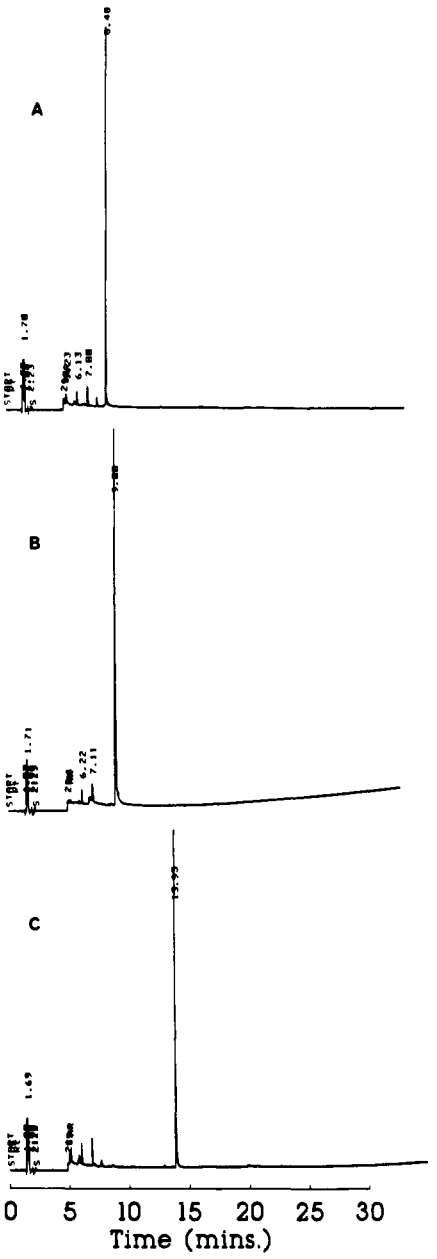

FIGURE 2: Gas chromatographic analysis of TMS derivatives of polyhydroxyindolizidine alkaloids from *A. lentiginosus*. (A) Lentiginosine (**7a**); (B) 2-epilentiginosine; (C) swainsonine.

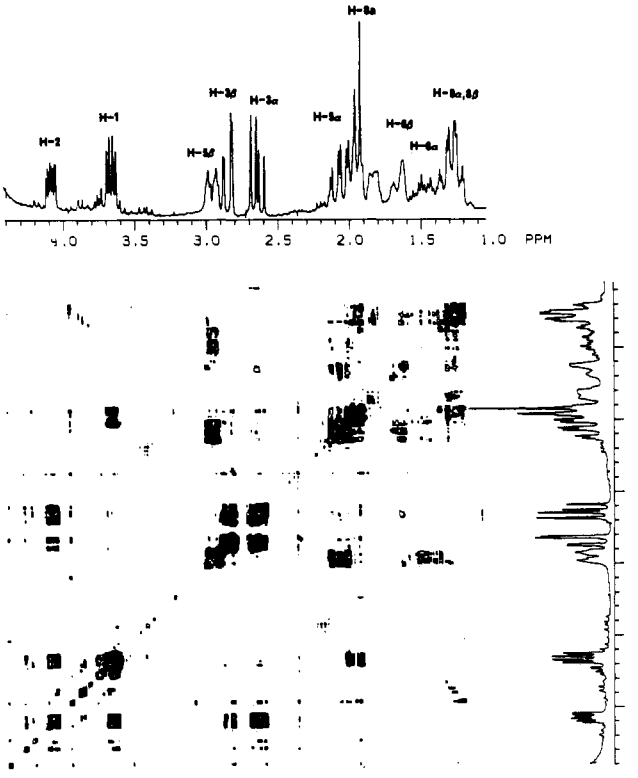

FIGURE 3: Two-dimensional ^1H–^1H COSY shift correlation NMR spectrum of lentiginosine (**7a**).

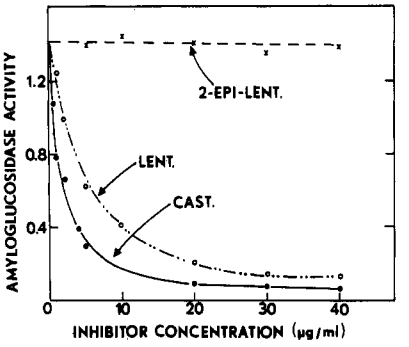

FIGURE 4: Comparison of the effect of lentiginosine, castanospermine, and 2-epilentiginosine on amyloglucosidase activity. Incubations were as described in the text and contained *p*-nitrophenyl α-glucoside as substrate. Various amounts of the inhibitors were added as indicated. The release of *p*-nitrophenol was measured at 410 nm.

for the diol group moiety, establishing a trans, trans configuration for H8a–H1–H2. The coupling constant (J = 4 Hz) for H1–H2 is quite small for protons having a trans relationship but similar small values have been observed in related alkaloids (Molyneux et al., 1988) due to the sensitivity of J values to variations in θ caused by endo or exo flipping of the five-membered ring. The diacetate derivative exhibited an even smaller coupling constant (2.7 Hz) for H1–H2, possibly due to greater distortion of the ring caused by the bulky acetoxy groups. Extensive decoupling experiments and a ^1H–^1H COSY NMR spectrum (Figure 3) confirmed the complete connectivity and substitution pattern of the alkaloid.

The structure of lentiginosine is therefore established as (1*S*,2*S*,8a*S*)-1,2-dihydroxyindolizidine (Figure 6, **7a**), or its enantiomer. On the basis of biosynthetic considerations (see Figure 6 and Discussion), we prefer the structure **7a**.

Glycosidase-Inhibitory Activity of Lentiginosine. The initial isolation of lentiginosine was prompted by the observation that extracts of *A. lentiginosus* contained an inhibitor of amyloglucosidase. Thus, it was of interest to examine the purified alkaloid to determine how potent an inhibitor of amyloglucosidase it was and also to determine the type and specificity of the inhibition. Figure 4 shows a concentration curve comparing the degree of inhibition of amyloglucosidase by castanospermine, lentiginosine, and 2-epilentiginosine. It can be seen that castanospermine was the most effective inhibitor of the amyloglucosidase with 50% inhibition requiring about 1 or 2 μg/mL of this alkaloid. Lentiginosine was also a fairly good inhibitor of the enzyme, but in this case about 5 μg/mL of alkaloid was necessary to give 50% inhibition. Finally, the 2-epimer of lentiginosine was also tested and found to be completely inactive against amyloglucosidase, even when tested at 40 μg/mL. Thus, a dihydroxyindolizidine alkaloid with the appropriate stereochemistry of the two hydroxyl groups is able to inhibit amyloglucosidase, although not as effectively as indolizidine alkaloids with more hydroxyl groups.

To determine whether lentiginosine was a competitive or noncompetitive inhibitor of the amyloglucosidase, a series of experiments were done where the substrate (*p*-nitrophenyl α-D-glucoside) concentration was varied, and several different

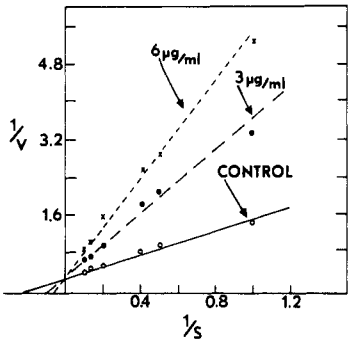

FIGURE 5: Effect of substrate concentration on lentiginosine inhibition of amyloglucosidase. Incubations were as described in the text except that the amount of substrate was varied as indicated. Substrate concentration studies were done in the presence of 3 or 10 μg of lentiginosine. The liberation of *p*-nitrophenol was determined, and the data were plotted by the method of Lineweaver and Burk.

concentrations of lentiginosine were used. As shown in Figure 5, when the data were plotted by the method of Lineweaver and Burk, the intercept of the plot of $1/V$ versus $1/S$ was the same in the presence or absence of inhibitor, indicating that the inhibition is of the competitive type.

Lentiginosine and its 2-epimer were also tested against a number of other enzymes to determine the specificity of inhibition of these alkaloids. These experiments showed that neither lentiginosine nor its 2-epimer, even at concentrations as high as 100 μg/mL, had any inhibitory activity against a variety of other glycosidases, including the glycoprotein processing glucosidase I or glucosidase II, intestinal sucrase, intestinal maltase, yeast α-glucosidase, β-glucosidase, α- or β-galactosidase, and α- or β-mannosidase (data not shown).

Discussion

The data presented in this paper describe the structure of a new indolizidine alkaloid from *A. lentiginosus* which we have named lentiginosine. This compound has the configuration (1*S*,2*S*,8a*S*)-1,2-dihydroxyindolizidine. In addition, we were also able to isolate and characterize the 2-epimer of lentiginosine. This latter compound [(1*S*,2*R*,8a*S*)-1,2-dihydroxyindolizidine] has previously been isolated from the fungus *Rhizoctonia leguminicola* and its structure established from the NMR of its diacetate derivative (Harris et al., 1987). This fungus also elaborated swainsonine, as does the plant *A. lentiginosus*. The alkaloid has also been identified as a metabolite of *Astragalus oxyphysus* by isolation of its diacetate (Harris et al., 1988).

The *cis*-diol, 2-epilentiginosine (**4a**), and its diacetate (**4b**) have been synthesized in racemic form (Colegate et al., 1984; Harris et al., 1987). More recently, both enantiomers have been synthesized by an asymmetric enantiodivergent route (Heitz & Overman, 1989). Examination of the NMR spectrum of the alkaloid from *A. lentiginosus* established a trans configuration between C1 and C8a and a cis configuration between C1 and C2. Comparison of the ^1H and ^{13}C NMR spectra with literature data for the synthetic racemates and with reference spectra of the synthetic enantiomer of **4a** confirmed these assignments. Gas chromatography of the TMS derivatives of the *cis*-diol and an authentic sample of the enantiomer of **4a** gave identical retention times. Comparison of the optical rotations of the *cis*-diol and synthetic **4a** showed that both were laevorotatory with similar magnitudes. The structure of the natural product is therefore established as (1*S*,2*R*,8a*S*)-1,2-dihydroxyindolizidine (**4a**).

The biosynthesis of swainsonine has been shown to follow a parallel course in the fungus *R. leguminicola* (Harris et al., 1987) and in *A. oxyphysus* (Harris et al., 1988), suggesting that these two groups of organisms have the same pathway. The biosynthetic sequence is illustrated in Figure 6. Pipecolic acid (**1**), derived from L-lysine, is transformed to 1-oxoindolizidine (**2**), which undergoes reduction to (1*R*,8a*S*)-1-hydroxyindolizidine. The latter is hydroxylated at the 2-position to give (1*S*,2*R*,8a*S*)-1,2-dihydroxyindolizidine (**4a**), the *cis*-diol, which has been isolated as the diacetate (**4b**) (Harris et al., 1988). Subsequent transformation to swainsonine requires epimerization at C8a, with replacement of the hydrogen atom. This most probably occurs via the iminium ion (**5**), which undergoes hydroxylation to swainsonine (**6**).

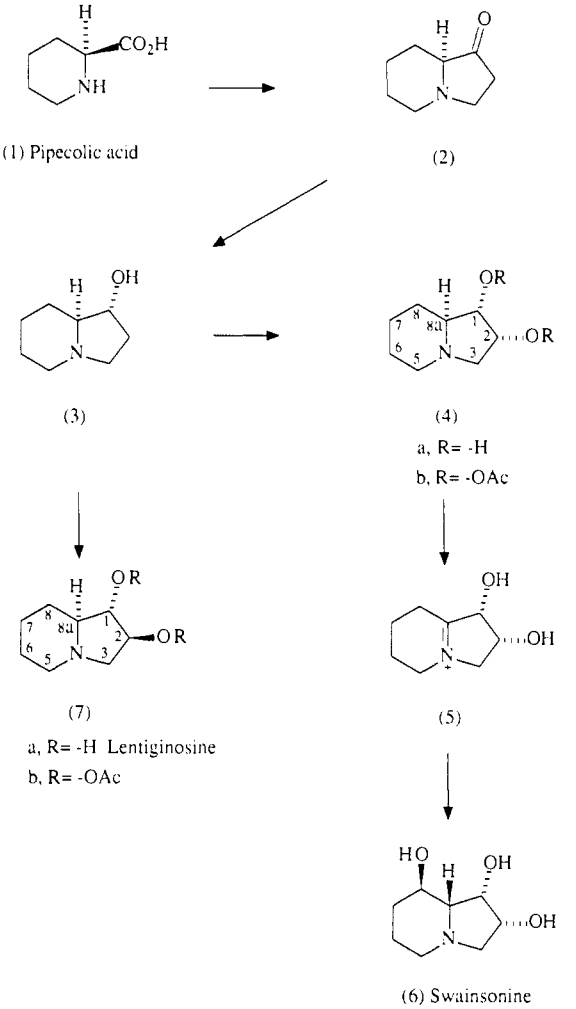

FIGURE 6: Biosynthetic pathway to swainsonine and structures of polyhydroxyindolizidine alkaloids of *A. lentiginosus*.

Lentiginosine (**7a**) [(1*S*,2*S*,8a*S*)-1,2-dihydroxyindolizidine], the *trans*-diol which we have now isolated from *A. lentiginosus*, together with the 2-epimer, is probably biosynthesized from 1-hydroxyindolizidine (**3**) by hydroxylation at C2, trans to the existing hydroxyl group at C1. Alternatively, both diols could be generated by oxidation at C2 to keto derivative and subsequent reduction to give both C2 diastereomers. For this reason, we believe lentiginosine to be enantiomer **7a**. Nevertheless, establishment of the absolute configuration must await the asymmetric synthesis of both enantiomers.

Lentiginosine is an interesting compound from a biochemical standpoint since it is the first glycosidase inhibitor that has been found that has only two hydroxyl groups. Until now, all of the other reported glycosidase inhibitors have three or more hydroxyl functions. An important goal from these studies on inhibitors is to determine the basic structural features that are necessary for a compound to be a glycosidase inhibitor. It

seems likely that the nitrogen in the ring is an essential requirement since compounds such as lentiginosine, swainsonine, castanospermine, australine, deoxynojirimycin, 1,4-dideoxy-1,4-imino-D-mannitol, and 2,5-(dihydroxymethyl)-3,4-dihydroxypyrrolidine all have a secondary or tertiary nitrogen atom in the ring system (Elbein & Molyneux, 1987; Elbein, 1987). However, a six-membered ring system is apparently not necessary for a compound to have glycosidase-inhibitory activity since some of the above compounds have two fused five-membered rings (australine), some have a single five-membered ring (1,4-dideoxy-1,4-iminomannitol and 2,5-(dihydroxymethyl)-3,4-dihydroxypyrrolidine), and others have a six-membered ring (deoxynojirimycin) or a fused five/six-membered ring system (lentiginosine, swainsonine, and castanospermine). However, it is likely that there are differences in the relative extent of biological activity depending on the ring system.

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