Geographic Distribution of Three Alkaloid Chemotypes of *Croton lechleri*

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Three known alkaloids, isoboldine (**2**), norisoboldine (**1**), and magnoflorine (**8**), have been isolated for the first time from *Croton lechleri*, a source of the wound healing latex "sangre de grado". An HPLC system was developed, and a large number of latex and leaf samples of *C. lechleri* from 22 sites in northern Peru and Ecuador were analyzed to gain an understanding of the natural variation in alkaloid content for the species. Up to six alkaloids were found to occur in the leaves including, in addition to those listed above, thaliporphine (**3**), glaucine (**4**), and taspine (**9**), whereas the latex contained only **9**. Taspine (**9**) is the component that has been previously found to be responsible for the wound healing activity of *C. lechleri* latex, and its mean concentration throughout the range examined was found to be 9% of the latex by dry weight. In addition, three chemotypes are defined based on the alkaloid content of the leaves, and the geographic distribution of these chemotypes is discussed along with a quantitative analysis of the alkaloid content as a function of chemotype.

A number of species in the genus *Croton* (Euphorbiaceae) have a long history of use in South and Central America for the treatment of wounds. The mature trees produce a blood-red to yellowish-orange colored sap, or latex, which is highly regarded for its ability to speed the healing of wounds. On account of its blood-like appearance, the latex is called either "sangre de drago" or "sangre de grado" in Spanish, translated into English as "dragon's blood" and "blood of the tree", respectively. The earliest written account of the use of "sangre de grado" comes from the 1600s, at which time its use was widespread as it remains today.[1] A search of the literature has revealed that at least 10 species of *Croton* are used presently, or were used in the past, for wound healing throughout South and Central America. Included among these, and perhaps the most widely utilized species, is *C. lechleri* Muell. Arg., which is the subject of this paper. *Croton lechleri* is a pioneer tree species which occurs in disturbed areas of tropical forest below 1000 m in Peru and Ecuador.

Over the last two decades a chemical examination of the latex has led to the isolation of the major wound healing constituent of *C. lechleri*, which has been identified as the benzylisoquinoline-derived alkaloid taspine (**9**).[2−4] Aside from *Croton*, **9** has been isolated from only two other genera both in the Berberidaceae. Thus, taspine (**9**) was first isolated and characterized from *Leontice ewersmannii* Bge.[5] and recently has been found in small yield in blue cohosh, *Caulophyllum thalictroides* (L.) Michx.[6] *Croton* as a whole produces a wide range of benzylisoquinoline-derived alkaloids possessing morphinan, aporphine, and proaporphine skeletons, but **9** is unique among these and probably represents an endpoint along a biosynthetic pathway. A biosynthetic pathway has been proposed for the production of **9** from the quaternary aporphine alkaloid magnoflorine (**8**).[7] Although the conversion of magnoflorine (**8**) into taspine (**9**) has been achieved synthetically in the laboratory, this biosynthetic pathway remains to be demonstrated in vivo. The genus *Croton* as a whole has been subjected to extensive alkaloid analysis, but little work has been done on the alkaloids of species producing "sangre de grado".

Previous work on the alkaloids of *C. lechleri* has led to the isolation of taspine (**9**) in the latex and thaliporphine (**3**) and glaucine (**4**) in the leaves.[2,8] Beyond the isolation of the above three alkaloids, little was known at the onset of this study about the natural variation in alkaloid content in this species and whether other alkaloids occur in this species. Herein we report on the isolation of three additional alkaloids from *C. lechleri* which allow the alkaloids isolated from this species to date to be placed within a biosynthetic framework. The natural variation in alkaloid content at the species level is also discussed. Additionally, three chemotypes of *C. lechleri* have been defined, and their distribution in northern Peru and Ecuador has been examined.

Results and Discussion

Lyophilized, powdered latex from *C. lechleri* was suspended in aqueous acid and extracted continuously with $CHCl_3$ to give a neutral/acidic fraction that showed no alkaloidal material by TLC, when visualized with Dragendorff's or iodoplatinate reagents. After basification, the aqueous layer was extracted again with $CHCl_3$ to afford the crude alkaloid fraction. Taspine (**9**) was obtained from the crude alkaloid fraction as the major component by precipitation from MeOH. The mother liquors remaining after precipitation of **9** were examined for several batches of latex collected from mature trees, and all were negative for additional alkaloids.

The alkaloid workup for leaf material was similar to that for the latex. An initial methanolic extract was taken up in aqueous acid and partitioned with $CHCl_3$ (neutral/acidic fraction) followed by basification and partitioning with $CHCl_3$ to yield the aqueous and crude alkaloid fractions. In contrast to the latex, several iodoplatinate-positive components were detected in the crude alkaloid fraction of leaf material by TLC, in addition to taspine (**9**), which has not previously been shown to occur in the leaves.

Chromatographic separation of a leaf crude alkaloid fraction from greenhouse-grown plants led to the isolation of isoboldine (**2**) and a small amount of norisoboldine (**1**)

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Scheme 1. Probable biosynthetic relationship of alkaloids isolated from *C. Lechleri*.

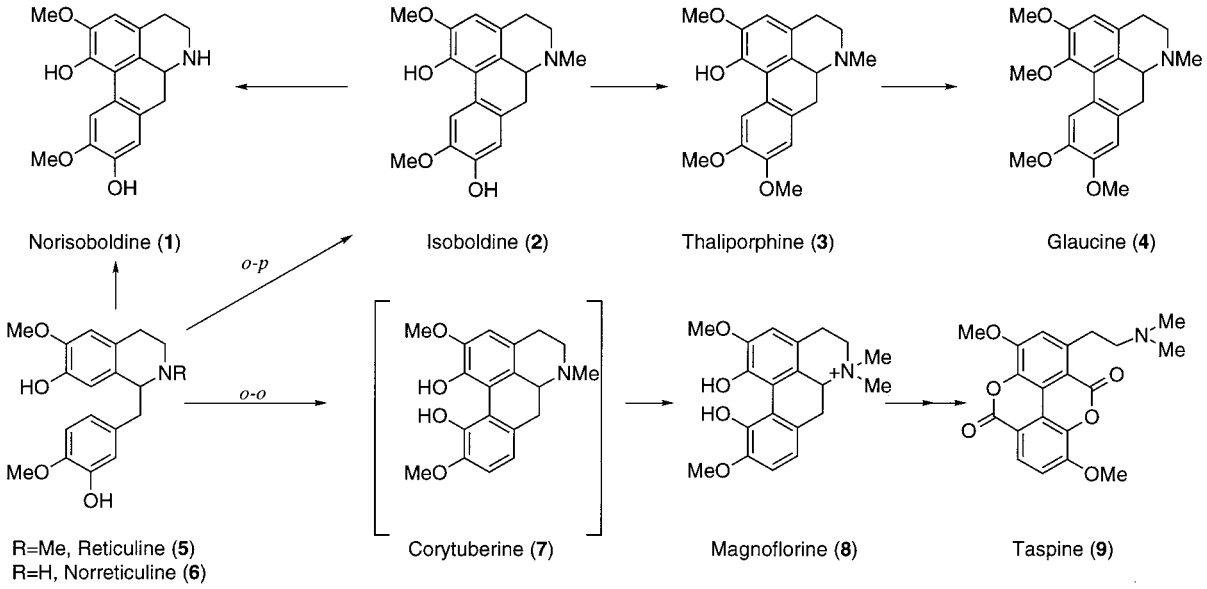

Table 1. Alkaloid Content of Leaves and Latex in *Croton lechleri*

chemo-type[a]	*N*	noriso-boldine (**1**)	isobol-dine (**2**)	thalipor-phine (**3**)	glau-cine (**4**)	magno-florine (**8**)	taspine (**9**)	pathway I	pathway II	total alkaloids
					Leaf					
1	4	0.116 ± 0.072[b]	0.538 ± 0.385	2.002 ± 0.509	2.653 ± 1.090	2.526 ± 0.828	2.154 ± 1.467	5.310 ± 1.521	4.680 ± 2.097	9.990 ± 3.592
2	233	0.059 ± 0.108	0.428 ± 0.419	1.676 ± 0.912		2.458 ± 1.131	1.516 ± 0.725	2.164 ± 1.104	3.974 ± 1.605	6.137 ± 2.478
3	27	0.011 ± 0.040	1.249 ± 0.773			2.612 ± 1.110	1.328 ± 0.535	1.260 ± 0.788	3.940 ± 1.548	5.200 ± 2.146
total	264	0.055 ± 0.104	0.514 ± 0.527	1.682 ± 0.907	2.653 ± 1.090	2.475 ± 1.123	1.506 ± 0.725	2.119 ± 1.181	3.981 ± 1.603	6.100 ± 2.517
					Latex					
1	1						94.144			
2	226						87.219 ± 42.089			
3	27						86.990 ± 32.116			
total	493[d]						90.075 ± 41.902			

[a] Chemotype 1: isoboldine (**2**), thaliporphine (**3**), and glaucine (**4**); chemotype 2: isoboldine (**2**) and thaliporphine (**3**); chemotype 3: isoboldine (**2**) only. [b] mg/g dry weight. [c] Taspine (**9**) is the only alkaloid to accumulate in the latex. [d] Total *N* is greater than the sum of the chemotype *N*'s because there was no leaf sample for a large number of trees from which latex samples were obtained, and thus it was not possible to determine the chemotype for those individuals.

identified by comparison (UV, NMR, EIMS) with reported spectra.[9,10] A second batch of leaf material from greenhouse-grown plants was extracted as above to yield a crude alkaloid fraction which showed one iodoplatinate-positive zone by TLC that did not correspond to the compounds above and was subsequently isolated and identified as thaliporphine (**3**) by comparison (UV, NMR, EIMS) with published data.[9] A collection of leaf material from Peru, Department of Loreto, gave an additional alkaloid, glaucine (**4**), identified by comparison (UV, NMR, EIMS) with published spectra[9] and HPLC and TLC comparison with an authentic sample. TLC and HPLC analysis of the aqueous fractions revealed that one alkaloid remained in the basified aqueous layers after partitioning with $CHCl_3$, and this was subsequently identified as the quaternary aporphine, magnoflorine (**8**), by comparison (TLC, HPLC) with an authentic sample.

Of the six alkaloids identified in *C. lechleri*, magnoflorine (**8**), isoboldine (**2**), and norisoboldine (**1**) have not previously been reported from this species. Taspine (**9**) has been recorded from the latex but was previously not known to occur in the leaves.

The *C. lechleri* alkaloids can be placed into a probable biosynthetic framework all arising from the benzylisoquinoline precursor, reticuline (**5**) (Scheme 1). Two distinct biosynthetic pathways are apparent in this species depending upon whether the initial phenolic coupling is *ortho-ortho* or *ortho-para*. An initial *ortho-para* coupling leads to the 1,2,9,10-substituted aporphines: norisoboldine (**1**), isoboldine (**2**), thaliporphine (**3**), and glaucine (**4**). Compounds **2**−**4** differ only in the number of *O*-methyl groups present, while **1** could be formed either by *N*-demethylation of **2** or directly from *N*-nor-reticuline (**6**).

A second biosynthetic pathway arises if the initial phenolic coupling is *ortho-ortho* and results in the formation of the 1,2,10,11-substituted aporphines (Scheme 1). The initial compound produced after phenolic coupling would be corytuberine (**7**), which has not been isolated or detected in *Croton lechleri*, or any other species in the genus. *N*-Methylation of **7** leads to the formation of the quaternary aporphine magnoflorine (**8**). It has been proposed that taspine (**9**) could then be derived from **8**,[7] which is supported by the co-occurrence of these two compounds in the leaves. The conversion of magnoflorine (**8**) into taspine (**9**) and the exact mechanism by which this would occur remain to be demonstrated in vivo.

A large number of leaf samples were analyzed by reversed-phase ion-pairing HPLC from throughout the range of *C. lechleri*, to examine the natural variation in alkaloid content at the species level. Two trends are readily observed in the data (Table 1). First, all leaf samples had readily detectable alkaloid levels, with taspine (**9**) and magnoflorine (**8**) found in all samples. Second, although **8** and **9** were detected in all individuals, the biosynthetically related aporphines isoboldine (**2**), thaliporphine (**3**), and glaucine (**4**) (biosynthetic pathway I) were not always

detected. An analysis of the data from leaf extracts has indicated that there are three chemotypes of *C. lechleri*, which differ in the degree to which the aporphines along pathway I are *O*-methylated. Chemotype 1 has the fully *O*-methylated aporphine, glaucine (**4**), along with thaliporphine (**3**) and isoboldine (**2**). Chemotype 2 has thaliporphine (**3**), which lacks the final *O*-methyl group of glaucine (**4**), together with isoboldine (**2**). Chemotype 3 has only isoboldine (**2**), which is the first product formed by phenolic coupling of reticuline (**5**). Repeated sampling of individuals in the wild indicated that these chemotypes were maintained over time. Also, seeds collected from a single individual from a population exhibiting two chemotypes were germinated in the greenhouse to give individuals of both chemotypes present in the wild population. Typically, all alkaloids produced along pathway I by a given chemotype were detectable, with the final alkaloid produced along pathway I being the predominant component. In a minority of samples, isoboldine (**2**) or thaliporphine (**3**) were not detected when they were not the final product produced along pathway I. This is presumably because these individuals produce very low levels of these alkaloids (below the level of detection under standard protocol) and not because they are incapable of producing these alkaloids. Norisoboldine (**1**) is found in a proportion of individuals from each chemotype, although it is found at lower levels than the other alkaloids.

The mean (± SD) taspine (**9**) concentration of *C. lechleri* latex across all samples was 90.075 ± 41.902 mg/g dry wt, with a range of 13.858−204.942 mg/g dry wt. This corresponds to a mean of approximately 9% taspine (**9**) by dry weight and a range of 1.3−20.4%. As can be seen from the data, **9** is a major component of the latex. When collecting samples from trees with a high latex taspine (**9**) content, this alkaloid could be observed as a flocular white precipitate in the samples. The mean (± SD) total alkaloid content of the leaves was 6.100 ± 2.517 mg/g dry wt, with a range of 1.142−14.617 mg/g dry wt (0.1−1.4%). Alkaloid content along pathway II was higher than that along pathway I, with means of 3.981 ± 1.603 and 2.119 ± 1.181 mg/g dry wt, respectively.

Of the individual alkaloids along biosynthetic pathway I, norisoboldine (**1**) had the lowest concentration, with a mean (± SD) of 0.055 ± 0.104 mg/g dry wt. Norisoboldine (**1**) was only detectable in 106 of the 264 individuals sampled, although all individuals are assumed to be capable of producing this alkaloid. Isoboldine (**2**) had a mean (± SD) concentration approximately 10-fold greater than that of **1** at 0.514 ± 0.527 mg/g dry wt and was detected in most individuals (250 of 264). Like norisoboldine (**1**), all individuals are assumed to be capable of producing this alkaloid. Thaliporphine (**3**) had a mean (± SD) concentration higher than that of isoboldine (**2**), at 1.682 ± 0.907 mg/g dry wt, with glaucine (**4**) higher still at 2.653 ± 1.090 mg/g dry wt. Thaliporphine (**3**) was detected in 237 individuals, while glaucine (**4**) was only detected in four individuals. This makes chemotype 2 (**2**, **3**) the most common of the chemotypes, with 233 of 264 individuals, while the other chemotypes were encountered much less frequently, with $N = 27$ for chemotype 3 (**2** only) and $N = 4$ for chemotype 1 (**2**−**4**).

Along biosynthetic pathway II, magnoflorine (**8**) and taspine (**9**) were detected in the leaves of all individuals with means (± SD) of 2.475 ± 1.123 and 1.506 ± 0.725 mg/g dry wt, respectively. The taspine (**9**) content of the leaves was 60-fold less than that of the latex by dry weight.

Table 1 and Figure 1 illustrate the relative alkaloid content for chemotypes 2 and 3. Only data from sites that had individuals of both chemotypes were included in the statistical analysis (Figure 1) due to a significant difference in alkaloid content by collection site. Also, chemotype 1 was excluded from analysis because of the small number of individuals for which quantitative data were available. Isoboldine (**2**) content was significantly lower ($p < 0.0001$) in individuals of chemotype 2, where **2** is further *O*-methylated to give thaliporphine (**3**). In contrast, total leaf alkaloids were significantly higher ($p = 0.044$) in chemotype 2. The concentration of magnoflorine (**8**) and taspine (**9**) in the leaves did not differ significantly between chemotypes. Taken together, these data provide insight into the distribution of the alkaloid content of the leaves among the chemotypes. With the addition of a second *O*-methyl derivative in chemotype 2, one might expect an increase in total alkaloid content of the leaves equal to the concentration of the additional derivative, in this case thaliporphine (**3**). This was not observed, however, and instead only a small increase in total leaf alkaloids was observed, indicating that with the addition of the capacity for production of thaliporphine (**3**) there is a shift in accumulation such that much of the increase due to thaliporphine (**3**) is offset by decreased accumulation of isoboldine (**2**).

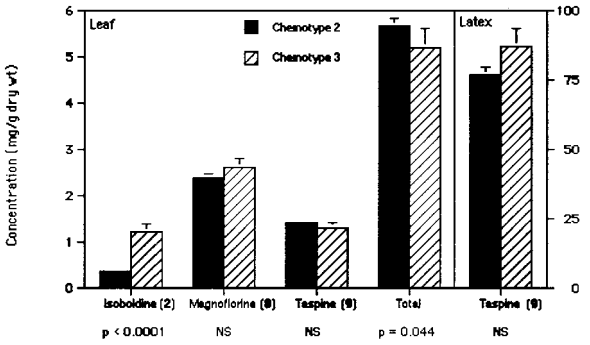

Figure 1. Relative alkaloid content (mean ± SEM) for leaves of *C. lecheri* by chemotype for individual and total alkaloids.

Latex samples, in contrast to the leaves, showed the same alkaloid profile regardless of chemotype. Taspine (**9**) was the only alkaloid detected in the latex of mature individuals and was readily detected in all individuals (a few, very young, ca. 1 yr old, individuals had detectable levels of the other alkaloids). There was no significant difference in latex taspine (**9**) content between chemotypes.

The analyses mentioned thus far focused on leaf and latex alkaloids of *C. lechleri*. Other plant tissues were also examined by HPLC, and these showed an alkaloid profile that was more similar to that of the leaves than of the latex. Samples of *C. lechleri* flowers, inflorescences, and trunk bark were found to have an alkaloid profile the same as that for leaves from the same individual. Root bark samples differed in that only magnoflorine (**8**) and taspine (**9**) were detected. An extract of *C. lechleri* seeds also showed the accumulation of only **8** and **9**. The only sample that showed no accumulation of alkaloids was that from the leaf basal gland exudate. The leaf basal glands secrete a sugary exudate that serves to attract ants, and as such, it is not surprising that alkaloids, which are potentially toxic to insects including ants, were absent from the gland exudate.

An analysis of collections from a range of collection sites was undertaken to examine the geographic distribution of the three chemotypes. Specimens were examined from 20 sites (Figure 2), with multiple individuals sampled at 13 of these sites. At six of the sites that provided multiple

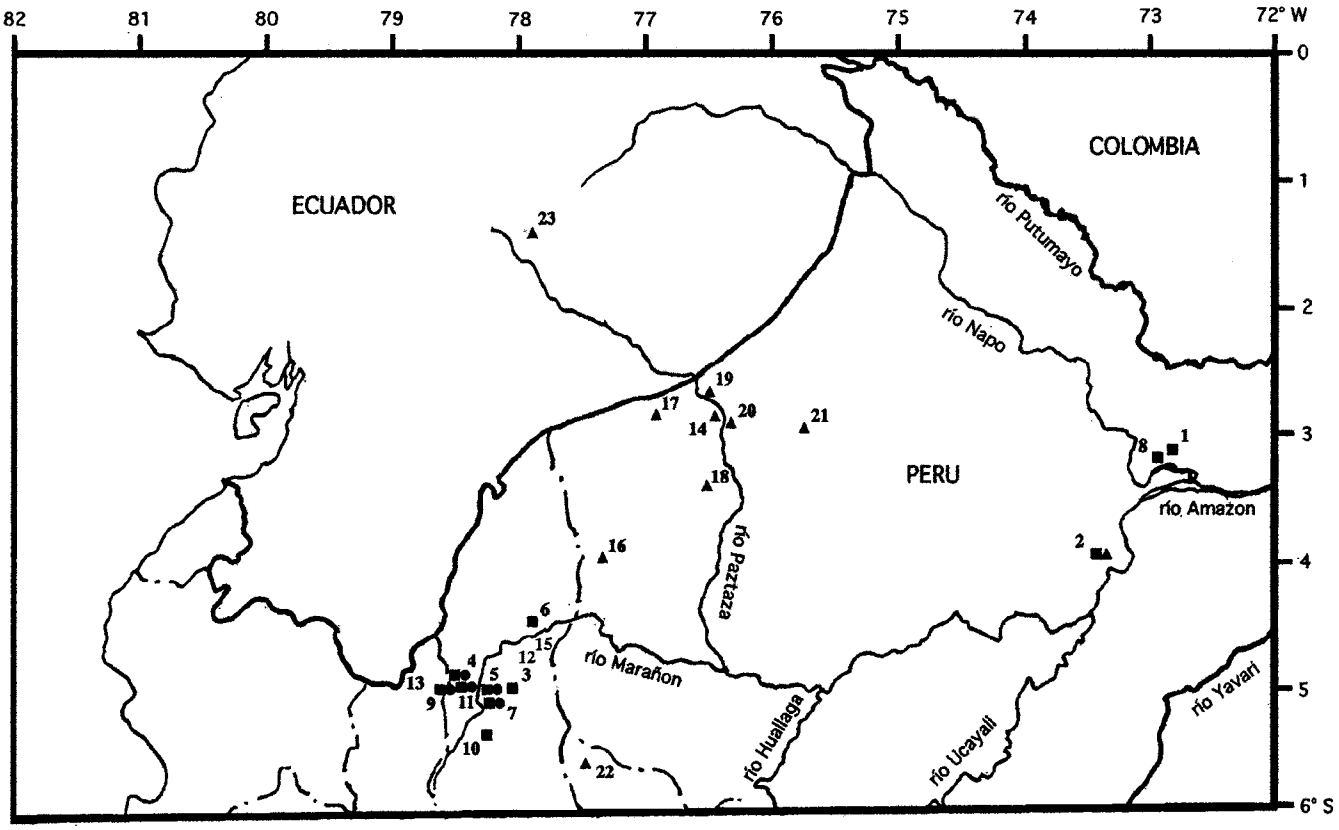

Figure 2. Map showing the distribution of *C. lechleri* collections by collection site and chemotype (▲ = chemotype 1, ■ = chemotype 2, ● = chemotype 3). Numbered sites with no symbols indicate locations where latex samples, but no leaf samples, were collected, and hence no chemotype determinations could be made for those sites.

collections, individuals representing two chemotypes were present, but no site had individuals of all three chemotypes.

The range of *C. lechleri* extends west to the slopes of the Andes below 1000 m. To the north the range extends into Ecuador and Colombia to 1° N latitude. The range extends south to the Departments of Cusco and Madre de Dios in Peru (ca. 12° S). The easternmost limit of the range is unclear, as no collections from Brazil were available at the Missouri Botanical Garden Herbarium (MO) or the Herbarium at the University of San Marcos, Lima (USM), although the range likely extends into westernmost Brazil at least in the central portion of the range. In the north, the range extends east to 72° W, and in the south the range extends to ca. 69° W. Collections for this study were made in the northern half of the range in northern Peru and Ecuador.

An inspection of Figure 2 reveals that the distribution of the chemotypes is not uniform. In the north-central portion of the range all individuals sampled were of chemotype 1, being able to fully *O*-methylate along pathway I to yield glaucine (**4**). To the east, lower in the Amazon basin, chemotype 1 gives way to chemotype 2, but no individuals of chemotype 3 were observed in the eastern portion of the range of *C. lechleri*. A similar pattern (i.e., chemotype 1 giving way to chemotype 2) is observed in the western portion of the range as one moves into the foothills of the Andes. Several sites were observed that were composed entirely of individuals of chemotype 2. Moving further to the west, chemotype 3 begins to be encountered together with chemotype 2. At sites where it occurs, chemotype 3 made up 10−25% of individuals, with chemotype 2 making up the remainder. No site was repesented solely by individuals of chemotype 3. Sites with individuals of chemotype 3 have the most restricted range, being found exclusively in the Alto Marañon region at around 5° S and between 78° and 79° W.

The observed pattern fits well with the probable biosynthetic relationships of the alkaloids involved. The ability to *O*-methylate isoboldine (**2**) to thaliporphine (**3**), or thaliporphine (**3**) to glaucine (**4**), would require a single *O*-methyltransferase in each case. The observed gradient with successive loss of the ability to *O*-methylate as one moves from the central portion of the range represents the successive loss of function for the individual methyltransferases involved. What is unknown is which level of methylation represents the ancestral state for this species and what the contribution to the fitness of the individual is for each alkaloid. The simplest model would be an ancestral state represented by chemotype 3 in which isoboldine (**2**) is produced but not further *O*-methylated. The data can then be interpreted by successive gain of the methyltransferases needed to produce thaliporphine (**3**) and glaucine (**4**) in the central portion of the range and subsequently spreading east and west toward the extremities of the range.

A more complicated explanation would be required if the ancestral state was represented by chemotype 1, with subsequent loss of the *O*-methyltransferases. If this is the case, then the observed pattern would require two centers for the diversification of this species, with one in the eastern and the other in the western extremes of the range. Although a pattern involving loss of function may seem counterintuitive, such a pattern could arise if isoboldine (**2**) contributes more to the fitness of the individuals than do the other alkaloids. Overall, the first scenario seems the more likely, but more data will be required to demonstrate this conclusively.

Experimental Section

General Experimental Procedures. The HPLC apparatus consisted of a Perkin-Elmer series 410 bio pump, a Bio-Rad As-100 autosampler fitted with a 20 μL injection loop, a Pharmacia Single Path Monitor UV-1 UV detector with detection at 254 nm, and a Hewlett-Packard 3390A integrator. Samples were analyzed by reversed-phase ion-pairing HPLC using a C_{18} column (250 × 4.6 mm, 5 μm, Alltech Econosphere) fitted with a guard column (20 × 2 mm, 35−50 μm pellicular C_{18}, Alltech Econosil).

Column chromatography employed 230−400 mesh silica (Sigma). Thin-layer chromatography (TLC) was performed on Kieselgel 60 F254 (Merck). Detection of alkaloids was by Dragendorff's reagent[11] or iodoplatinate reagent.[12] Preparative thin-layer chromatography was performed on Si gel (1000 μm, 60 Å, Whatman). Authentic glaucine (**4**) was purchased from Sigma. Authentic magnoflorine (**8**) and taspine (**9**) came from laboratory collections.

Melting points were determined on a Laboratory Devices Mel-Temp apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. UV spectra were obtained on a Beckman DU-40 spectrophotometer in methanol. NMR specta were recorded on a Varian Unity 300 spectrometer. EIMS were obtained on a JEOL Mstation (JMS 700) at 70 eV.

Plant Material. Latex for bulk extraction was obtained from Peru, Department of Amazonas. Leaf material for bulk extraction came from greenhouse-grown or wild-collected individuals. The seed source for greenhouse-grown plants was Peru, Department of Amazonas (site 5 in Figure 2, 5° 4.2′ S, 78° 18.9′ W, W.H. Lewis 16660, voucher: MO, USM). Wild-collected leaf material was obtained from Peru, Department of Loreto (site 14 in Figure 2, 2° 43.9′ S, 76° 31.6′ W, Elvin-Lewis 08, voucher: MO, USM).

For the quantitative alkaloid survey, samples were collected (by D.J.M.) from 15 field sites in northern Peru during three field trips in February/March 1996, March/April 1997, and September 1997 (Figure 2, sites 1−15). For latex collections, one sample was obtained from each individual. The latex samples were obtained by making a minimum of five slashes around the trunk at breast height (ca. 1.5 m) and combining the latex from each slash to produce one aggregate sample. Typical latex sample volume was 2−5 mL. A small volume of medicinal grade alcohol (purchased in Peru) was added to each sample in the field as a preservative. In the laboratory the samples were air-dried to remove the alcohol and then frozen and lyophilized. The dried samples were powdered prior to analysis and stored frozen (−20 °C). For leaf collections, two independent samples were obtained by clipping separate branches and removing one mature, intact leaf from each branch. For each sample only the leaf blade was collected by removal of the petiole at the leaf base. Leaves were maintained in a plant press for several days to remove the bulk of the leaf moisture and then transferred to small envelopes. The leaves were fully dried by placing on top of a plant press drying oven (30−40 °C) at the end of each field trip. The leaf samples were ground to a coarse powder in a coffee mill and stored frozen (−20 °C) prior to analysis. The leaf samples were maintained and analyzed individually, and the mean of the two samples from each individual was used in analysis.

For the chemotype distribution map, the quantitative samples above were supplemented with leaf samples from herbarium vouchers collected by W.H.L. (Figure 2, sites 16−22). Leaf samples from herbarium vouchers were prepared as above with the exception that only a single leaf was sampled from each individual.

Isolation and Identification of Alkaloids. A 1 L specimen of *C. lechleri* latex was lyophilized to yield 161 g of reddish-white powdered latex. A portion of the powdered latex (128 g) was combined with 2 L of distilled H_2O and made strongly acidic with concentrated HCl. The acidified aqueous layer was extracted continuously with $CHCl_3$ for 48 h to yield the chloroform-soluble neutral/acidic fraction (1.25 g) as a brown oil. The neutral/acidic fraction showed no Dragendorff's reagent-positive components. The aqueous layer was then adjusted to pH 8 with concentrated NH_4OH and extracted continuously with $CHCl_3$ over 24 h to yield the first basic fraction. The aqueous layer was adjusted to pH 10 with NH_4OH and extracted continuously an additional 24 h to yield the second basic fraction. TLC examination of the two basic fractions, visualized with Dragendorff's reagent, did not reveal any differential extraction, and so the basic fractions were combined (1.697 g). The basic fraction was dissolved in MeOH with warming, and taspine (**9**) (963 mg) was obtained as a white precipitate upon refrigeration.

Leaf material from a ca. 6-month-old greenhouse-grown plant was dried and ground in a Waring blender to give 70 g of a coarse powder. The ground plant material was extracted with MeOH and the resulting extract concentrated. The concentrated MeOH extract (50 mL) was added into $CHCl_3$ (200 mL) and partitioned (4 × 150 mL) with 1 N HCl. The aqueous layer was made basic with concentrated NH_4OH (pH 11) and partitioned (4 × 200 mL) with $CHCl_3$. The $CHCl_3$ layers were combined to give the crude alkaloid fraction (108 mg). The crude alkaloid fraction was subjected to column chromatography over Si gel (2 g, 1 × 25 cm column), eluting with $CHCl_3$−MeOH−concentrated NH_4OH (25:5:0.5) and collecting 1 mL fractions (after pass of solvent front). Fractions 4−6 contained small amounts of taspine (**9**) and thaliporphine (**3**) (TLC). Fractions 7−10 accounted for the bulk of the crude alkaloid fraction and were dissolved in a minimum of MeOH and left overnight at −20 °C to afford the recovery of crystalline isoboldine (**2**) (43 mg). Fractions 11−16 were subjected to preparative TLC developed with $CHCl_3$−MeOH−concentrated NH_4OH (25:5:0.5), resulting in the recovery of norisoboldine (**1**) (3 mg).

A batch of dried leaf material from a second greenhouse-grown plant was ground in a Waring blender to give 152 g of a coarse powder. The plant material was extracted with MeOH and worked up as above to give a crude alkaloid fraction. The crude alkaloid fraction was chromatographed over Si gel (10 g, 3 × 50 cm) and eluted with 100 mL each of $CHCl_3$, 1% MeOH, 5% MeOH, 10% MeOH, 20% MeOH, and 100% MeOH, collecting one fraction at each solvent change. The 1% and 5% MeOH-eluted fractions were combined and chromatographed by preparative TLC developed with $CHCl_3$−MeOH (9:1). The major UV quenching and iodoplatinate-positive component was rechromatographed [preparative TLC, $CHCl_3$−MeOH (9:1)] to yield thaliporphine (**3**) (30 mg).

A leaf sample of *C. lechleri* collected in Peru, Department of Loreto, was ground in a Waring blender to give 100 g of a coarse powder. The powdered leaf material was extracted with MeOH and worked up as previously to yield 62 mg of a crude alkaloid fraction. The crude alkaloid fraction was chromatographed over Si gel (10 g, 2 × 30 cm), eluting with 100 mL each of $CHCl_3$, 5% MeOH, 10% MeOH, 20% MeOH, and 50% MeOH and collecting 8 mL fractions. Fractions 3−11 contained the unknown iodoplatinate-positive component and were cleaned up by preparative TLC, developed with $CHCl_3$−MeOH (9:1) to give glaucine (**4**) (8 mg).

An additional, iodoplatinate-positive component remained in the basified aqueous layers of each of the leaf extracts and was subsequently identified as magnoflorine (**8**) by TLC and HPLC comparison with an authentic sample from laboratory collections. For HPLC standardization, **8** was obtained as the iodide from *Croton ruizianus* Muell. Arg., and details of this isolation will be published elsewhere.

Norisoboldine (1): $[\alpha]_D^{25}$ +43.7° (*c* 0.1, EtOH), lit.[9] +47° (*c* 1, EtOH); spectral data (UV, ^{1}H NMR, and EIMS) consistent with literature values.[9]

Isoboldine (2): mp 118−120 °C, lit.[10] 122−123 °C; $[\alpha]_D^{25}$ +50.3° (*c* 0.33, EtOH), lit.[9] +54° (*c* 0.2, EtOH); spectral data (UV, ^{1}H NMR, and EIMS) consistent with literature values.[9,10]

Thaliporphine (3): mp 169−172 °C, lit.[9] 170−172 °C; $[\alpha]_D^{25}$ +39.8° (*c* 0.31, EtOH), lit.[9] +44° (*c* 0.97, EtOH); spectral data (UV, ^{1}H NMR, and EIMS) consistent with literature values.[9]

Glaucine (4): $[\alpha]_D^{25}$ +102.0° (*c* 0.1, EtOH), lit.[9] +116° (*c* 0.75, EtOH); spectral data (UV, ^{1}H NMR, and EIMS) consis-

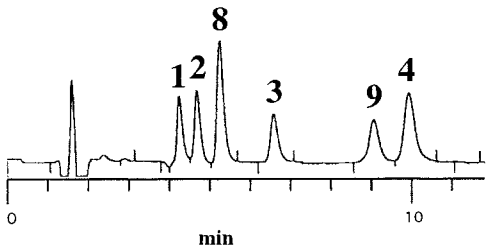

Figure 3. Representative HPLC trace of a standard mix of the alkaloids of *C. lechleri*. The standard mix consisted of 0.5 mg/mL of each alkaloid. Peak designations are norisoboldine (**1**) (t_R = 4.2), isoboldine (**2**) (t_R = 4.6), magnoflorine (**8**) (t_R = 5.2), thaliporphine (t_R = 6.5) (**3**), taspine (**9**) (t_R = 9.0), and glaucine (**4**) (t_R = 9.8).

tent with literature values;[9] chromatographic behavior (TLC, HPLC) identical to an authentic sample.

Taspine (9): mp 345−348 °C (dec), lit.[8] 350 °C (dec); $[\alpha]_D^{25}$ +5.2° (*c* 0.33, $CHCl_3$), lit.[2] +7.6° (pyridine); spectral data (UV, ^{1}H NMR, and EIMS) consistent with literature values;[7,13] chromatographic behavior (TLC, HPLC) identical to an authentic sample.

HPLC Analysis. Samples for HPLC analysis were prepared by extraction in the same buffer used for eluting the HPLC column. Powdered latex (10 mg) or 100 mg of ground leaf material was extracted in a test tube with 10 mL of buffer A or buffer B, respectively (see below). After addition of buffer the samples were sonicated for 15 min, macerated 24 h, and then sonicated an additional 15 min. After allowing the extracted plant material to settle (leaves only, the latex dissolved completely in the buffer), a 1 mL aliquot was transferred to an autosampler tube and centrifuged at 15 000 rpm for 5 min. Latex samples were eluted with buffer A consisting of 45:55 acetonitrile−aqueous buffer [0.1 M PO_4^-, pH 2.5, 10 mM sodium dodecyl sulfate (SDS), 0.1% triethylamine (TEA)]. Leaf samples were eluted with buffer B consisting of 43:57 acetonitrile−aqueous buffer (0.1 M PO_4^-, pH 2.5, 10 mM SDS, 0.08% TEA). For both leaf and latex samples, elution was isocratic at 2 mL/min over 12 min with detection at 254 nm. Injection volume was 20 μL. A representative HPLC trace for the leaf alkaloids is shown in Figure 3.

Quantitation was by the external standard method. Standard curves were prepared for isoboldine HCl (**2**), magnoflorine iodide (**8**), thaliporphine (**3**), taspine (**9**), and glaucine (**4**) at 2-fold dilutions between 0.1 and 6.25 × 10^{-3} mg/mL for latex samples and 0.05 and 3.125 × 10^{-3} mg/mL for leaf samples. Three sample injections were made at each concentration. UV absorptions for preparation of standards were taken in methanol. Standard solutions of each alkaloid were prepared on the basis of the following absorptions: isoboldine (**2**) λ_{max} 219 (4.54), thaliporphine (**3**) λ_{max} 220 (4.52), glaucine (**4**) λ_{max} 219 (4.58), magnoflorine iodide (**8**) λ_{max} 226 (4.65), and taspine (**9**) λ_{max} 246 (4.67). Because insufficient material was available for norisoboldine (**1**), the concentration of **1** was estimated using the standard curve for isoboldine (**2**). Standard curves were rerun when reinjection of a standard mix deviated from the standard curve.

Statistical Analysis. Since there was a significant effect of site on alkaloid concentration, only those sites with individuals of both chemotypes 2 and 3 were included in the analysis (Figure 2, sites 4, 5, 7, 9, 11). There were too few individuals of chemotype 1 for which quantitative data were available to allow inclusion of chemotype 1 in the analysis. A two-way analysis of variance (ANOVA) was performed to examine the effect of chemotype on alkaloid content using collection site as a blocking factor. Individual ANOVAs were performed for each alkaloid and for total alkaloids. Homogeneity of variance was tested using Bartlett's test. Statistical analysis was performed with the Statview SE software package (Abacus Concepts, Inc.).

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References and Notes

(1) Cobo, P. B. In *Biblioteca de Autores Españoles* (Series); Mateos, P. F., Ed.; Atlas: Madrid, 1956; Vols. 91−92.
(2) Persinos Perdue, G.; Blomster, R. N.; Blake, D. A.; Farnsworth, N. R. *J. Pharm. Sci*. **1979**, *68*, 124−126.
(3) Porras-Reyes, B. H.; Lewis, W. H.; Roman, J.; Simchowitz, L.; Mustoe, T. A. *Pro. Soc. Exp. Biol. Med*. **1993**, *203*, 18−25.
(4) Vaisberg, A. J.; Milla, M.; del Carmen Planas, M.; Cordova, J. L.; Rosas de Agusti, E.; Ferreyra, R.; del Carmen Mustiga, M.; Carlin L.; Hammond, G. B. *Planta Med*. **1989**, *55*, 140−143.
(5) Platanova, T. F.; Kuzovkov, A. D.; Sheinker, Y. N. *J. Gen. Chem. USSR* **1956**, *26*, 2957−2961.
(6) Kennelly, E. J.; Flynn, T. J.; Mazzola, E. P.; Roach, J. A.; McCloud, T. G.; Danford, D. E.; Betz, J. M. *J. Nat. Prod*. **1999**, *62*, 1385−1389.
(7) Shamma, M.; Moniot; J. L. *Chem. Commun*. **1971**, 1065−1066.
(8) Bettolo, R. M.; Scarpati, M. L. *Phytochemistry* **1979**, *18*, 520.
(9) Guinaudeau, H.; Leboeuf, M.; Cavé, A. *Lloydia* **1975**, *38*, 275−338, and references therein.
(10) Wu, W.-N.; Beal, J. L.; Doskotch, R. W. *J. Nat. Prod*. **1980**, *43*, 372−381.
(11) Stahl, E. *Thin-layer Chromatography, a Laboratory Handbook*; Springer-Verlag: Berlin, 1969; reagent no. 97.
(12) Wagner, H.; Bladt, S.; Zgainski, E. M. *Plant Drug Analysis*; Springer-Verlag: Berlin, 1984.
(13) Pieters, L.; De Bruyne, T.; Claeys, M.; Vlietinck, A.; Calomme, M.; vanden Berghe, D. *J. Nat. Prod*. **1993**, *56*, 899−906.

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