Alkaloids from Bufonid Toads (Melanophryniscus): Decahydroquinolines, Pumiliotoxins and Homopumiliotoxins, Indolizidines, Pyrrolizidines, and Quinolizidines

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ALKALOIDS FROM BUFONID TOADS (*MELANOPHRYNISCUS*): DECAHYDROQUINOLINES, PUMILIOTOXINS AND HOMOPUMILIOTOXINS, INDOLIZIDINES, PYRROLIZIDINES, AND QUINOLIZIDINES

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ABSTRACT.—Skins of bufonid toads of the genus *Melanophryniscus* contain several classes of alkaloids: decahydroquinolines, pumiliotoxins, allopumiliotoxins, homopumiliotoxins, both 3,5- and 5,8-disubstituted indolizidines, 3,5-disubstituted pyrrolizidines, and a 1,4-disubstituted quinolizidine. Tricyclic alkaloids, including precoccinelline **[193A]** and alkaloid **236**, an oxime methyl ether, are present in one population of *Melanophryniscus stelzneri.*

A wide range of biologically active alkaloids occur in amphibians (1, 2). A particularly rich source of such alkaloids has been skin extracts of frogs of the family Dendrobatidae. Major classes of alkaloids from dendrobatid frogs include the following: batrachotoxins (novel steroidal alkaloids), histrionicotoxins (highly unsaturated azaspiro[5.5]undecanols), 2,5-disubstituted decahydroquinolines, the pumiliotoxin-A class, consisting mainly of pumiliotoxins (8-hydroxy-8-methyl-6-alkylidenylindolizidines) and allopumiliotoxins (7,8-dihydroxy-8-methyl-6-alkylidenylindolizidines), and both 3,5- and 5,8-disubstituted indolizidines. These have been termed dendrobatid alkaloids, since all had appeared unique to this family of amphibians. However, during the screening of skin extracts from various genera of other amphibian families, it was discovered that alkaloids of the pumiliotoxin-A class were present in skin from a bufonid toad of the new world genus *Melanophyrniscus*, ranid frogs of the Madagascan genus *Mantella,* and a myobatrachid frog of the Australian genus *Pseudophryne* (3). This has prompted further study on toads/frogs of these three nondendrobatid genera. The pumiliotoxins were found to be accompanied in frogs of the genus *Pseudophryne* by a new class of amphibian alkaloids, the pseudophrynamines (3a-prenylpyrrolo[2,3*b*]indoles) (4).

The present report documents the occurrence of decahydroquinolines, pumiliotoxins, and allopumiliotoxins, and both 3,5- and 5,8-disubstituted indolizidines in skin extracts of bufonid toads of the genus *Melanophryniscus*. In addition, homopumiliotoxins (in which the indolizidine moiety of pumiliotoxins and allopumiliotoxins is replaced with a quinolizidine ring), 3,5-disubstituted pyrrolizidines, and 1,4-disubstituted quinolizidines are present. Three tricyclic alkaloids were detected. Gc in conjunction with ms or Ft-ir spectroscopy provided the basis for structural assignments.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Gc-ms analyses used capillary columns (25 m×0.32 mm, either OV-1 or HP-5) programmed from 100° to 280° at a rate of 10°/min, employing either a Finnigan model 4500 mass spectrometer [ei spectra, 70 eV, or ci spectra with NH_3 to obtain $[M+1]^+$ ions or ND_3 to obtain $[M+2]^+$ ions and the number of exchangeable H's (5)] or a Finnigan ion trap detector, model 800. A first analysis of the sample is done with a Finnigan TSP thermospray mass spectrometer, where a direct injection in MeCN/H_2O/NH_4OAc buffer gives $[M+1]^+$ peaks of all the alkaloids and provides a rough quantitation. A Hewlett-Packard model 5890 gas chromatograph fitted with an HP-5 fused silica capillary column (25 m×0.32 mm) with the same program as used above for the ms analyses, interfaced with a Hewlett-Packard 5965A ir detector and a 59970 IRD ChemStation, was used to generate the total response chromatogram and Ft-ir spectra of gc peaks.

SOURCE MATERIAL.—Voucher specimens are in the Museo Argentino de Ciencias Naturales, Buenos Aires. Extracts of skins were obtained from toads of the following localities: I, *Melanophryniscus stelzneri* (Weyenbergh), 23 skins, 3.2 g (wet wt), Tanti, Córdoba, Argentina, May 1989; II, *M. stelzneri*, 31 skins, 4.3 g (dry wt), Las Alpacas, Córdoba, Argentina, November, 1987; III, *Melanophryniscus stelzneri montevidensis* (Philippi), 39 skins, 4.5 g (dry wt), La Coronilla, Depto. Rocha, Uruguay, January–February 1987.

PREPARATION OF FROG/TOAD SKIN EXTRACT.—In the case of extract I the following procedure was used. Skins were macerated in MeOH and the extract was treated with equal volumes of 0.1 N HCl and $CHCl_3$ and shaken. The aqueous acidic layer was neutralized with aqueous NH_3 or $NaHCO_3$ and reextracted with $CHCl_3$. The $CHCl_3$ extract was washed with H_2O and dried with Na_2SO_4. It was carefully evaporated to dryness, and MeOH was added (1 ml for each gram wet wt of skins). With extracts II and III the following procedure was used. A MeOH extract was prepared as above and subjected to Al_2O_3 cc. The initial fractions, eluting with EtOH-H_2O (95:5), contained the alkaloids and were subjected to the above partitioning.

CHEMICAL PROCEDURES.—*Microhydrogenation.*—Samples of 0.01 to 0.1 mg in 50–100 μl MeOH were stirred under H_2 at 1–2 atm with a 5% Rh/Al_2O_3 catalyst for several h. After filtration, the solution was analyzed by gc-ms and gc-Ft-ir to detect the number of double and triple bonds that were reduced in each of the alkaloids.

Acetylation.—The MeOH extract (50 μl) was evaporated, and pyridine and Ac_2O (50 μl each) were added and left for 1–2 h at room temperature. The solution was treated with saturated aqueous $NaHCO_3$ and extracted with EtOAc. After drying (Na_2SO_4), alkaloids in the organic layer were analyzed by gc-ms and gc-Ft-ir to detect any acetylated hydroxyl or amino groups.

α-*Deuteration.*—MeOH-d_4 was treated with sodium, and the solution containing sodium deuteromethoxide was added to an evaporated aliquot of the MeOH extract. After 4 h the solution was treated with D_2O and EtOAc, and the organic layer was analyzed by ms to detect the number of H's adjacent to carbonyls that have exchanged with deuterium.

ALKALOIDS.—The following section provides a listing of the alkaloids detected in the *Melanophryniscus* extracts. Bold face numerical designations according to nominal mass with an identifying letter are used as in prior reports (3, 4). Molecular formula (given in quotes if not confirmed by hrms), eims or pseudo eims (obtained with the ion trap), the number of exchangeable hydrogens, Ft-ir spectrum with absorbances relative to the most intense band considered 100 (sharp bands unless indicated by "b" for broad), the number of hydrogens added to form the perhydro derivative, and other diagnostic data are presented for each alkaloid. High resolution data cited below were obtained in previous studies (3, 6). The structures are presented in Table 1.

DECAHYDROQUINOLINES.—*cis*-**223F**.—"$C_{15}H_{29}N$"; *m/z* 223 (3), 222 (3), 180 (100); 1D; ir 2935 (100), 2877 (51), 2801 (18), 1454 (17), 1321 (8, b), 1091 (6) cm^{-1}; Ft-ir see Figure 1.

trans-**223F**.—"$C_{15}H_{29}N$"; *m/z* 223 (2), 222 (2), 180 (100); 1D; ir 2934 (100), 2873 (47), 1455 (15), 1344 (7), 1131 (10) cm^{-1}; Ft-ir see Figure 1.

cis- **249D**.—"$C_{17}H_{31}N$"; *m/z* 249 (2), 248 (3), 206 (100), 180 (15); 1D; ir 3085 (6), 2935 (100), 2870 (44), 2804 (15), 1642 (4), 1450 (16), 1355 (77), 1320 (9), 1125 (5), 1090 (6), 992 (6), 913 (14) cm^{-1}; H_2-derivative *m/z* 251, 208, 180.

trans-**249D**.—"$C_{17}H_{31}N$"; ion trap *m/z* 250 (5), 248 (1), 206 (100), 180 (6), 164 (2), 136 (5), 67 (19), 55 (18); 1D; ir 3086 (6), 2933 (100), 2871 (45), 1641 (3), 1453 (14), 1345 (7), 1130 (8), 994 (2), 915 (7) cm^{-1}.

trans-**249E**.—"$C_{17}H_{31}N$"; ion trap *m/z* 250 (15), 244 (6), 220 (2), 206 (29), 180 (100), 150 (5), 124 (10), 121 (10), 111 (12), 95 (13), 81 (25), 67 (29), 56 (34), 55 (22); 1D; ir 3084 (5), 2934 (100), 2870 (44), 1646 (4), 1453 (14), 1343 (7), 1130 (7), 996 (4), 914 (8) cm^{-1}.

cis-**275B**.—"$C_{19}H_{33}N$"; *m/z* 275 (6), 274 (4), 232 (22), 220 (3), 206 (100), 178 (1), 176 (3), 136 (3), 124 (12), 111 (12), 98 (5), 96 (5), 95 (9), 81 (14), 67 (16), 56 (16), 55 (16); 1D; ir 3085 (11), 2934 (100), 2868 (42), 2804 (15), 1642 (7), 1448 (16), 1355 (6), 1321 (8), 1089 (5,b), 993 (8), 915 (16) cm^{-1} (terminal double bond absorptions 3085, 1642, 993, and 915 cm^{-1} are double the intensity of those of *cis*-**249D**). The presence of the fragment ion 232 (or 236 after hydrogenation) is not readily rationalized for the proposed structure. H_4-derivative *m/z* 279, 236, 208. The Ft-ir spectrum of the H_4-derivative is almost superimposable with that of the H_2-derivative from *cis*-**249D**. The structure is tentative.

PUMILIOTOXINS, ALLOPUMILIOTOXINS, AND HOMOPUMILIOTOXINS.—*Pumiliotoxins.*—**251D**.—$C_{16}H_{29}NO$; *m/z* 251 (18), 250 (10), 208 (10), 206 (6), 194 (40), 176 (6), 166 (100), 70 (48), 1D; ir 3543 (11), 2967 (100), 2886 (41), 2797 (35), 1460 (13), 1385 (17), 1313 (23), 1155 (19), 1097 (16), 965 (16) cm^{-1}; H_2-derivative.

TABLE 1. Profile of Alkaloids[a] in Four Populations of *Melanophryniscus*.

Alkaloids	Population[b]				Structures
	I	II	III	IV	
Decahydroquinolines					
cis-**223F**	++	+++			
trans-**223F**	+++	+++			
cis-**249D**	+++	++			
trans-**249D**	+	+			
trans-**249E**	+	+			
cis-**275B**	+	+			
Pumiliotoxin-A Class					
251D	++	+	+++		
267C				+++	
323B				+	
319A			+		
319B			+		
321B			+		

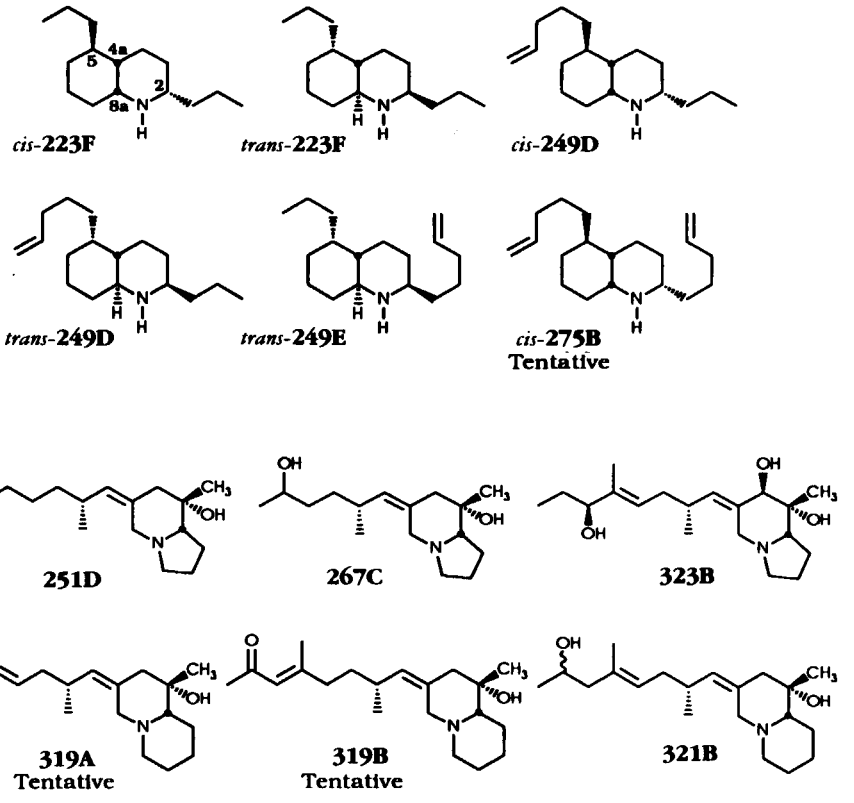


TABLE 1. Continued.

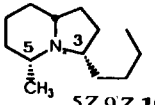
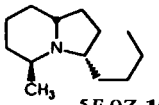
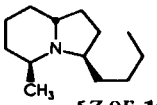
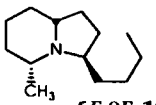
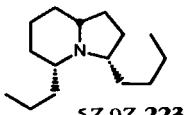
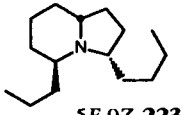
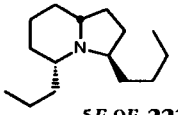
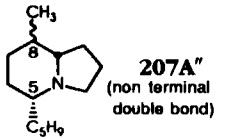
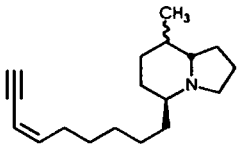
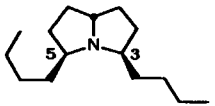
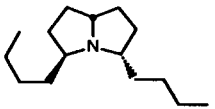
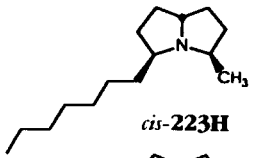
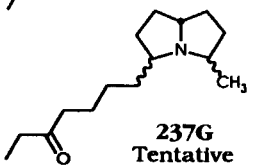
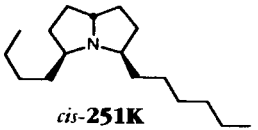
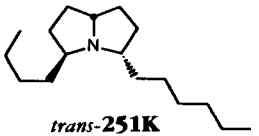
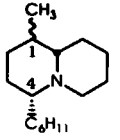
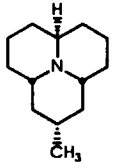
Alkaloids	Population[b]				Structures
	I	II	III	IV	
Indolizidines					
5*Z*,9*Z*-**195B**	+	+			 5*Z*,9*Z*-**195B**
5*E*,9*Z*-**195B**	++	++	+		 5*E*,9*Z*-**195B**
5*Z*,9*E*-**195B**	+++	++	+++		 5*Z*,9*E*-**195B**
5*E*,9*E*-**195B**	+	+			 5*E*,9*E*-**195B**
207A″	+	++			
5*Z*,9*Z*-**223AB**	++	+++			 5*Z*,9*Z*-**223AB**
5*E*,9*Z*-**223AB**		++			 5*E*,9*Z*-**223AB**
5*E*,9*E*-**223AB**		+			 5*E*,9*E*-**223AB**
259B	+++	+			 **207A″** (non terminal double bond)  **259B**
Pyrrolizidines					
cis-**223B**	++				 *cis*-**223B**
trans-**223B**	+	+			 *trans*-**223B**
cis-**223H**		+	+		 *cis*-**223H**
237G			+		 **237G** Tentative
cis-**251K**	++				 *cis*-**251K**
trans-**251K**	+++	+			 *trans*-**251K**

TABLE 1. Continued.

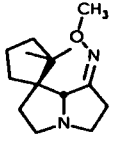
Alkaloids	Population[b]				Structures
	I	II	III	IV	
Quinolizidines					
235E'	+++	+			
Tricyclics					
193A	++	+			
235I	++	+			
236	++				
Unknowns					
219G	++				



235E' (non terminal double bond)



193A



236

[a]+++=Major alkaloid. ++=Minor alkaloid. +=Trace alkaloid.

[b]I, *Melanophryniscus stelzneri*, Tanti, Córdoba, Argentina; II, *Melanophryniscus stelzneri*, Las Alpacas, Córdoba, Argentina; III, *Melanophryniscus stelzneri montevidensis*, La Coronilla, Depto. Rocha, Uruguay; IV, *Melanophryniscus moreirae*, Serra da Mantiquerira, State of Rio de Janeiro, Brazil.

267C.—$C_{16}H_{29}NO_2$; *m/z* 267 (16), 266 (7), 252 (5), 250 (2), 234 (3), 224 (7), 222 (9), 194 (12), 176 (5), 166 (100), 112 (8), 84 (18), 70 (75); 2D; ir 3655 (5), 3545 (11), 2970 (100), 2889 (45), 2798 (39), 1458 (15), 1384 (30), 1312 (30), 1160 (29), 1095 (28), 963 (20) cm^{-1}; Ft-ir see Daly *et al.* (4); H_2-derivative.

Allopumiliotoxins.—**323B**.—$C_{19}H_{33}NO_3$; *m/z* 323 (3), 306 (7), 210 (7), 209 (9), 182 (43), 114 (22), 70 (100); 3D; ir 3647 (14), 3523 (12), 2970 (100), 2887 (49), 2803 (38), 1458 (19), 1383 (21), 1310 (36), 1154 (32), 1095 (29), 1011 (47) cm^{-1}; H_4- derivative.

Homopumiliotoxins.—**319A**.—$C_{20}H_{33}NO_2$; an unconjugated ketone in the side chain; ion trap *m/z* 320 (15), 319 (3), 302 (5), 276 (35), 261 (37), 220 (34), 208 (30), 190 (27), 180 (90), 164 (6), 162 (12), 148 (9), 136 (8), 135 (7), 134 (10), 107 (12), 98 (20), 84 (100), 67 (30), 56 (36), 55 (36); 1D; non acetylatable OH; α- deuteration shows a maximum of 7D exchanged; ir 3557, 2946, 2757, 1728, 1451, 1381, 1271, 1116, 1059 cm^{-1} (intensities are not reported since **319A** does not completely separate from **321B**).

319B.—$C_{20}H_{33}NO_2$; a conjugated ketone in the side chain; ion trap *m/z* 320 (7), 319 (3), 276 (12), 261 (12), 220 (9). 208 (1), 190 (2), 180 (100), 164 (3), 162 (6), 148 (4), 136 (5), 135 (3), 134 (4), 122 (5), 109 (6), 107 (4), 105 (3), 98 (17), 95 (12), 84 (83), 67 (20), 56 (22), 55 (29); 1D; non-acetylatable OH; longer Rt than **319A**; ir 3554 (10), 2946 (100), 2756 (22), 1702 (35), 1622 (40), 1450 (19), 1379 (30), 1270 (24), 1164 (26), 1119 (31), 960 (17) cm^{-1}; Ft-ir see Figure 1.

321B.—$C_{20}H_{35}NO_2$; a homoallylic alcohol in the side chain; ion trap *m/z* 322 (13), 321 (6), 304 (4),

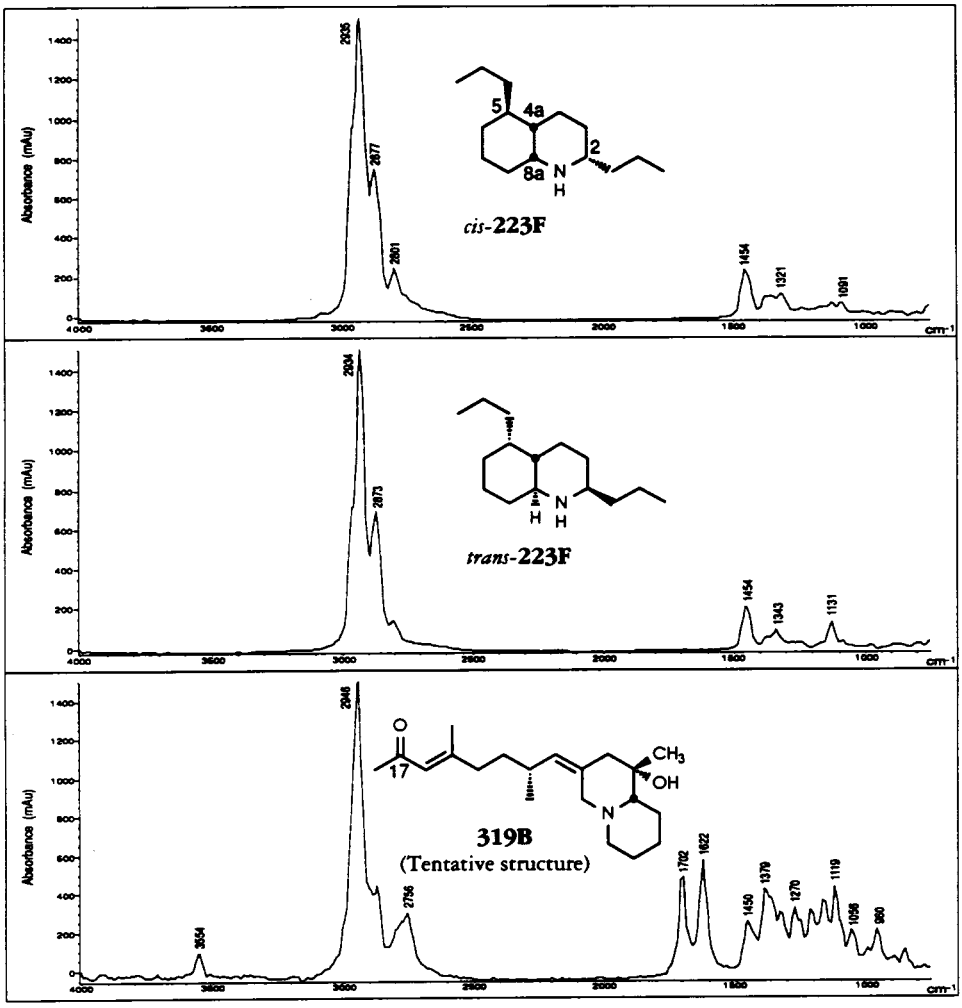


FIGURE 1. Selected vapor phase Ft-ir spectra.

276 (8), 258 (1), 220 (22), 208 (16), 190 (18), 180 (100), 164 (4), 162 (5), 148 (4), 136 (6), 135 (4), 134 (5), 110 (5), 109 (4), 107 (6), 105 (6), 98 (12), 84 (52), 67 (17), 56 (21), 55 (22); 2D; one acetylatable OH; almost the same Rt as **319A**; ir 3640, 3600, 3556, 2945, 2757, 1451, 1384, 1270, 1115, 1059, 940 cm^{-1} (intensities are not reported since **321B** does not completely separate from **319A**); monoacetate ion trap *m/z* 363 (3), 304 (10), 220 (22), 208 (17), 190 (18), 180 (100), 148 (4), 136 (7), 134 (3), 95 (11), 84 (85), 67 (39), 56 (23), 55 (35); monoacetate ir 3551 (7), 2944 (91), 2860 (27), 2752 (17), 1754 (70), 1455 (14), 1376 (34), 1240 (100), 1117 (32), 1063 (29), 951 (15), 853 (8) cm^{-1}.

INDOLIZIDINES.—5*Z*,9*Z*-**195B**.—"$C_{13}H_{25}N$"; monomorine-I, (5*Z*,9*Z*)-3-butyl-5-methylindolizidine; *m/z* 195 (3), 194 (2), 180 (1), 163 (1), 138 (100), 95 (6), 84 (12), 70 (10); 0D; ir 2974 (80), 2939 (100), 2874 (38), 2788 (19), 1458 (12), 1381 (12), 1318 (13), 1212 (9), 1125 (10) cm^{-1}.

5*E*,9*Z*-**195B**.—"$C_{13}H_{25}N$"; (5E,9*Z*)-3-butyl-5-methylindolizidine; *m/z* 195 (2), 194 (3), 180 (8), 138 (100), 122 (3), 108 (2), 95 (3); 0D; ir 2961 (63), 2936 (100), 2873 (31), 2800 (13), 1461 (8), 1377 (14), 1191 (8), 1144 (9) cm^{-1}.

5*Z*,9*E*-**195B**.—"$C_{13}H_{25}N$"; (5*Z*,9*E*)-3-butyl-5-methylindolizidine; ion trap *m/z* 196 (50), 195 (3), 194 (6), 138 (100), 122 (4), 121 (5), 95 (22), 68 (18); 0D; ir 2937 (100), 2878 (41), 1459 (11), 1400 (8), 1292 (5), 1088 (4, b), 986 (3) cm^{-1}.

5*E*,9*E*-**195B**.—"$C_{13}H_{25}N$"; (5*E*,9*E*)-3-butyl-5-methylindolizidine. This isomer coeluted with another alkaloid; therefore ms and Ft-ir are not reported.

207A".—"$C_{14}H_{25}N$"; an 8-methyl-5-pentenylindolizidine, the double bond in the side chain being internal; *m/z* 208 (cims), 206 (1), 138 (100), 136 (5), 122 (3), 108 (2), 96 (22), 69 (4), 67 (5); 0D; ir 2972 (100), 2949 (87), 2895 (58), 2787 (45), 1458 (15), 1384 (22), 1301 (9), 1234 (8), 1145 (11), 1081 (14), 967 (18) cm^{-1}. Configuration at C-8 is unknown. Ft-ir see Figure 3. Indolizidine **207A**, isolated from a dendrobatid frog, has a terminal double bond (3080 cm^{-1}) in the five carbon side-chain, and the 8-Me is equatorial (7).

5*Z*,9*Z*-**223AB**.—"$C_{15}H_{29}N$"; (5*Z*,9*Z*)-3-butyl-5-propylindolizidine; *m/z* 223 (2), 222 (3), 180 (30), 166 (100), 124 (10); 0D; ir 2975 (94), 2942 (100), 2876 (44), 2792 (19), 1458 (15), 1322 (15, b), 1212 (13), 1118 (15) cm^{-1}; Ft-ir see Figure 2.

5*E*,9*Z*-**223AB**.—"$C_{15}H_{29}N$"; (5*E*,9*Z*)-3-butyl-5-propylindolizidine; *m/z* 223 (4), 222 (5), 180 (100), 166 (80), 124 (10); 0D; ir 2939 (100), 2876 (34), 2803 (12), 1458 (9), 1367 (9), 1138 (7) cm^{-1}. Ft-ir see Figure 2.

5*E*,9*E*-**223AB**.—"$C_{15}H_{29}N$"; (5*E*,9*E*)-3-butyl-5-propylindolizidine; *m/z* 223 (3), 180 (40), 166 (100), 124 (10); 0D; ir 2939 (100), 2875 (34), 2795 (15), 1460 (10), 1370 (9, b), 1180 (8) cm^{-1}; Ft-ir see Figure 2.

259B.—"$C_{18}H_{29}N$"; 8-methyl-5-(non-6-en-8-ynyl)indolizidine; *m/z* 259 (3), 244 (1), 138 (100), 96 (20); 0D; ir 3327 (16), 3025 (10), 2963 (100), 2930 (80), 2880 (56), 2812 (14), 1461 (16), 1380 (13), 1340 (8), 1208 (13), 1143 (9), 963 (6) cm^{-1}. Configuration at C-8 is unknown. Ft-ir see Figure 3.

PYRROLIZIDINES.—*cis*-**223B**.—"$C_{15}H_{29}N$"; *exo,exo*-3,5-dibutylpyrrolizidine; ion trap *m/z* 224 (10), 222 (3), 194 (2), 180 (1), 166 (100), 138 (3), 124 (7), 110 (5), 96 (3), 81 (8), 70 (12), 55 (8); 0D; ir 2959 (100), 2879 (44), 1464 (11), 1353 (8), 1104 (11) cm^{-1}; Ft-ir see Figure 4.

trans-**223B**.—"$C_{15}H_{29}N$"; *exo,endo*-3,5-dibutylpyrrolizidine; ion trap *m/z* 224 (8), 194 (1), 166 (100), 152 (1), 138 (3), 124 (20), 110 (12), 81 (10), 70 (15); 0D; ir 2958 (100), 2876 (45), 1463 (11), 1360 (8), 1156 (7, b) cm^{-1}; Ft-ir see Figure 4.

cis-**223H**.—"$C_{15}H_{29}N$"; *exo,exo*-3-heptyl-5-methylpyrrolizidine; ion trap *m/z* 223 (1), 208 (2), 194 (1), 180 (1), 152 (1), 138 (2), 124 (100), 110 (4), 96 (4), 81 (10), 68 (10); 0D; ir 2960 (100), 2940 (94), 2875 (47), 1462 (12), 1361 (10), 1107 (10) cm^{-1}; Ft-ir see Figure 4.

237G.—$C_{15}H_{27}NO$; a 3-(ketoheptyl)-5-methylpyrrolizidine; ion trap *m/z* 238 (2), 222 (1), 194 (1), 180 (3), 138 (6), 124 (100), 110 (5), 96 (5), 81 (12), 68 (13); 0D; ir 2957 (100), 2878 (42), 1731 (32), 1457 (13), 1361 (25), 1165 (19, b) cm^{-1}. Structure is tentative.

cis-**251K**.—"$C_{17}H_{33}N$"; *exo,exo*-3-butyl-5-hexylpyrrolizidine; ion trap *m/z* 252 (10), 251 (8), 250 (8), 222 (3), 208 (1), 194 (80), 166 (100), 138 (4), 124 (12), 110 (10), 70 (20); 0D; ir 2964 (100), 2942 (92), 2879 (47), 1464 (13), 1359 (9, b), 1106 (12), 1055 (9) cm^{-1}.

trans-**251K**.—"$C_{17}H_{33}N$"; *exo,endo* (or *endo,exo*)-3-butyl-5-hexylpyrrolizidine; ion trap *m/z* 252 (20), 251 (3), 250 (10), 222 (1), 194 (90), 166 (100), 138 (3), 124 (10), 110 (20), 70 (12); 0D; ir 2965 (100), 2946 (94), 2874 (50), 1463 (12), 1365 (9), 1151 (8, b) cm^{-1}.

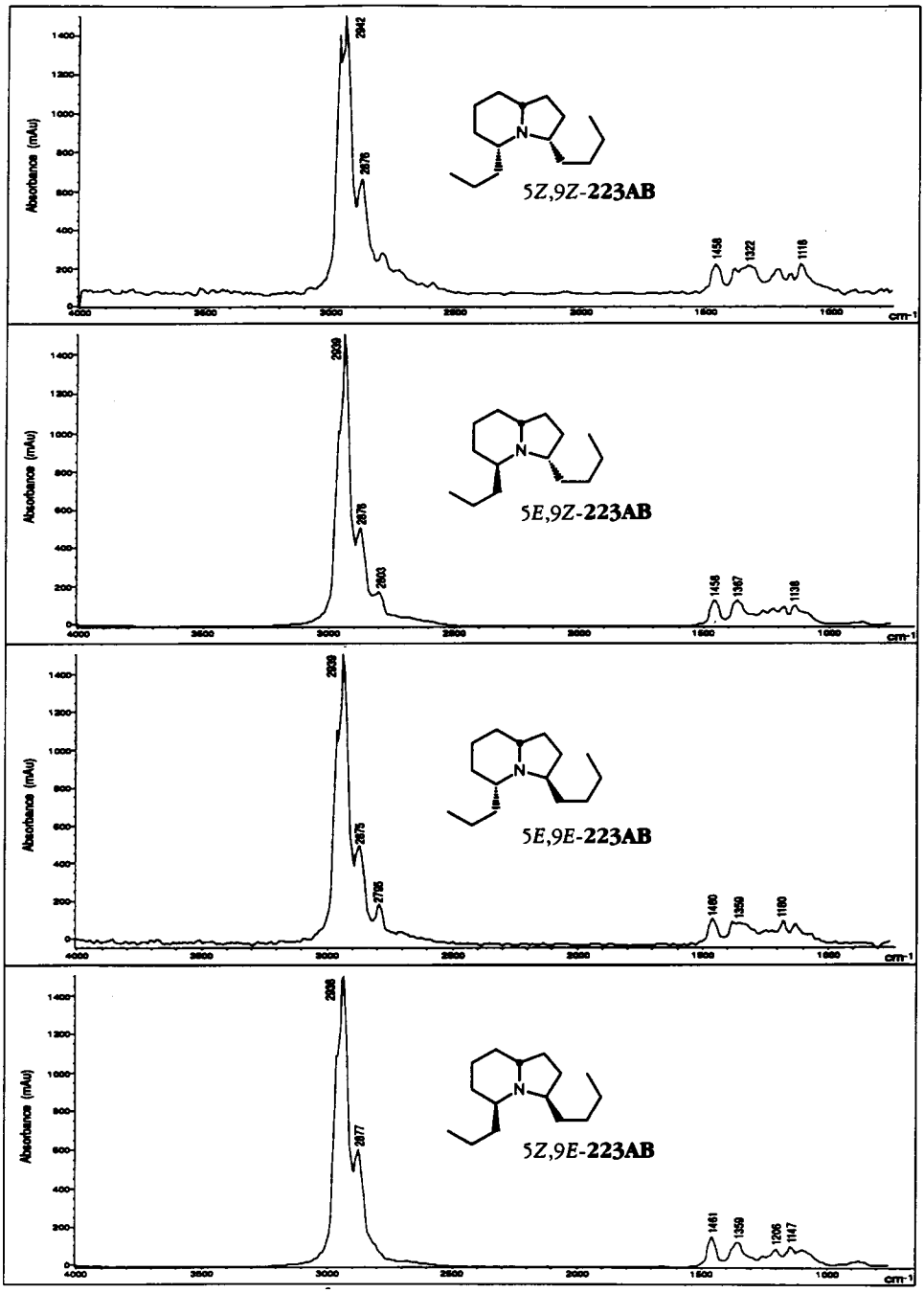


FIGURE 2. Selected vapor phase Ft-ir spectra.

QUINOLIZIDINES.—**235E′**.—"$C_{16}H_{29}N$"; a quinolizidine with a Me group at C-1 and a hexenyl group at C-4, the double bond being interior; *m/z* 235 (4), 234 (1), 220 (1), 206 (1), 178 (2), 165 (3), 164 (3), 152 (100), 150 (5), 136 (2), 122 (2), 110 (15), 96 (7), 81 (3), 70 (5), 67 (5), 55 (10); 0D; ir 2967 (100), 2787 (33), 1460 (17), 1381 (14), 1294 (11), 1227 (8), 1156 (14), 914 (3) cm^{-1}. H_2-derivative. Stereochemistry at C-1 and position of double bond are not known. Ft-ir see Figure 5. An alkaloid with a similar mass spectrum was reported from dendrobatid frogs (1).

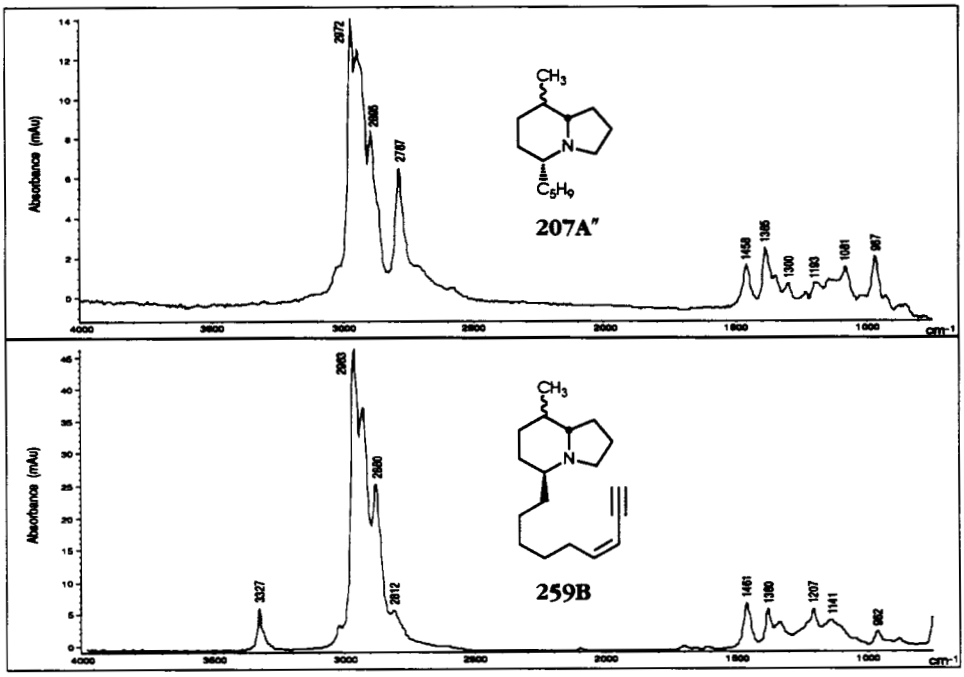


FIGURE 3. Selected vapor phase Ft-ir spectra.

TRICYCLICS.—**193A.**—"$C_{13}H_{23}N$"; precoccinelline; *m/z* 193 (45), 192 (95), 178 (35), 164 (55), 151 (80), 150 (100), 137 (40), 136 (50), 122 (40), 110 (15), 108 (30), 96 (20), 94 (30), 82 (20), 80 (20), 67 (27), 55 (28); 0D; ir 2936 (100), 2876 (39), 1454 (9), 1364 (9), 1283 (6), 1138 (8) cm^{-1}; Ft-ir see Figure 5.

235I.—"$C_{16}H_{29}N$"; *m/z* 235 (63), 234 (100), 220 (61), 208 (12), 206 (21); 192 (26), 178 (11), 150 (20), 138 (18), 136 (50), 122 (21), 110 (20), 96 (20), 84 (27), 70 (30), 67 (20), 55 (30); 0D; ir 2960 (100), 2910 (80), 2791 (35), 1464 (15), 1367 (16), 1232 (9), 1169 (17) cm^{-1}; probably related to precoccinelline but ir differs. Ft-ir see Figure 5.

236.—$C_{14}H_{24}N_2O$; an oxime methyl ether; *m/z* 236 (5), 205 (2), 135 (3), 126 (100), 111 (4), 95 (9), 81 (6), 68 (6), 67 (7), 55 (6); 0D; ir 2962 (100), 2886 (53), 2825 (22), 1470 (17), 1390 (7), 1322 (8), 1244 (7), 1055 (74), 864 (28) cm^{-1}. Ft-ir see Tokuyama *et al.* (16).

UNKNOWN.—**219G.**—"$C_{15}H_{25}N$"; *m/z* [ion trap 220 (20)], 164 (100), 162 (46), 134 (7), 120 (30); two double bonds; ir 2969 (100), 2930 (78), 2885 (55), 2787 (35), 1459 (14), 1374 (14),1239 (11), 1145 (15), 965 (17) cm^{-1}. Structural class is unknown.

RESULTS AND DISCUSSION

Gc-ms and ir spectral analyses of individual alkaloids in fractions from amphibian skins provide in most cases sufficient information to allow an assignment by class. For example, histrionicotoxins exhibit a major loss of the side chain adjacent to nitrogen and a major fragment ion of *m/z* 96 ($C_6H_{10}N^+$). They have two exchangeable hydrogens and an intramolecular H-bonded hydroxyl group absorbance in the ir at 3300 cm^{-1}. Ir spectra also show, very clearly, terminal double bonds (3084, 1642, 994, and 915 cm^{-1}), triple bonds [3326, 2115 (weak) cm^{-1}], conjugated terminal double bonds (3032 cm^{-1}), allenes (1951, 846 cm^{-1}), and conjugated en-ω-yne bonds (3326, 3038, 980 cm^{-1}), all commonly occurring unsaturation patterns found in histrionicotoxins and certain other classes of amphibian alkaloids (1, 8). Histrionicotoxins were, however, not detected in *Melanophryniscus*.

Decahydroquinolines exhibit a dominant fragment due to loss of the side chain from

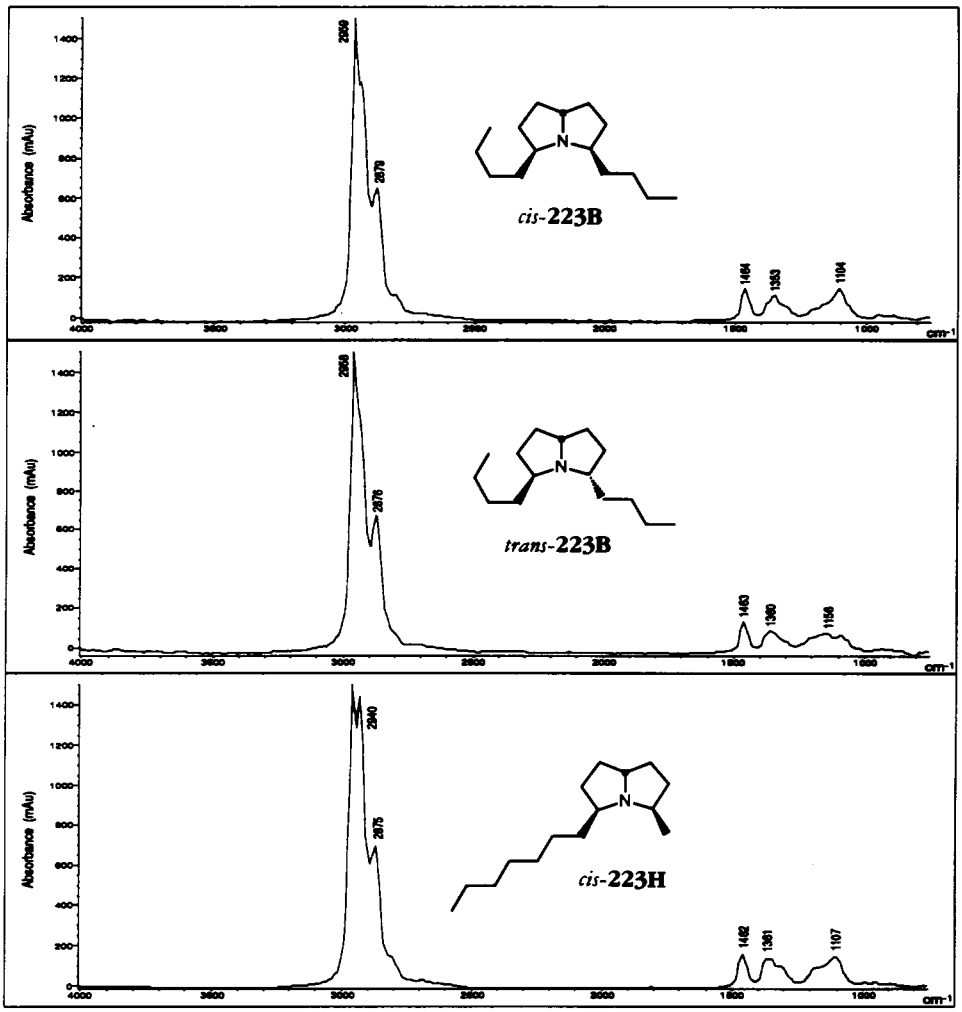


FIGURE 4. Selected vapor phase Ft-ir spectra.

C-2 adjacent to nitrogen with a minor fragment resulting from loss of the C-5 substituent. There is one exchangeable hydrogen. Acetylation yields an amide. Ir spectral absorptions can distinguish cis and trans ring fusion isomers (9). Six decahydroquinolines were detected in *Melanophryniscus.*

The pumiliotoxin-A class has three subgroups: pumiliotoxins, allopumiliotoxins and homopumiliotoxins. The pumiliotoxins exhibit two diagnostic mass spectral ions of *m/z* 166 ($C_{10}H_{16}NO^+$) and *m/z* 70 ($C_4H_8N^+$). The allopumiliotoxins exhibit ions of *m/z* 182 ($C_{10}H_{16}NO_2^+$) and *m/z* 70 ($C_4H_8N^+$), the former due to the presence of the additional hydroxyl group at C-7. The homopumiliotoxins show ions of *m/z* 180 ($C_{11}H_{18}NO^+$) and *m/z* 84 ($C_5H_{10}N^+$), which are 14 mass units greater than those of the pumiliotoxins due to the additional methylene. Ir absorbance patterns are diagnostic for all three subgroups (10, and this paper). Pumiliotoxins, allopumiliotoxins, and homopumiliotoxins were detected in *Melanophryniscus.*

The 3,5-disubstituted indolizidines show, in general, two diagnostic mass spectral ions as a result of fragmentations α to the nitrogen. However, when one substituent is a Me group, the corresponding fragmentation $[M-15]^+$ is weak. Several 3,5-disubsti-

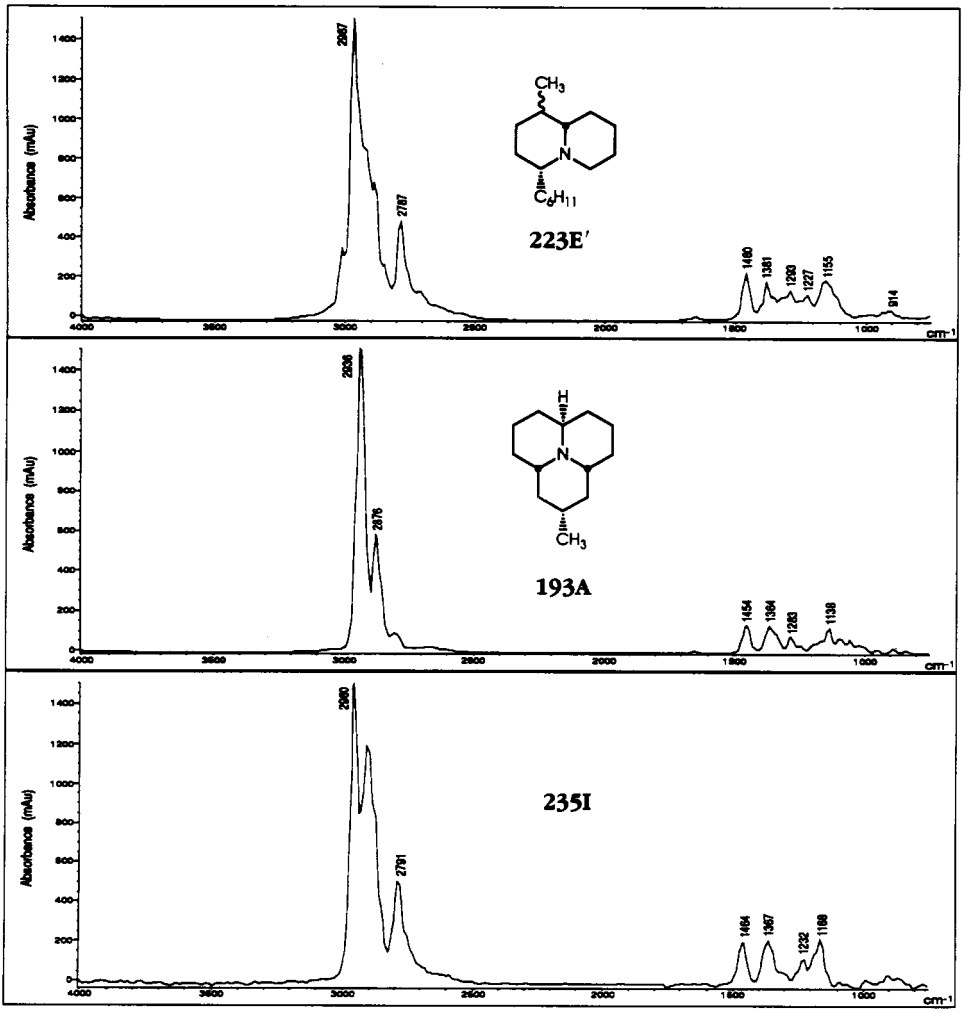


FIGURE 5. Selected vapor phase Ft-ir spectra

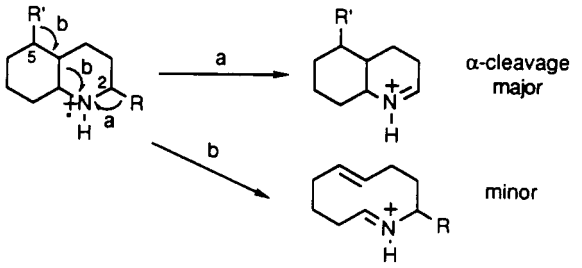
tuted indolizidines were detected in *Melanophryniscus*. The 5,8-disubstituted indolizidines have one important fragmentation whereby the side chain at C-5 is lost. In most cases, the base peak is *m/z* 138 ($C_9H_{16}N^+$) since the 8-substituent is generally a Me group. Two 5,8-disubstituted indolizidines were detected in *Melanophryniscus*.

In the same way, mass and ir spectral analyses are diagnostic for the two new classes of amphibian alkaloids now detected in *Melanophryniscus*, the pyrrolizidines and the 1,4-disubstituted quinolizidines. The pyrrolizidines, all having a 3,5-disubstitution pattern, give two mass spectral fragmentations α to the nitrogen, although the cleavage is very weak for an α-Me substituent. The ir spectral absorptions are less informative, as discussed below. Several pyrrolizidines were detected in *Melanophryniscus*. The 1,4-disubstituted quinolizidines lose the side chain at C-4 by α cleavage and exhibit one dominant fragment ion. In most cases the C-1 substituent is a Me, as in the equivalent indolizidines, yielding a base peak of *m/z* 152 ($C_{10}H_{18}N^+$). Ir spectra with significant Bohlmann bands are characteristic of this class. One quinolizidine is reported from *Melanophryniscus*, but traces of other quinolizidines appear also to be present.

In addition to the five major classes (decahydroquinolines, pumiliotoxin-A class,

indolizidines, pyrrolizidines, quinolizidines) of amphibian alkaloids that were detected in *Melanophryniscus*, there were three tricyclic alkaloids: precoccinelline and an apparent higher homologue and a pyrrolizidine oxime methyl ether. These alkaloids exhibit characteristic mass spectral fragmentations and ir spectra.

DECAHYDROQUINOLINES.—Six 2,5-disubstituted decahydroquinolines (DHQs) were characterized: *cis*-**223F**, *trans*-**223F**, *cis*-**249D**, *trans*-**249D**, *trans*-**249E**, and *cis*-**275B** (Table 1). The ms fragmentations characteristic of the DHQ class are: $[M]^+ \mapsto$ a major fragment (a) and a minor fragment (b) as indicated in Scheme 1.



SCHEME 1

There are four asymmetric carbons in these DHQs and the mass spectral fragmentations are very similar for the different diastereomers so far detected. The ir spectrum provides information for the cis or trans stereochemistry of the ring fusion and for the relative configuration at C-2. Only at C-5 are we unable to assign the relative configuration by ms or ir. In the cis ring-junction there are two main minimum energy conformations of the molecule, whereas in the trans only one conformation is important. That is reflected in the ir spectrum as doublets in the regions 1300 cm^{-1} and 1100 cm^{-1} for the cis DHQs, and singlets in the same regions for the trans DHQs (9). This is illustrated for *cis*-**223F** and *trans*-**223F** in Figure 1.

The configuration at C-2 relative to the one at C-8a can be postulated on the basis of the presence or absence of a small Bohlmann band. Only when the hydrogens at C-2 and C-8a are axial and antiparallel to the N lone-pair (*i.e.*, H-2 and H-8a are cis to one another), does this absorption appear (unpublished results). *Trans*-**223F** is identical with a synthetic compound obtained by hydrogenation of *trans*-**219A**. For the other five DHQs, the relative configuration at C-5 remains unknown. However, ir spectra of the three cis-fused DHQs reported here are very similar to that of *cis*-**195A** (formerly referred to as pumiliotoxin C) (9), and ir spectra of the other two trans-fused DHQs are very similar to that of *trans*-**223F**. Because of this, we tentatively assign the same stereochemistry of *cis*-**195A** for *cis*-**223F**, *cis*-**249D**, and *cis*-**275B**, and the same sterochemistry of *trans*-**223F** for *trans*-**249D** and *trans*-**249E**.

These decahydroquinolines may be considered to originate from 2,6-disubstituted piperidines, where the side chain at C-6 forms another ring by a bond to the C-5 position of the piperidine. Remarkably, all of the DHQs found in these extracts could arise from cis-piperidines, three of them forming cis-fused DHQs and the other three forming trans-fused DHQs. But not all the natural DHQs could be formed from cis-piperidines, since *cis*-**219A** and *cis*-**243A**, found in a dendrobatid frog (9) have a trans-piperidine moiety.

PUMILIOTOXIN-A CLASS.—Pumiliotoxin **251D**, of known structure (6), is the only alkaloid of the pumiliotoxin subclass present in *Me. stelzneri*. Allopumiliotoxins were not

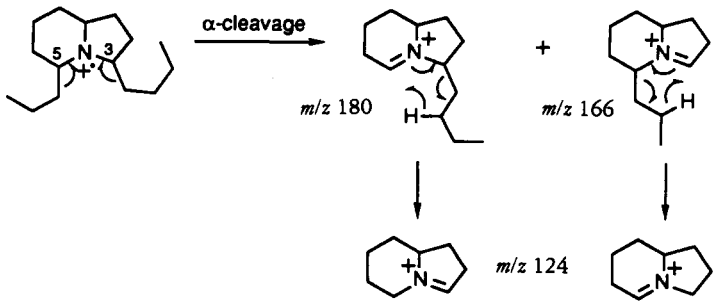
detected in *Me. stelzneri*. We now report that three previously unknown homopumiliotoxins are also present as traces in extracts of *Me. stelzneri* from Uruguay. These are **319A**, **319B**, and **321B** (Table 1).

In the homopumiliotoxins, the indolizidine moiety of the pumiliotoxin A structure is replaced by a quinolizidine ring. Two of these compounds have a side-chain keto group, one conjugated, the other unconjugated, while the third has a side-chain hydroxyl group. On the basis of deuterium exchange of the ketones with deuteromethoxide and a $NaBH_4$ reduction, we conclude that these three alkaloids are functionalized at the 17 position (see Table 1). Alkaloids **319A** (unconjugated ketone) and **321B** (acetylatable alcohol) have almost the same retention time in gas chromatography, but could be separated after acetylation of **321B**. Deuteration α to the carbonyl, with deuteromethoxide in MeOH-d_4, should show the maximum number of hydrogens in that position. In this case we expected to see three exchanges for the conjugated ketone **319B** due to the α-Me and five exchanges for the unconjugated ketone **319A** due to the α-methylene and α-Me groups. With the new homopumiliotoxins, we observed a total of seven exchanges in both ketones, a result that can only be explained if we invoke allylic exchanges as well. Because of this ambiguous result, the main evidence in favor of these structures remains their hrms, with cleavages of 43 amu (MeCO) for the Me ketones and 45 amu (MeCHOH) for the alcohol and a loss of 58 amu (C_3H_6O) from both ketones, suggesting either a direct loss of acetone in a McLafferty-type cleavage arising from a Me ketone, or a loss of a Me after the acetyl cleavage (43+15 amu). A reduction was attempted with $NaBH_4$ and $CeCl_3$ on the *Me. stelzneri montevidensis* extract containing these compounds, in hopes of transforming **319A** into **321B**, but it was inconclusive. Simpler evidence to support the homoallylic alcohol structure for **321B** is the presence in its spectrum of a double absorption in the region of 3650–3600 cm^{-1}, which is typical of homoallylic but not of allylic alcohols, due probably to partial H-bonding between the alcohol and the double bond (11). The ir data also support the conjugated and unconjugated ketone structures for the **319** compounds. See Ft-ir of **319B** in Figure 1.

INDOLIZIDINES.—Two types of indolizidines were found: 3,5- and 5,8-disubstituted. 3,5-Disubstituted indolizidines included all four diastereomers of 3-butyl-5-methylindolizidine, characterized by mass and Ft-ir spectra as 5*Z*,9*Z*-**195B**, 5*E*,9*Z*-**195B**, 5*E*,9*E*-**195B** and 5*Z*,9*E*-**195B** (Table 1). The four diastereomers elute on gc in that order. The 5*E*,9*E* diastereomer cochromatographed with another trace alkaloid in *Me. stelzneri* from both Argentine localities, thus complicating ms and Ft-ir analyses. The 3,5-disubstituted indolizidines also included three diastereomers of 3-butyl-5-propylindolizidine characterized by comparison to synthetic standards as 5*Z*,9*Z*-**223AB**, 5*E*,9*Z*-**223AB** and 5*E*,9*E*-**223AB** (Table 1). The Ft-ir are shown in Figure 2, together with the Ft-ir of a synthetic standard of 5*Z*,9*E*-**223AB** (12). The last diastereomer was not detected from *Melanophryniscus*.

The mass spectral fragmentations characteristic of these structures are $[M]^+ \mapsto$ two major fragments and a weaker *m/z* 124 ion as shown in Scheme 2.

The 3,5-disubstituted indolizidines, after α- cleavage, give a fragmentation of the McLafferty type yielding *m/z* 124 rather than undergoing a retro-Diels-Alder process, especially when using the ion trap mass detector. The ion trap shows *m/z* 124 as a prominent, characteristic peak, while an ei spectrum on a quadrupole instrument scarcely shows this ion. All four diastereomers of **223AB** have been synthesized (12) and show differences in the relative intensities of the fragment ions in eims. Comparison with these synthetic materials permitted the identification of the three diastereomers of **223AB** reported here. Their Ft-ir spectra also showed differences, particularly in the region of the Bohlmann bands. Relying on such Bohlmann bands, it is possible to

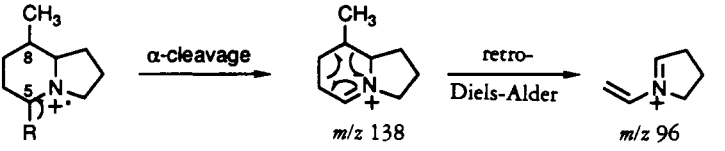


SCHEME 2

determine the relative configuration of the carbons vicinal to nitrogen, since it has been established that distinctive C-H stretching bands appear below 2800 cm^{-1}, if there are two or more α-C-H's oriented antiparallel to the nitrogen lone-pair (13). These characteristic differences also pertained to the **195B** diastereomers and allowed the identification of the three diastereomers of **195B** detected in *Melanophryniscus*. Thus, ir provides the means to assign stereochemistry in the 3,5-disubstituted indolizidines.

Two 5,8-disubstituted indolizidines were characterized: **259B** with a non-6-en-8-yne side-chain at C-5, and **207A″**, with a pentenyl side chain at C-5 (Table 1). As is the case for many 5,8-disubstituted indolizidines, these two alkaloids have a Me at C-8.

The mass spectral fragmentations characteristic of these structures are as follows: $[M]^+ \rightarrow$138 (100), 96 (20) as shown in Scheme 3.

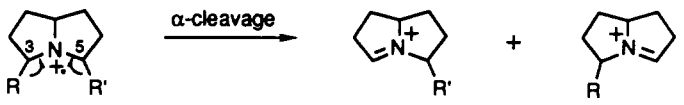


SCHEME 3

The *m/z* 138 base peaks where the C-8 substituent is a Me, is replaced with higher mass base peaks, when other C-8 substituents are present (2). The 5,8-disubstituted indolizidines, after α cleavage and loss of the side chain at C-5, generally give a fragmentation ion of *m/z* 96 ($C_6H_{10}N^+$), coming from the base peak ion after a retro-Diels-Alder process (losing a propene molecule, when an 8-Me is present). Indolizidine **259B** clearly showed the terminal en-yne side-chain in the ir (3327, 3025, 2100 cm^{-1}), while the double bond in **207A″** was not seen (see Ft-ir in Figure 3). Major Bohlmann bands are seen in **207A″** (2700–2850 cm^{-1} region) but not in **259B**. Thus these alkaloids differ in their relative configuration at C-5, leading to the tentative structures of Table 1. The relative configuration at C-8, a position remote from the N, is unknown. In the case of several 5,8-disubstituted indolizidines from dendrobatid frogs, the 8-Me substituent is equatorial (2).

PYRROLIZIDINES.—Six 3,5-disubstituted pyrrolizidines were characterized in these extracts: *cis*-**223B**, *trans*-**223B**, *cis*-**223H**, **237G**, *cis*-**251K**, and *trans*-**251K** (Table 1). The ms fragmentatons for this group are $[M]^+ \rightarrow$2α-cleavages as shown in Scheme 4.

The ir spectra are relatively uninformative, although for 3,5-disubstituted pyrrolizidines only the cis (endo, endo) diastereomers display significant Bohlmann



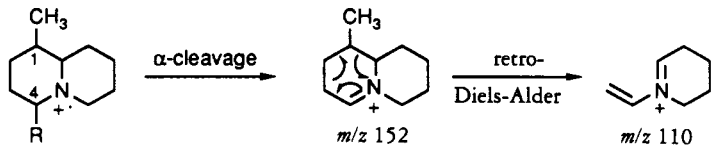
SCHEME 4

bands (2700–2850 cm^{-1} region): The cis (exo, exo) diastereomers display only small Bohlmann bands, and both trans diastereomers show none (Figure 4). Cis (endo, endo) 3,5-disubstituted pyrrolizidines have not been found in nature.

Using synthetic standards (14, and unpublished results) we were able to identify the two 3,5-dibutylpyrrolizidines, one as being exo, exo, i.e., cis-disubstituted (*cis*-**223B**) and the other being exo, endo, i.e., trans-disubstituted (*trans*-**223B**), and two butylhexylpyrrolizidines, also being exo, exo and exo, endo (or endo, exo) for the butyl and hexyl substituents, respectively (*cis*-**251K** and *trans*-**251K**, the latter representing one or both of the two possible exo, endo isomers). A sample (14) containing the two possible synthetic diastereomers of *trans*-**251K** could not be separated by capillary gc, so we could neither identify the isomer in the extracts nor ascertain whether it was a single isomer. A fifth pyrrolizidine found was *exo, exo*-3-heptyl-5-methylpyrrolizidine (*cis*-**223H**), identical with a known synthetic compound (15).

Another minor pyrrolizidine, **237G**, found in these extracts and evidently related to *cis*-**223H**, had a ketone-containing side chain. Although its stereochemistry is uncertain, its ir spectrum is very similar to that of *cis*-**223H**, with the exception of the 1731 cm^{-1} absorption.

QUINOLIZIDINES.—The only quinolizidines that have been found as yet in frogs or toads are the 1,4-disubstituted quinolizidines. In extracts of *Melanophryniscus*, **235E′**, with a non-terminal double bond in the side chain, was characterized (Table 1). The characteristic ms fragmentations of this class, when the C-1 substituent is a Me, are $[M]^+ \mapsto$152 (100), 110 (20), as shown in Scheme 5.



SCHEME 5

A fragment ion at *m/z* 110 ($C_7H_{12}N^+$) is generally seen coming from the base peak after the retro-Diels-Alder process. The Bohlmann bands in the ir spectra of 1,4-disubstituted quinolizidines are also very characteristic, being broader but less intense than in 5,8-disubstituted indolizidines. The Ft-ir spectrum of **235E′** is shown in Figure 5. From the Ft-ir spectrum it is possible to assign the relative configuration for quinolizidine **235E′** at C-4 and C-10, α to the N, but not at C-1 where the relative configuration of the Me remains unknown.

TRICYCLIC ALKALOIDS.—Precoccinelline [**193A**] was found in minor amounts. This alkaloid has no side chain α to the N, and thus there is no single fragmentation by α-cleavage except for an H loss. This results in an eims with a significant molecular ion and many fragmentation peaks. The ir spectrum shows only weak Bohlmann bands. See Ft-ir in Figure 5.

Alkaloid **235I** and precoccinelline have very similar fragmentation patterns, with

prominent parent and $[M-1]^+$ peaks and many significant fragment ions, but different ir spectra (see Ft-ir in Figure 5). The structure is unknown, but it is a tricyclic alkaloid, perhaps related to precoccinelline.

Another tricyclic alkaloid, **236**, has been found in these extracts. Alkaloid **236**, whose structure has recently been proposed (16), is a spiro-fused pyrrolizidine oxime methyl ether (Table 1).

SUMMARY

The present study illustrates the potential of gc-ir combined with gc-ms for identification of mixtures containing literally dozens of alkaloids, many at the trace level, in extracts of less than 1 mg total alkaloid wt. Fortunately for our current work on amphibian alkaloids, prior studies with nmr or X-ray crystallography on alkaloids isolated from extracts containing much more material have characterized in detail one or more members of the various alkaloid classes. Gc-ms of unknown alkaloids, in many instances, now permits identification of the alkaloid class. Illustrating the value of gc-ms and gc-Ft-ir for further identification of an alkaloid are the diastereomers of 3-butyl-5-methylindolizidine [**195B**]. The eims clearly indicates the presence of a butyl side-chain at C-3 and an Me at C-5 in an indolizidine. Three out of four possible diastereomers have different Rt on gc, but the ms fragmentations are similar and the minor differences in ms are difficult to generalize. However, the stereochemistry of a side chain adjacent to the N is readily assigned using Bohlmann band patterns in gc-Ft-ir spectra, not only in indolizidines but in other ring systems. On the other hand, the ir spectra of homologues with different length side-chains, but having the same relative configuration, are nearly superimposable. Such homologues are readily distinguished by gc-ms. Thus, the two techniques are complementary. In addition, gc-cims with NH_3 and ND_3 is invaluable for assignment of mol wt and number of exchangeable hydrogens, while high resolution gc-eims allows formulae to be assigned to the parent ion and to fragment ions.

The present study further documents the occurrence of dendrobatid alkaloids in non-dendrobatid anurans. The profile of these alkaloids in the toads of the genus *Melanophryniscus* is tabulated in Table 1, as major, minor, and trace alkaloids. Some other trace alkaloids were detected but are not documented because of insufficient data to characterize them. In *Melanophryniscus moreirae* (Mirando-Ribeiro) from Brazil, only pumiliotoxin **267C** and allopumiliotoxin **323B** were detected (3). In *Me. stelzneri,* on the other hand, there is a wider array of alkaloids, and this varies markedly for the three populations examined. The two populations from Argentina come from one province, and both have decahydroquinolines and indolizidines as major alkaloids. One also has pumiliotoxin **251D**, pyrrolizidines, a quinolizidine, and tricyclics as significant alkaloids, while these are trace alkaloids in the other population. The third population of *Me. stelzneri,* from Uruguay, has no decahydroquinolines, only two indolizidines and trace amounts of two pyrrolizidines. It contains pumiliotoxin **251D** and three homopumiliotoxins.

It is remarkable that suites of diastereomers, for example of indolizidines **195B** and **223AB**, are found together in extracts from one population, since one might expect a high selectivity in the biosynthesis of a particular alkaloid. Definitive characterization of diastereomers was impossible before the advent of gc-Ft-ir, and even at present it is difficult in some cases. In addition, certain sets of diastereomers probably could not have been separated on the micro-bore or packed, less polar OV-1 columns used in earlier investigations, and thus, more examples of complex mixtures of diastereomers undoubtedly have gone unreported for dendrobatid frogs.

Another major finding was the presence in amphibian skin extracts of a number of

alkaloids that also occur in arthropods. The indolizidine monomorine-I, 5*Z*,9*Z*-**195B**, occurs in ants (17). Pyrrolizidines *cis*-**223H** (15) and *trans*-**251K** (14) also occur in ants. Precoccinelline is a major alkaloid in certain beetles (18). Alkaloid **236**, the oxime methyl ether, is closely related to nitropolyzonamine, an alkaloid found in a millipede (19). The fact that ants and other insects represent the diet of bufonid toads and dendrobatid frogs, together with the wide variety of alkaloids found in such toads and frogs, raises the possibility of two processes, an alkaloid intake and de novo biosynthesis, as the source of amphibian skin alkaloids. The total lack of alkaloids in captive-raised dendrobatids (20) poses further questions as to the origin of amphibian alkaloids.

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